The Temperate Marine Phage Φ HAP-1 of *Halomonas aquamarina* Possesses a Linear Plasmid-Like Prophage Genome[∇]

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A myovirus-like temperate phage, Φ HAP-1, was induced with mitomycin C from a *Halomonas aquamarina* strain isolated from surface waters in the Gulf of Mexico. The induced cultures produced significantly more virus-like particles (VLPs) (3.73×10^{10} VLP ml⁻¹) than control cultures (3.83×10^7 VLP ml⁻¹) when observed with epifluorescence microscopy. The induced phage was sequenced by using linker-amplified shotgun libraries and contained a genome 39,245 nucleotides in length with a G+C content of 59%. The Φ HAP-1 genome contained 46 putative open reading frames (ORFs), with 76% sharing significant similarity (E value of $<10^{-3}$) at the protein level with other sequences in GenBank. Putative functional gene assignments included small and large terminase subunits, capsid and tail genes, an N6-DNA adenine methyltransferase, and lysogeny-related genes. Although no integrase was found, the Φ HAP-1 genome contained ORFs similar to protelomerase and *parA* genes found in linear plasmid-like phages with telomeric ends. Southern probing and PCR analysis of host genomic, plasmid, and Φ HAP-1 DNA indicated a lack of integration of the prophage with the host chromosome and a difference in genome arrangement between the prophage and virion forms. The linear plasmid prophage form of Φ HAP-1 begins with the protelomerase gene, presumably due to the activity of the protelomerase, while the induced phage particle has a circularly permuted genome that begins with the terminase genes. The Φ HAP-1 genome shares synteny and gene similarity with coliphage N15 and vibriophages VP882 and VHML, suggesting an evolutionary heritage from an N15-like linear plasmid prophage ancestor.

The marine environment is one of the largest reservoirs of viruses, where concentrations range from 10^7 virus-like particles (VLPs) per liter to 10^{11} VLPs per cubic centimeter of sediment (5, 65). Viruses are believed to contribute significantly to the marine microbial loop and nutrient cycling in the oceans, and may also serve as agents of gene transfer in the marine environment (19, 43). They may also contribute to the environmental adaptation of their host, as in the case of photosynthetic genes on phages infecting marine cyanobacteria, as well as constrain host diversity (37, 62).

Temperate phages can exist either in a lytic or lysogenic state. In the lysogenic state, the prophage is replicated along with the host genome. Jiang and Paul (28) found that more than 40% of marine bacterial isolates screened contained inducible phages (28). Polylysogeny may also be abundant in the marine environment. For example, the genome of *Silicibacter* sp. strain TM1040 was found to contain five prophage-like elements, three of which were inducible temperate phages (15). Studies of natural marine populations have indicated that environmental cues, such as host density and temperature, may influence the incidence of lysogeny (36, 64, 66). Although temperate phages are abundant in bacterial isolates and natural environments, little is known about the molecular control of lysogeny in marine bacteria. Sequencing and experimental characterization of temperate marine phage genomes may of-

fer insights into novel lysogenic interactions that occur in the ocean.

Most temperate bacteriophages integrate into the host chromosome during lysogeny. However, some phages, such as *Escherichia coli* phage P1 and phage cp32 from *Borrelia burgdorferi*, exist as low-copy-number plasmids (18, 27). *E. coli* phage N15, *Klebsiella oxytoca* phage Φ KO2, and *Yersinia enterocolitica* phage PY54 are a group of closely related phages that exist as linear plasmid-like prophages, with covalently closed hairpin ends (telomeres) due to the activity of a phage-encoded protein, protelomerase (12, 24, 54). During lysogeny, the protelomerase cuts the prophage DNA at an inverted repeat generally located near the protelomerase gene itself. The protelomerase protein resolves the ends of the prophage genome into telomeres. The resulting plasmid prophage gene order is 50% circularly permuted with respect to the virion DNA, such that the terminase genes are found toward the middle of the prophage conformation (24, 54). In addition to a protelomerase, the genomes of these linear plasmid-like phages contain similar lysogeny modules and replication genes, as well as plasmid-partitioning genes, to ensure that daughter cells receive a copy of the phage genome (12, 25, 55). The presence of protelomerase genes in the genomes of the *Vibrio harveyi* temperate phage VHML and the uncharacterized *Vibrio parahaemolyticus* phage VP882 indicates that linear plasmid-like prophages may be common among cultivated marine lysogens (39).

Halomonas aquamarina, formerly known as *Deleya aquamarina*, is a gram-negative halophilic gammaproteobacterium that has been isolated from a variety of marine and hypersaline environments, including the pelagic ocean, deep-sea hydrothermal vents, the brine-seawater interface of deep-sea brine

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pools, and coastal surface waters (31, 41, 58). The phage-host interactions of two temperate myoviruses infecting *Halomonas* species from the Great Salt Plains in Oklahoma and two temperate siphoviruses from *Halomonas halophila*, isolated from hypersaline soil, have been characterized, but these phages have not been sequenced (11, 60).

To increase our knowledge of marine prophage genomics, we characterized a temperate phage, ΦHAP-1, in an *H. aquamarina* isolate from the Gulf of Mexico with respect to morphological characteristics, nucleotide sequence, and overall phage-host relationship. Prophage induction resulted in tailed-phage particles resembling members of the *Myoviridae*. Finally, the genomic properties of the phage were experimentally analyzed, including the presence of telomeric ends and its existence as a linear plasmid.

MATERIALS AND METHODS

Isolation of host and phage particles. *Halomonas aquamarina* was isolated from samples of the surface waters of the Gulf of Mexico (latitude 26°00'N, longitude 83°35.6'W) collected on 15 July 2001. Vortex flow filtration was used to concentrate the water sample (29). The retentate was heated at 80°C for 10 min and then plated onto artificial seawater nutrient agar plates (ASWJP+PY) (45), a procedure employed to select for spore-forming bacteria. The *H. aquamarina* isolate was identified by partial sequencing of a cloned PCR product obtained by using a bacterial 16S universal primer set (23). The bacterium was maintained in pure culture by monthly plating on ASWJP+PY plates, as well as in 25% glycerol stocks kept at -80°C.

H. aquamarina phage particles were isolated by a mitomycin C induction procedure. Fifty milliliters of overnight *H. aquamarina* culture was inoculated into 450 ml ASWJP+PY and grown to log phase (optical density at 600 nm, 0.4). Mitomycin C (1 µg ml⁻¹; Sigma) was added to the culture and incubated for 24 h at 28°C. A bacteria-free viral lysate was obtained by centrifugation at 11,000 × rpm and filtering of the supernatant through 1-µm, 0.4-µm, and 0.2-µm filters. DNase (DNase I) and RNase (RQ1) (Promega, Madison, WI) were each added (1 µg ml⁻¹), and the reaction mixtures were incubated at room temperature for 30 min. The phage particles were further concentrated with polyethylene glycol 6000 and purified through discontinuous cesium chloride gradients as described by Sambrook and Russell (57) with the following modifications to maximize particle concentration from a marine phage. After the polyethylene glycol 6000 precipitation, the phage pellets were eluted in 2.0 ml of 0.02-µM-filtered 75% artificial seawater (ASWJP) (29). The phage particles were purified by centrifugation at 29,000 rpm for 2 h at 4°C through discontinuous cesium chloride gradients of 1.7 ρ, 1.5 ρ, and 1.35 ρ in polycarbonate tubes, and the purified phage band was collected from the 1.5 ρ-1.35 ρ interface by using a syringe.

Phage particles were enumerated from the cesium chloride viral lysate by using Sybr gold stain (Molecular Probes, Eugene, OR) as described by Chen et al. (14), with a staining time of 12 min. VLPs were enumerated by using blue-light excitation with an Olympus BH-2 epifluorescence microscope. At least 200 phage particles in 10 fields per slide were counted.

Time series mitomycin C induction of phage particles. Phage production in *H. aquamarina* was monitored over a 24-h time series following induction with mitomycin C. Five milliliters of an overnight culture of *H. aquamarina* was diluted into 45 ml of fresh ASWJP+PY in separate flasks, one for a control and one for treatment. A 5-ml amount was removed from each flask to enumerate bacteria and viruses (time zero). When the optical density at 600 nm reached 0.4, mitomycin C (1.0 µg ml⁻¹) was added to the treatment flask and returned to the shaker. Absorbance readings were taken every 2 h until 8 h and at 24 h after induction, and 5 ml of culture was removed from each flask for phage enumeration. Samples were stained with Sybr gold, and VLPs were enumerated as described above. The direct-counting method was also used to enumerate the number of bacteria (BDC). Statistical analysis was done with Microsoft Excel using the pooled Student *t* test for equal variances ($\alpha = 0.05$). The equation $B_z = (V_m - V_c)/(B_c - B_m)$, where B_z is burst size, V_m is VLP per ml in mitomycin C cultures, V_c is VLP per ml in control cultures, B_c is BDC per ml in mitomycin C cultures, and B_m is BDC per ml in control cultures, was used on the results of each replicate from each time point after the addition of mitomycin C ($t = 3$) to calculate the mean burst size.

TEM. Transmission electron microscopy (TEM) was used to determine the morphology of the ΦHAP-1 particle from a cesium chloride-purified phage

lysate. A formvar carbon-coated grid (Electron Microscopy Sciences, Hatfield, PA) was floated on a large drop of lysate for 30 min. The grid was air dried and then negatively stained with 2% uranyl acetate for 1 min. The phage particles were visualized with a Hitachi 7100 TEM. The tail length, tail width, and head width of the ΦHAP particles were determined by measuring each feature on 14 different phage particles from several TEM micrographs.

Extraction and characterization of phage DNA. Phage DNA was extracted from the cesium chloride-purified phage lysate by the formamide extraction protocol adapted from Sambrook and Russell (57) as described below. The lysate was heated at 65°C for 30 min in 0.1 volume 2 M Tris HCl, 0.5 volume 0.5 M EDTA, 1.0 volume deionized formamide, and 2 µl/ml sample glycogen. After the ethanol washes, the phage DNA pellet was resuspended in 0.02 µm filtered 1× Tris-EDTA and DNA was quantified by using the Hoechst 33258 fluorometric method (45).

In order to identify the presence of a *cos* site, 300 ng of *Halomonas* phage DNA was isolated from a mitomycin C-induced lysate by using a Qiagen lambda DNA isolation kit according to the manufacturer's instructions (52). The restriction enzymes that gave relatively simple restriction patterns were *Ava*I, *Bam*HI, *Hpa*I, and *Kpn*I (New England Biolabs, Ipswich, MA). For all these, digestion was performed for 3 h at 37°C, and then one half of the digest was placed on ice, while the second was heated to 65°C and then immediately placed on ice before loading. A 1% Tris-borate-EDTA agarose gel was used and run at 80V in 1× Tris-borate-EDTA to prevent denaturation of the sticky ends.

Library construction and sequencing. Most of the DNA sequence of the ΦHAP genome obtained was from two types of libraries. The first was made by the Nanoclone method at Lucigen (Middleton, WI); this entails physical shearing of phage DNA purified from mitomycin C-induced cultures of the *Halomonas* host, end repair, selection of fragments of between 1 and 2 kb, and cloning into the pSMART vector (46). The second library was made by complete restriction digestion with *Nhe*I of phage DNA from a mitomycin C-induced *Halomonas* lysogen culture and cloning into pBR322 cut with *Nhe*I. Clones from these libraries were sequenced by using an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA). The software program Sequencer (Gene Codes, Ann Arbor, MI) was used to assemble the contigs. Gaps between the contigs were filled in by generating fragments using multiplex PCR and by primer walking using phage DNA as the template. Direct sequencing was also used to sequence the ends of the phage genome.

Sequence analysis of the ΦHAP-1 genome. Open reading frames (ORFs) were defined by using KODON software (Applied Maths, Austin, TX) and the ORF Finder software from the National Center of Biotechnology Information (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>), using the bacterial code option. Putative ORFs were compared to the nonredundant GenBank database using BLASTP (<http://www.ncbi.nlm.nih.gov/BLAST/>) in January 2008 (3). All annotation was performed by using the KODON software. For genomic comparisons with the protelomerase-containing phages N15, PY54, ΦKO2, and VHML, the ORFs were assigned functions based on the published genomes (12, 25, 39, 55). For phage VP882, ORF functions were assigned based on putative function from the GenBank annotation as of January 2008. BLASTN and BLASTP searches were used to search the CAMERA database (<http://camera.calit2.net>) for the presence of ΦHAP-1 protelomerase in environmental metagenomic data (61). The GenBank environmental database and SDSU Center for Universal Microbial Sequencing database (<http://scums.sdsu.edu>) were also BLAST searched for the presence of protelomerase from environmental samples, including metaviromes.

Protein analysis by mass spectroscopy. Phage proteins were separated on a one-dimensional 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel run at 4 mA for 16.5 h by the Laemmli method (33). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining with Sypro ruby (Bio-Rad, Hercules, CA). Protein-containing bands were numbered and excised from the gel with a scalpel. The excised samples were sent to the Proteomics Core Facility at Moffitt Cancer Research Center (Tampa, FL) for de novo peptide analysis by matrix-assisted laser desorption/ionization-time-of-flight-time-of-flight (MALDI-TOF-TOF) mass spectroscopy as described in Paul et al. (46). The tandem mass spectral analysis produces a collision-induced dissociation spectrum that can be used for protein identification based on peptide sequence (68).

Pulsed-field gel electrophoresis (PFGE). Phage genomic DNA was extracted from 500 ml of induced lysate as described above. Host chromosomal and plasmid DNA were extracted by using a Wizard genomic DNA purification kit and Wizard plus SV minipreps DNA purification system (Promega, Madison, WI), respectively, following the manufacturer's protocol (49, 50).

For PFGE analysis, approximately 325 ng of each DNA fraction was digested with 5 U of the specified restriction enzymes (Promega, Madison, WI). Digestions were performed with *Nar* I, *Xba*I, *Bam*HI, and double digests of *Nar* I plus

FIG. 1. Growth of host (A) and prophage production (B) during the 24-h mitomycin C induction experiment. Asterisks indicate when mitomycin C was added. Mean BDC for each time point was used to chart bacterial growth. Error bars indicate the standard deviations of the results from triplicate slides. VDC, enumeration of viruses by the direct-count method.

XbaI for 3 h at 37°C. PFGE was performed as described in reference 43 with a run time of 22.5 h.

Riboprobe labeling and Southern hybridization. A phage riboprobe was created by cloning a PCR-amplified fragment of Φ HAP-1 ORF 37 into the pCRII cloning vector by using a TOPO cloning kit per the manufacturer's instructions (Invitrogen, Carlsbad, CA). The probe was created by in vitro transcription using a Riboprobe combination system SP6/T7 (Promega, Madison, WI) according to the manufacturer's instructions (48). Probes were labeled with 35 S-UTP (GE Healthcare, Piscataway, NJ).

A Southern transfer of the pulsed-field gel to charge nylon membranes was done by using the standard method (57). The DNA was cross-linked to the membrane by using an FB-UVXL-1000 UV cross-linker (Fisher Scientific, Pittsburgh, PA). The Southern transfer blot was hybridized and washed as previously described (26). The probed blot was exposed to Biomax high-sensitivity film (Kodak, Rochester, NY) for 3 days at 4°C.

PCR. The two different genomic arrangements of Φ HAP-1 that are due to the activity of the protelomerase were confirmed by designing specific oligonucleotide PCR primer sets (Operon, Huntsville, AL). The primer set for the virion form (genomic arrangement starting with the putative terminase gene) is as follows: primer 1, 5' AGAAGTGCAGCTCAACACC 3', and primer 2, 5' ATC CTCTACCGCTTCATCCA 3'. The PCR product size for this primer set was 2,441 base pairs. The primer set used for the prophage arrangement (genomic arrangement starting with the protelomerase) is as follows: primer 3, 5' CGTC TTCTGTTTCGTCGTCA 3', and primer 4, 5' GGTGTCTGCCAATGTT GATG 3'. The PCR product size for this primer set was 2,681 base pairs. The DNA preparations were the same as those used in the Southern blot experiment. In order to account for the differing amount of phage DNA present in each preparation, 14 ng of host chromosomal DNA and 2 ng of plasmid or phage DNA were used in the reaction mixtures. GoTaq green mastermix (Promega, Madison, WI) was used as recommended, and the primer sets were added to a final concentration of 0.5 μ M per primer. The PCR was performed in a MyCycler thermal cycler (Bio-Rad, Hercules, CA) with the following PCR program: initial annealing of 2 min at 95°C, 25 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 2 min, and a final extension of 72°C for 10 min. The amplicons were loaded onto a 1% agarose gel with 0.5 μ g μ l⁻¹ of ethidium bromide and run at 89 V for 45 min. The gel was imaged by using an AlphaImager 2200 imaging system (Alpha Innotech, San Leandro, CA).

Nucleotide sequence accession numbers. The sequence of Φ HAP-1 has been deposited into GenBank under accession number EU399241. The GenBank accession numbers for the phage genomes used in this study are as follows: N15, NC_001901; Φ KO2, NC_005857; PY54, NC_005069; VHML, NC_004456; and VP882, NC_009016 (12, 25, 39, 55).

RESULTS

Characterization of the Φ HAP-1 virion. The results of the *H. aquamarina* phage (Φ HAP-1) mitomycin C prophage in-

duction experiments are shown in Fig. 1. In the mitomycin C-treated cultures, lytic activity was observed, as shown by the number of VLPs being significantly larger in the supernatant than in the control culture ($P = 0.01$) (Fig. 1B). Based on our sampling regimen, phage production was greatest at 8 h after the addition of mitomycin C (9×10^{10} VLP ml⁻¹) and decreased slightly at 24 h (3.73×10^{10} VLP ml⁻¹). Bacterial growth also decreased after the addition of mitomycin C ($P = 0.01$) (Fig. 1A). The mean burst size was calculated to be 38 ± 8 (mean $\pm$ standard deviation) phages per bacterium.

Electron micrographs of negatively stained Φ HAP-1 particles showed icosahedral capsids with long, thick tails consistent with the *Myoviridae* family (Fig. 2A and B). The capsid diameter was 49.81 ± 4.89 nm, while the tail was 254.67 ± 17.13 nm in length and 16.09 ± 2.65 nm in width ($n = 14$).

Analysis of the Φ HAP-1 genome. The Φ HAP-1 genome was found to be 39,245 nucleotides in length and had a G+C content of 59%. No comparisons between the G+C content in the phage and host DNA could be made as the *H. aquamarina* genome has not been sequenced. The G+C content in Φ HAP-1 is higher than in other protelomerase-containing phages, including PY54 (44%), Φ KO2 (52%), N15 (51%), VHML (50%), and VP882 (56%). No *cos* site was found, since

FIG. 2. Transmission electron micrographs of Φ HAP-1 particles. Black scale bars represent 50 nm.

FIG. 3. Genomic map of ΦHAP-1. KODON was used to construct the gene map. ORFs were numbered based on the arbitrary start of the genome at the terminase small subunit. Patterns were assigned based on functional assignments of the ORFs as indicated in the key.

there was no difference in the pattern of migration observed in the restriction enzyme-digested phage preparations with or without cooling, except for the phage lambda DNA/HindIII digest which served as the positive control (data not shown).

The ΦHAP-1 genome contains 46 putative ORFs, based on KODON analysis (Fig. 3). Thirty-five of the ORFs (76%) shared significant similarity (E value of $<10^{-3}$) at the protein level with other sequences in GenBank. Putative functional assignments and significant similarities to other sequences are listed in Table 1. The ORFs identified were similar to genes from other phages or genes encoding phage-related proteins from annotated bacterial genomes. The top BLASTP hits for 29 of the ORFs were similar to those for genes in *V. parahaemolyticus* phage VP882, 20 of which were also similar at the nucleotide level (E value of $<10^{-3}$) (Table 1).

ORFs 1 and 2: large and small terminase. Terminase genes are responsible for ATP-dependent packaging of concatameric DNA in phage capsids. The small subunit possesses DNA recognition specificity, while the large subunit has catalytic activity (7). The ORF 1 product is a hypothetical protein that is similar to vibriophage VP882 on both the protein (E value, $2e^{-52}$; 55% identity) and nucleotide level ($5e^{-87}$; 77% identity). This ORF's product also had weak similarity to phage terminase small-subunit nu1 from bacteriophage λ ($5e^{-4}$; 30% identity). The ORF 2 product is similar to the putative terminase large subunit in VP882 on both the protein (0; 62% identity) and nucleotide level ($2e^{-88}$; 75% identity). The ORF 2 product also shares similarity with the terminase large-subunit proteins from *Vibrio cholerae* AM-19226 ($2e^{-174}$; 53% identity) and *Wolbachia* phage WO ($4e^{-27}$; 23% identity).

ORFs 4 and 5: portal and capsid proteins. Portal proteins are responsible for forming a ring that enables DNA to pass into the major capsid during assembly and out during infection,

and they serve as a junction between the capsid and tail proteins (4). ORF 4 encodes a protein similar to the lambda-like portal proteins found in the putative prophage regions in the genomes of *Neisseria meningitidis* FAM18 ($4e^{-77}$; 35% identity) and *Silicibacter* sp. strain TM1040 ($1e^{-59}$; 29% identity) and in the *Wolbachia* phage WOcauB1 ($2e^{-44}$; 29% identity). This ORF shares significant similarity with those found in the vibriophages VP882 ($2e^{-132}$; 52% identity) and VHML ($6e^{-13}$; 23% identity). The expected size of the ΦHAP-1 portal protein, based on the amino acid composition, is 59.4 kDa, which is smaller than the expressed protein identified by MALDI-TOF-TOF from the 65.6-kDa excised band from the SDS-PAGE gel shown in Fig. 4. ORF 5 shares similarity with VP882 ($2e^{-179}$; 53% identity) and with genes encoding major capsid proteins found in the genomes of *Neisseria meningitidis* FAM18 ($2e^{-95}$; 33% identity) and *Silicibacter* sp. strain TM1040 ($7e^{-78}$; 33% identity). Its product also shares weaker identity with the major capsid proteins from the plasmid-like prophage ΦKO2 ($3e^{-7}$; 24% identity). MALDI-TOF-TOF analysis of the structural protein profile of ΦHAP-1 found protein fragments from the major capsid protein in two locations on the SDS-PAGE gel (Fig. 4). Based on the amino acid composition, the predicted size of ORF 5 is 66.6 kDa, while the bands of the gel correspond to 31.5 kDa (ORF 5A) and 28.4 kDa (ORF 5B).

ORFs 9, 10, 11, 12, 13, 16, 20, 21, 23, 24, and 25: tail proteins. The ΦHAP-1 genome encoded 11 putative tail proteins, including three baseplate assembly proteins, a tail fiber protein, a tail tape measure protein, a tail sheath, and a tail tube protein (Fig. 3; Table 1). Tail sheath and tail tubes are components that make up the contractile tail that is the hallmark of the *Myoviridae* family (10). ORFs encoding tail proteins were similar to tail protein genes from myoviruses, in-

TABLE 1. ORFs of the Φ HAP-1 genome and BLASTP hits

ORF no./orientation ^a	Nucleotide position	Predicted function	Related BLASTP hit(s) (accession no.; E value)
1/+	1–576	Terminase, small subunit ^c	Hypothetical protein of vibriophage VP882 (YP_001039813; 2e ⁻⁵²); nu1 of phage λ (AAA96533; 5e ⁻⁴)
2/+	602–2458	Terminase, large subunit ^c	Phage terminase large subunit of vibriophage VP882 (YP_001039815; 0); phage terminase large subunit of <i>Vibrio cholerae</i> AM-19226 (EDN16714; 1e ⁻¹⁷⁴); phage terminase large subunit of phage WO (BAA89640; 4e ⁻²⁷)
3/+	2552–3067	Hypothetical protein ^c	Phage-related protein of vibriophage VP882 (YP_001039816; 7e ⁻⁴⁰); hypothetical protein TM1040_1301 of <i>Silicibacter</i> sp. strain TM1040 (YP_613296; 1e ⁻¹⁴)
4/+	3071–4627	Portal protein ^{b,c}	Phage-related protein of vibriophage VP882 (YP_001039817; 2e ⁻¹³⁵); phage-related protein of <i>Neisseria meningitidis</i> FAM 18 (YP_974935; 4e ⁻⁷⁷); phage portal protein of <i>Silicibacter</i> sp. strain TM 1040 (YP_613295; 1e ⁻⁵⁹); portal protein of phage WOcauB1 (BAD16786; 2e ⁻⁴⁴); ORF 23 of <i>Vibrio harveyi</i> phage VHML (NP_758916; 6e ⁻¹³)
5/+	4731–6539	Major capsid ^{b,c}	Phage-related protein of vibriophage VP882 (YP_001039818; 2e ⁻¹⁷⁹); phage-related protein of <i>Neisseria meningitidis</i> FAM 18 (YP_974936; 2e ⁻⁹⁵); phage prohead of <i>Silicibacter</i> sp. strain TM1040 (YP_613294; 7e ⁻⁷⁸); major capsid head protein of Φ KO2 (YP_006586; 3e ⁻⁷)
6/+	6820–7182	Hypothetical protein ^c	Hypothetical protein of vibriophage VP882 (YP_001039822; 4e ⁻¹⁶)
7/+	7211–7690		Hypothetical protein of vibriophage VP882 (YP_001039824; 2e ⁻⁴³); ORF 28 of <i>Vibrio harveyi</i> phage VHML (NP_758921; 1e ⁻⁴)
8/+	7671–8231		Hypothetical protein of vibriophage VP882 (YP_001039825; 3e ⁻⁴³); hypothetical protein Tcr_0686 of <i>Thiomicrospira crunogena</i> XCL-2 (YP_390956; 2e ⁻⁶); ORF 29 of <i>Vibrio harveyi</i> phage VHML (NP_758922; 5e ⁻⁵)
9/+	8240–8863	Baseplate assembly protein V	Phage baseplate assembly protein V of <i>P. fluorescens</i> PfO-1 (YP_346871; 3e ⁻³³); phage baseplate assembly protein V of vibriophage VP882 (YP_001039827; 8e ⁻²⁹); baseplate protein of Φ CTX (NP_490615; 2e ⁻¹¹); gpV of phage P2 (NP_046771; 3e ⁻⁹); ORF 30 of <i>Vibrio harveyi</i> phage VHML (NP_758923; 1e ⁻⁷)
10/+	8869–9192	Baseplate assembly ^c	Phage-related protein of vibriophage VP882 (YP_001039829; 4e ⁻³⁴); hypothetical protein of <i>P. fluorescens</i> PfO-1 (YP_346872; 3e ⁻²³); phage-related baseplate protein of <i>Xanthomonas campestris</i> pv. <i>campestris</i> strain ATCC 33913 (NP_638335; 4e ⁻⁴)
11/+	9192–10184	Baseplate assembly ^c	Baseplate assembly protein of vibriophage VP882 (YP_001039830; 3e ⁻⁹⁸); baseplate J-like protein of <i>P. fluorescens</i> PfO-1 (YP_346873; 6e ⁻⁸⁷); gpJ of phage P2 (NP_046773; 3e ⁻³¹); ORF 32 of <i>Vibrio harveyi</i> phage VHML (NP_758925; 2e ⁻²²); baseplate protein of Φ CTX (NP_490617; 2e ⁻²¹); phage baseplate J-like protein of <i>Thiomicrospira crunogena</i> XCL-2 (YP_390959; 7e ⁻¹⁷)
12/+	10253–10906	Tail protein ^c	Phage tail protein I of vibriophage VP882 (YP_001039831; 4e ⁻⁴⁴); phage tail protein I of <i>P. fluorescens</i> PfO-1 (YP_346874; 1e ⁻²⁰); ORF 33 of <i>Vibrio harveyi</i> phage VHML (NP_758926; 2e ⁻⁷)
13/+	10910–11428	Tail protein ^c	Putative pyocin R2_PP of vibriophage VP882 (YP_001039832; 1e ⁻⁵⁶); ORF 34 of <i>Vibrio harveyi</i> phage VHML (NP_758927; 2e ⁻²²)
14/+	11437–11799	Tail fiber	Hypothetical protein of vibriophage VP882 (YP_001039833; 4e ⁻²⁵); hypothetical protein of <i>Acidovorax avenae</i> subsp. <i>Citrulli</i> AAC00-1 (YP_970083; 3e ⁻⁷)
15/+	11792–12139		Hypothetical protein of vibriophage VP882 (YP_001039834; 6e ⁻¹⁴)
16/+	12148–12525		Hypothetical protein of vibriophage VP882 (YP_001039835; 4e ⁻¹³); tail fiber S protein of phage P1 (YP_006502; 2e ⁻⁹); gpH of phage P2 (NP_046775; 3e ⁻⁶); phage tail fiber protein of <i>Thiomicrospira crunogena</i> XCL-2 (YP_390961; 2e ⁻⁵)
17/+	12532–13365	Tail fiber assembly	No significant hits
18/+	13377–13856		Hypothetical protein HCH_06783 of <i>Hahella chejuensis</i> KCTC 2396 (YP_437832; 3e ⁻⁷); tail fiber assembly-like protein of <i>Ralstonia solanacearum</i> phage RSA1 (BAF52402; 8e ⁻⁵)
19/+	13853–14158		Hypothetical protein of <i>Escherichia coli</i> CFT073 (NP_753380; 9e ⁻⁵); hypothetical protein of <i>Escherichia coli</i> APEC O1 (YP_852555; 1e ⁻⁴)
20/+	14420–15586	Tail sheath ^{b,c}	Phage tail sheath protein of vibriophage VP882 (YP_001039839; 2e ⁻¹⁴⁵); phage tail sheath protein FI-like of <i>Pseudomonas fluorescens</i> PfO-1 (YP_346877; 9e ⁻¹²³); ORF 39 of <i>Vibrio harveyi</i> phage VHML (NP_758931; 3e ⁻²⁸); phage tail sheath protein of <i>Silicibacter</i> sp. strain TM1040 (YP_613279; 2e ⁻²⁵); phage tail sheath protein of <i>Thiomicrospira crunogena</i> XCL-2 (YP_390965; 2e ⁻¹⁶); gpFI of phage P2 (NP_046778; 2e ⁻¹⁴)
21/+	15587–16093	Tail tube	Tail tube protein of vibriophage VP882 (YP_001039840; 4e ⁻³⁷); phage-related contractile tail tube of <i>Xylella fastidiosa</i> 9a5c (NP_298018; 1e ⁻⁷); phage tail tube protein of <i>Thiomicrospira crunogena</i> XCL-2 (YP_390966; 2e ⁻⁶); ORF 40 of <i>Vibrio harveyi</i> phage VHML (NP_758932; 5e ⁻⁴)
22/+	16131–16805	Hypothetical protein ^c	Putative phage-related protein of vibriophage VP882 (YP_001039841; 1e ⁻³⁷); hypothetical protein of <i>P. fluorescens</i> PfO-1 (YP_346879; 2e ⁻²⁶)
23/+	16930–19515	Tail tape measure ^c	Phage-related tail protein of vibriophage VP882 (YP_001039842; 2e ⁻¹⁰⁷); putative tail protein of phage Φ P27 (NP_543099; 1e ⁻³⁹); gpT of phage P2 (NP_046782; 7e ⁻¹⁰); phage tail tape measure protein of <i>Thiomicrospira crunogena</i> XCL-2 (YP_390968; 8e ⁻⁸); ORF 43 of <i>Vibrio harveyi</i> bacteriophage VHML (NP_758934; 1e ⁻⁶)

Continued on facing page

TABLE 1—Continued

ORF no./ orientation ^a	Nucleotide position	Predicted function	Related BLASTP hit(s) (accession no.; E value)
24/+	19516–19929	Tail protein ^c	Phage protein U-like of vibriophage VP882 (YP_001039843; 2e ⁻³⁵); phage protein U-like of <i>P. fluorescens</i> PfO-1 (YP_346881; 1e ⁻³²); F protein of phage 186 (NP_052274; 9e ⁻⁷)
25/+	20107–21135	Tail protein ^c	Phage protein D-like of vibriophage VP882 (YP_001039846; 3e ⁻¹⁰²); phage protein D-like of <i>P. fluorescens</i> PfO-1 (YP_346883; 8e ⁻⁹⁷); ORF 46 of <i>Vibrio harveyi</i> bacteriophage VHML (NP_758937; 6e ⁻²⁶); gpD of phage P2 (NP_046784; 3e ⁻¹⁸); phage protein D-like of <i>Thiomicrospira crunogena</i> XCL-2 (YP_390970; 4e ⁻¹²)
26/+	21276–22031	DNA adenine methyltransferase ^c	DNA adenine methyltransferase of vibriophage VP882 (YP_001039848; 5e ⁻¹⁰⁹); DNA adenine methyltransferase of ΦE125 (NP_53683; 1e ⁻⁹³); DNA adenine methyltransferase of phage F116 (YP_164275; 2e ⁻²²)
27/+	22511–22858		No significant hits
28/-	23336–23794		No significant hits
29/-	23835–24248		No significant hits
30/-	24358–24954		Hypothetical protein of phage P27 (NP_543056; 3e ⁻²¹); hypothetical protein of <i>Escherichia coli</i> E22 (ZP_00728122; 1e ⁻¹⁰)
31/-	25072–25494		No significant hits
32/-	25561–25860		Hypothetical protein of vibriophage VP882 (YP_001039863; 2e ⁻¹¹)
33/-	25853–26617	Partitioning protein ^c	ParA protein of vibriophage VP882 (YP_001039864; 1e ⁻⁶⁵); ORF 58 of <i>Vibrio harveyi</i> phage VHML (NP_758949; 4e ⁻⁵⁵); ParA of <i>Pseudomonas alcaligenes</i> (YP_025341; 3e ⁻⁵¹)
34/+	27075–28637	Protelomerase ^c	Protelomerase of vibriophage VP882 (YP_001039865; 3e ⁻¹⁵⁵); protelomerase of phage N15 (NP_046924; 3e ⁻⁶⁴); protelomerase of ΦKO2 (YP_006606; 7e ⁻⁵⁴); protelomerase of phage PY54 (NP_892077; 2e ⁻⁵⁰); ORF 1 of <i>Vibrio harveyi</i> phage VHML (NP_758894; 1e ⁻¹⁴)
35/-	28613–28900		No significant hits
36/-	29849–32992	Phage replication ^c	Replication protein RepA of vibriophage VP882 (YP_001039868; 0); replication protein RepA of phage PY54 (NP_892081; 1e ⁻¹²²); replication protein RepA of phage N15 (NP_046932; 1e ⁻¹¹³); replication protein RepA of phage ΦKO2 (YP_006615; 2e ⁻¹¹³); ORF 4 of <i>Vibrio harveyi</i> phage VHML (NP_758897; 1e ⁻⁵)
37/-	33292–33996	Prophage repressor	Repressor protein of vibriophage VP882 (YP_001039870; 8e ⁻²³); putative prophage repressor protein of <i>Burkholderia</i> phage BcepC6B (YP_024964; 4e ⁻⁷); repressor protein cl of phage 434 (S32822; 6e ⁻⁶)
38/+	34092–34724	Prophage antirepressor	ORF 7 of <i>Vibrio harveyi</i> bacteriophage VHML (NP_758900; 1e ⁻²⁹); hypothetical protein of vibriophage VP882 (YP_001039871; 5e ⁻²⁹); Cro of phage PY54 (NP_892087; 4e ⁻¹⁸)
39/+	34986–35312		No significant hits
40/+	35312–35842		No significant hits
41/+	35839–36066		No significant hits
42/+	36104–36712	Prophage transcriptional antiterminator	Antiterminator Q of vibriophage VP882 (YP_001039873; 3e ⁻²⁹); antiterminator Q of phage 82 (P13870; 9e ⁻⁹); antiterminator Q of phage PY54 (NP_892088; 1e ⁻⁸)
43/+	36954–37259		No significant hits
44/+	37468–37665		No significant hits
45/+	37790–38425	Hypothetical protein ^c	Hypothetical protein of vibriophage VP882 (YP_001039881; 3e ⁻⁵⁷); ORF 19 of <i>Vibrio harveyi</i> phage VHML (NP_758912; 3e ⁻³⁵)
46/+	38536–38973		Hypothetical protein of vibriophage VP882 (YP_001039882; 8e ⁻¹⁴); ORF 20 of <i>Vibrio harveyi</i> phage VHML (NP_758913; 2e ⁻⁸)

^a +, forward orientation; –, reverse orientation.^b Experimentally characterized.^c Nucleotide similarity to VP882 is <e⁻³.

cluding P2, P1, VHML, and ΦCTX. Six of the ΦHAP-1 ORFs (11, 16, 20, 21, 23, 25) were significantly similar to genes of the inducible prophage from *Thiomicrospira crunogena* XCL-2 (Table 1). Many of the ORF products were similar to proteins encoded in a prophage-like region on the *Pseudomonas fluorescens* PfO-1 genome. All the putative ΦHAP-1 tail proteins shared significant similarity with proteins from the VP882 phage. The tail sheath protein (encoded by ORF 20) was identified from the structural protein profile of ΦHAP-1; the predicted size of the protein (41.7 kDa) is smaller than the expressed size of the protein (43.6 kDa) (Fig. 4).

ORF 26: DNA-methylating protein. ORF 26, which is immediately downstream of the tail genes, putatively encodes a

DNA adenine methyltransferase protein. Methyltransferase genes are believed to methylate phage DNA in order to protect it from host restriction endonucleases (1). This ORF's product has significant similarity to proteins from other temperate phages infecting gram-negative bacteria, including from phage VP882 (5e⁻¹⁰⁹; 72% identity), and the N6 adenine methyltransferases from the *Burkholderia mallei* phage ΦE125 (1e⁻⁹³; 62% identity) and *Pseudomonas aeruginosa* phage F116 (2e⁻²²; 31% identity). Additionally, ORF 26 shares 73% nucleotide identity across 90% of the gene with VP882 and 68% identity over 92% of the gene with ΦE125.

ORFs 33, 34, and 36: genome maintenance module. This group of ORFs is similar in sequence and organization to those

FIG. 4. SDS-PAGE of proteins associated with Φ HAP-1 particles. Molecular-mass standard is in lane 1, with sizes in kilodaltons to the left. Φ HAP-1 proteins are in lane 2, with ORF designations based on peptide sequence to the right. The contrast of the image was digitally enhanced to increase the visibility of the bands.

found in other plasmid-like temperate phages. ORF 33 potentially encodes a partitioning protein (ParA) that was similar to gene products from phage VP882 ($1e^{-65}$; 60% identity), the product of ORF 58 of phage VHML ($4e^{-55}$; 55% identity), and *parA* from *Pseudomonas alcaligenes* plasmid pRA2 ($3e^{-51}$; 53% identity). ParA is part of the system that is responsible for plasmid segregation during cell division (21). ORF 34 is similar to protelomerase genes found in linear plasmid-like phages, including N15 ($3e^{-64}$; 33% identity), Φ KO2 ($7e^{-54}$; 32% identity), and PY54 ($2e^{-50}$; 32% identity). Protelomerases process phage DNA at a specific inverted repeat, resulting in linear phages with covalently closed ends (16). This ORF also shared significant similarity with a gene from VP882 ($3e^{-155}$; 54% identity). ORF 36 encodes a replication protein similar to those in other plasmid-like prophages, such as the *repA* in PY54 ($1e^{-119}$; 29% identity), N15 ($1e^{-112}$; 29% identity), and Φ KO2 ($2e^{-113}$; 29% identity). RepA is a multifunctional replication protein with primase and helicase activities that is found in plasmid-like phages (63). Additionally, these three ORFs

shared significant nucleotide similarity with genes from VP882, with at least 65% identity along 42 to 71% of the gene (Table 1).

ORFs 37, 38, and 42: lysogeny module. Φ HAP-1 contains lysogeny-related genes found in known temperate phages. The gene products include a putative phage repressor (encoded by ORF 37) that is upstream from and in reverse orientation to a conserved phage protein (encoded by ORF 38) and the transcription antiterminator Q protein (encoded by ORF 42). Phage repressors are the regulators of lysogeny by binding to promoter sites that prevent the transcription of lytic genes (51). The antiterminator Q protein is not involved in the lytic-lysogenic decision, but it must accumulate in order to allow the transcription of late lytic genes (32). The ORF 37 product contains a conserved helix-turn-helix DNA binding domain that is similar to a gene product from VP882 ($8e^{-23}$; 31% identity). It also shares similarity with a prophage repressor protein from *Burkholderia* phage BcepC6B ($4e^{-7}$; 26% identity) and the *cI* repressor of phage 434 ($6e^{-6}$; 21% identity). The ORF 38 product is a conserved phage protein similar to the products of ORF 7 of VHML ($1e^{-29}$; 37% identity) and ORF 60 of VP882 ($5e^{-29}$; 38% identity). ORF 38 also shares similarity with *cro* from PY54 ($4e^{-18}$; 26% identity), which competes with *cI* for binding sites for the operator to initiate the lytic cycle (51). ORF 42, encoding the putative transcriptional antiterminator Q, is located 1,380 base pairs downstream from ORF 38. The ORF 42 product is similar to proteins from VP882 ($3e^{-29}$; 43% identity), as well as *q* proteins from PY54 ($1e^{-8}$; 29% identity) and the enterobacterial phage 82 ($3e^{-7}$; 31% identity). No integrase or lytic genes were identified in the Φ HAP-1 genome by similarity searches.

Inverted repeat. Since N15 contains an inverted repeat site that enables the protelomerase to break the phage genome and rejoin it to make the linear telomeres, we searched the Φ HAP-1 genome for an inverted repeat. A 92-base-pair inverted, palindromic repeat was found between the regions encoding the partitioning protein (encoded by ORF 33) and the protelomerase (encoded by ORF 34) (Fig. 5). The end of the repeat was found to be 127 base pairs upstream from the start codon of the gene encoding the protelomerase protein.

Genomic organization of plasmid-like, double-stranded linear prophages. A comparison of the genomic organization of Φ HAP-1 with the genomes of temperate phages that harbor a protelomerase gene (e.g., linear plasmid prophages N15, PY54, and Φ KO2 and vibriophages VHML and VP882) is shown in Fig. 6. There was no nucleotide sequence homology between Φ HAP-1, N15, PY54, Φ KO2, or VHML, but the organization of structural, lysogeny, and replication modules in these phage genomes was strikingly similar. The linear plasmid

FIG. 5. Cartoon of the nucleotide sequence of the Φ HAP-1 92-base-pair inverted repeat found in the DNA region between the partitioning and protelomerase genes. The predicted cut site of the protelomerase is indicated by the dashed line. The sequences below the repeat are the predicted right and left telomeric ends of Φ HAP-1.

FIG. 6. Genomic comparisons of six completed phage genomes that contained a protelomerase gene. KODON was used to construct the gene maps. The genomes of N15, ΦHAP-1, PY54, VHML, and VP882 were collected from GenBank. The location of the protelomerase gene in each genome is denoted by a "P." Patterns were assigned based on functional assignment of the ORFs as indicated in the key.

prophages N15, PY54, and ΦKO2 are longer than ΦHAP-1 and the vibriophages and are the only ones that contain recognizable lytic genes. The nucleotide sequence similarity, with a minimum of 60% identity over at least 80 bases, between ΦHAP-1 and VP882 was seen across the genomes, including in most of the structural, DNA metabolism, and plasmid-like genes, but not in the lysogeny-related genes. VP882 contained genes for a putative transcriptional regulator and exonuclease that are not present in ΦHAP-1.

Integration of ΦHAP-1 in cellular replicons and different phage genomic arrangements. Southern hybridizations of host chromosomal, plasmid, and phage preparations were performed to determine if ΦHAP-1 integrates into the chromosome or exists as an extrachromosomal element. Nucleic acid preparations were restriction digested at unique sites or double digested to confirm assembly. Figure 7 illustrates the different digestion sites for the restriction enzymes based upon two hypothetical structures derived from similarities of phage N15 structure. The undigested DNA preparations hybridized at a molecular weight of 39 kb (Fig. 8, lanes 2, 7, and 12), indicating a lack of integration into the host chromosomal DNA. The BamHI-digested preparations served as a positive control for the presence of phage DNA in the fractions. In the host and plasmid fractions, these hybridized at around the same molecular weight (10 kb), with the fragment in the phage being slightly larger (Fig. 8, lanes 6, 11, and 16). The hybridization patterns of the rest of the host genomic and plasmid digests

were identical, while the phage virion digestion pattern was different (Fig. 8, lanes 3 to 5, 8 to 10, and 13 to 15).

The detection of the two different phage genomic arrangements was verified by PCR analysis of the host genomic, plasmid, and cesium chloride-purified phage preparations using the two form-specific primer sets (Fig. 7). The virion primer set (primers 1 and 2) resulted in an amplicon 2,441 base pairs in length, while the prophage primer set (primers 3 and 4) resulted in an amplicon 2,681 base pairs in length (Fig. 9). The host genomic (Fig. 9, lane 3) and plasmid (lane 5) amplicons were from the prophage primers, with no amplification with the virion primers. For the phage preparation, there was only amplification from the virion primer set (Fig. 9, lane 6). We interpret the similarity of the host genomic and plasmid preparations as being caused by the presence of plasmid DNA in the host genomic preparation.

DISCUSSION

Based on the TEM micrograph showing thick, contractile tails, ΦHAP-1 can be classified as a myovirus. The diversity and number of ΦHAP-1 ORFs encoding tail proteins, including ORFs with products similar to a tail sheath, tail tube, and several baseplate proteins from P2-like temperate myoviruses that have genomes below 50 kb in size, including coliphage P2, VHML, and ΦCTX, support this finding (35, 38, 39). The major structural components of the contractile tails that are

FIG. 7. Schematic representation of the two conformations of the ΦHAP-1 genome. The cut sites of restriction enzymes are indicated by vertical lines. The predicated sizes of fragments are noted to the right of each map. The numbered arrows below each map represent the primers used for PCR analysis. Figures are not drawn to scale. Pt, protelomerase; Pa, ParA; Ts, terminase small subunit; 46, ORF 46 hypothetical protein; cI, ORF 36 prophage repressor, which was used as the probe.

the hallmark of the *Myoviridae* family are the tail tube and tail sheath proteins, both of which are found on the ΦHAP-1 genome. The tail sheath protein, which contracts during infection of the host, was identified by peptide sequencing of a protein band from the ΦHAP-1 proteome expressed on an SDS-PAGE gel. The actual molecular mass of the tail sheath protein agrees with the predicted molecular mass, indicating little posttranslation modification. The tails of ΦHAP-1 particles are longer and have less variation in length than those of the other, related myoviruses P2 and VHML (6, 40). The length of the tail tape measure protein, which was identified in ΦHAP-1 as the product of ORF 23, is believed to be directly proportional to the phage tail length (30). This protein in ΦHAP-1 was 861 amino acids long, which puts its length between those of P2 and VHML.

The capsid-associated proteins were similar to those from lambda-like siphoviruses, such as WOcauB1, PY54, and ΦKO2 (12, 20, 24). The expression and identification of the major capsid and portal proteins on the SDS-PAGE gel confirmed our in silico annotation of these head genes and provided further information on the mature ΦHAP-1 virion. The major capsid protein is typically the most-abundant protein present in mature virions, and so it is highly expressed on SDS-PAGE gels, as is seen in Fig. 4. Sequencing of the peptide fragments from two different-sized protein bands suggests that the ΦHAP-1 major capsid protein is cleaved after translation into two smaller proteins. The sum of the two fragments is 59.9 kDa, which is close to the predicted size of 66.6 kDa for the major capsid protein. Proteolytic cleaving of the viral-coat proteins during assembly occurs in several phages, including PY54 and ΦKO2 (12, 25). The expressed ΦHAP-1 portal protein is 6 kDa larger than the molecular mass that is expected based on

the amino acid sequence, indicating either abnormal migration in the SDS-PAGE gel or posttranslation modification of the protein. The similarity of ΦHAP-1 capsid and tail proteins to those from mitomycin C-inducible phages found in the genomes of the deep-sea chemolithoautotroph *T. crunogena* and the *Pfisteria piscicida*-associated bacterium *Silicibacter* sp. strain TM1040, as well as to marine vibriophage VHML, illustrates the conservation of structural genes in marine prophages (15, 59).

ΦHAP-1 is a double-stranded DNA phage. No *cos* site was found in the genome, but due to the presence of terminase genes, we propose that ΦHAP-1 is packed by the headfull packaging mechanism. Packaging of phages via this mechanism begins from the rolling-circle replication intermediate at a sequence usually known as a *pac* site and proceeds until no more DNA fits into the capsid, at which point the phage DNA is cut and the rest is packaged into the next capsid (7). This results in DNA of different lengths with no specific sequence at the ends of the majority of the packaged phage genomes. These types of tailed phages have circularly permuted genomes; thus, the terminase small subunits are often selected as the arbitrary start points for genomic maps to ease comparison between phages (13, 46). This convention was followed when constructing the ΦHAP-1 genomic map.

The *H. aquamarina* host behaved like a lysogen when treated with the prophage-inducing chemical mitomycin C. Mitomycin C is an antibiotic that inhibits DNA synthesis in bacteria by cross-linking cDNA strands, eliciting the SOS response and causing prophage induction (42). The increase in the number of VLPs in the mitomycin C-treated culture in comparison to the lack of change in the number of VLPs in the control treatment was typical of a lysogenic bacterium.

FIG. 8. Southern gel transfer (right) and PFGE (left) of ΦHAP-1 DNA fractions. Lanes: 1, 8- to 48-kb standard; 2 to 6, host chromosomal DNA (2, undigested; 3, XbaI and Nar I digested; 4, Nar I digested; 5, XbaI digested; 6, BamHI digested); 7 to 11, *H. aquamarina* plasmid DNA (7, undigested; 8, XbaI and Nar I digested; 9, Nar I digested; 10, XbaI digested; 11, BamHI digested); 12 to 16, ΦHAP-1 DNA (12, undigested; 13, XbaI and Nar I digested; 14, Nar I digested; 15, XbaI digested; 16, BamHI digested). Numbers at left are sizes in kilobases.

FIG. 9. Gel electrophoresis of *H. aquamarina* DNA PCR amplicons. Lanes: 1, MidRange Plus DNA ladder, with sizes in base pairs on the left; 2, host chromosomal DNA amplified with primer set 1 and 2; 3, host chromosomal DNA amplified with primer set 3 and 4; 4, *H. aquamarina* plasmid DNA amplified with primer set 1 and 2; 5, *H. aquamarina* plasmid DNA amplified with primer set 3 and 4; 6, ΦHAP-1 DNA amplified with primer set 1 and 2; 7, ΦHAP-1 DNA amplified with primer set 3 and 4.

As ΦHAP-1 is mitomycin C inducible, the presence of several lysogeny-related genes on the ΦHAP-1 genome is not surprising. The prophage repressor and antirepressor are responsible for determining if the phage will enter the lytic or lysogenic cycle. The location and orientation of these two proteins in the ΦHAP-1 genome are characteristic of repressors in the model phage system λ (51). Functional studies of coliphage 434 and PY54, which share similarities with the prophage repressor and antirepressor, respectively, of ΦHAP-1 indicate that these gene products act as repressor and antirepressor in these model systems (25, 47). In ΦHAP-1, the ORF encoding the transcription antiterminator Q protein, which is part of the lytic switch that activates late gene expression, is located downstream from the repressor protein ORFs. This protein is similar to the antiterminator Q protein from enterobacterial phage 82, whose activity was shown during protein activity assays (22). The lack of identifiable lytic genes in ΦHAP-1 is not unprecedented. It is possible that some of the unknown ΦHAP-1 ORFs 27 through 31 could be lysis-associated genes, although functional protein studies would need to be undertaken to determine this. No lysis-associated genes were found in the genomes of the lytic roseophage SIO1 or the pseudotemperate phage ΦHSIC, both of which we isolated from ma-

rine bacteria (46, 56). These marine phages most likely have lytic genes, but they remain unknown due to the bias of genomic databases toward terrestrial and medical phages.

Although no recognizable lysis genes are present in the genome, the Φ HAP-1 genome contained an ORF encoding an N6-DNA adenine methyltransferase. Many tailed-phage genomes contain methyltransferase genes, whose products are believed to methylate phage DNA to protect it from host restriction endonucleases, providing a selective advantage over unmodified phages (1). Phage methyltransferases may also play a role in host pathogenicity through lysogenic conversion (39). For Φ E125, the function of the N6-DNA adenine methyltransferase was confirmed through protein expression (67). In N15 and Φ KO2, adenine methyltransferases were expressed late during lytic growth and were not detected during lysogeny (12, 55). In temperate phage genomes, methyltransferases are located in the lytic or DNA modification modules (39, 40, 55).

The presence of three genes in Φ HAP-1 that are similar to those in linear plasmid-like phages and bacterial plasmids and the absence of an integrase suggest that Φ HAP-1 exists as a linear plasmid. This was also supported by restriction digestion analysis and PCR of the plasmid and phage genomic preparations. Partitioning proteins, known generically as ParA and ParB, ensure that daughter bacteria will contain copies of bacterial chromosomal and low-copy-number plasmids (21). Both ParA and ParB are necessary for stable inheritance of the N15 prophage (53). Although the Φ HAP-1 *parA* was similar to those in the two vibriophages and a bacterial plasmid gene, there was no *parB* homolog. This may indicate that the partitioning mechanism in this phage is different from the ParA-ParB system in coliphage N15. In N15, RepA is a multidomain protein containing conserved primase, helicase, and replication origin sites whose activity is similar to the theta replication mechanism found in some plasmids (54, 63). The replication protein (RepA) of Φ HAP-1 is very similar to those of linear plasmid phages, such as N15. Ravin (54) also found that RepA was active during lytic and lysogenic growth, allowing maintenance of the plasmid state.

Protelomerase genes have been found in a small number of phages, including the temperate linear plasmid siphoviruses N15, PY54, and Φ KO2 and the vibriophages VHML and VP882 (12, 25, 39, 55). In N15, this gene was found to be necessary for the replication of the prophage form, but not the virion form (54). The protelomerase is responsible for breaking and resolving the single-stranded DNA ends into hairpin ends, allowing them to exist as a linear plasmid (16, 24). Although the exact mechanism is unknown, the results of studies with protelomerase from both N15 and Φ KO2 suggest that two protelomerase molecules form a dimer on a 10-base-pair core palindromic inverted repeat. Each molecule is responsible for cutting and rejoining the strands to form hairpin ends (17, 26). The inverted repeats in these linear phages are found about 150 base pairs upstream from the protelomerase gene and are between 42 and 56 base pairs in length. The 92-base-pair palindromic inverted repeat identified in Φ HAP-1 was much longer than those in the linear plasmid prophages. Like the one in PY54, it was also palindromic for its whole length, unlike those in N15 and Φ KO2, which are interrupted by a 2-base-pair insertion. These differences could result in differ-

ences in the binding affinities of the protelomerases, although further experiments are needed.

The results of Southern hybridization and PCR experiments indicate that Φ HAP-1 does not integrate into the host chromosome but exists as a linear episome. An integrated phage would have hybridized with the phage probe at a much-higher molecular weight in the undigested host DNA than in the phage and plasmid DNA fractions. This was not the case in the results of our Southern hybridization experiment, as seen by the presence of the 39-kb Φ HAP-1 genome-sized fragment in all of the DNA preparations in Fig. 8. The linear nature of the Φ HAP-1 phage genome was seen in the phage DNA fraction digested with the unique cutter restriction enzyme, Nar I. If Φ HAP-1 was a circular plasmid, the single restriction cut would linearize the molecule, causing it to migrate as an apparently higher-molecular-weight molecule. In the case of our Southern hybridization results, the target fragment size was the same as predicted by the linear virion restriction maps (Fig. 8).

As a prophage, Φ HAP-1 exists as a linear plasmid. In this arrangement, the genome starts with the protelomerase gene and ends with the gene for the partitioning-protein ParA, most probably due to the activity of the protelomerase. The prophage form was dominant in the host genomic and plasmid DNA fractions, as these cultures were not induced with mitomycin C treatment. This is apparent in the digestion patterns of the host genomic and plasmid DNA, where the hybridized fragments were the same as those predicted by the prophage restriction maps (Fig. 8). Additionally, only the prophage primer set (primers 3 and 4) amplified the host and plasmid DNA. The differences between the host genomic and plasmid fractions in the hybridization strength and amounts of PCR amplicon were indicative of the low concentration of phage DNA present in the whole-host DNA fractions relative to the amount in the plasmid preparations.

In the virion, the Φ HAP-1 genome is a linear, circularly permuted molecule; as such, the terminase genes were used as the start point. The phage DNA used in the hybridization and PCR experiments was isolated from a large-scale mitomycin C induction experiment, and so the virion form dominated. The differences between the results of the restriction digests and Southern hybridization of the phage DNA in comparison to those of the host genomic and plasmid DNA support this arrangement. In the PCR experiment, only the virion primer set (primers 1 and 2) amplified phage DNA. These findings are similar to what was seen in the results of the restriction digest experiments for other linear plasmid phages, including N15, PY54, and Φ KO2 (12, 25, 55).

Φ HAP-1 shares genetic characteristics with the other phages that contain a protelomerase gene, the N15-like linear plasmid phages N15, PY54, and Φ KO2 and two marine vibriophages, VP882 and VHML. The overall genomic organization of the functional modules was similar across all six phages, with packaging, structural, and phage metabolism genes present. Conservation of functional gene groups, even if the genes themselves are not similar, is common among temperate tailed phages, including those from the marine environment (9, 44). This conservation supports Botstein's modular theory of phage evolution, where evolution occurs through the exchange of groups of functional genes (modules) between phages, and these modules select for optimal biological activity in a partic-

ular environment (8). In ΦHAP-1, genes within the modules share similarity with those of a variety of siphoviruses and myoviruses. This mosaicism supports what is seen in other marine phages, such as the prophage of *Thiomicrospira crunigena* XCL-2 and vibriophages K139, VP16T, and VP16C (44, 59).

Phages that contain a protelomerase, with the exception of VP882 and VHML, which have not been characterized on that level, exist as stable plasmid prophages. It is possible that a plasmid-like prophage conformation could be advantageous to the phage, not requiring the constraints that a chromosomally integrated temperate phage possesses. That is, a plasmid-like prophage could be transferred by the processes of conjugation and transformation. Like N15, PY54, and ΦKO2, ΦHAP-1 exists in two different linear plasmid forms. While ΦHAP-1 and these phages are similar on the protein level, there is no significant nucleotide homology. The nucleotide similarity of 20 ΦHAP-1 genes with genes from VP882 and the similar G+C content of the phages suggest that our phage is more closely related to the vibriophages than to N15.

There are differences in the occurrence and organization of genes involved in lysogeny and lysis between ΦHAP-1, N15-like phages, VP882, and VHML. The N15 phage has three immunity regions, of which one is similar to the λ lysogeny module, one is for lysogenic conversion, and one is an antirepressor region similar to those from phages P1 and P4 (34, 53). Both ΦHAP-1 and VP882 only have a λ-type lysogeny region, while VHML also has an antirepressor similar to one from phage Φ80 (39). The coding region between the protelomerase and *repA* genes in ΦHAP-1 encodes a small hypothetical protein. In VP882 and VHML, there is a transcriptional regulator between the two genes, while N15 has an antirepressor immunity region located there. Additionally, the location of the N6 adenine methyltransferase gene, near the lysis module, is different from its location in ΦHAP-1 and the vibriophages (Fig. 6).

These findings suggest that N15 and ΦHAP-1 diverged from a common ancestor, while recent divergence may have occurred between ΦHAP-1, VP882, and, to a lesser extent, VHML. Since *Halomonas* and *Vibrio* species are cosmopolitan in the ocean, it is tempting to hypothesize that their respective phages may have encountered each other in the environment, resulting in gene transfer between them. However, it should be noted that our knowledge of the VP882 and VHML systems is limited. VHML has only been sequenced, and there is limited knowledge of the host-phage system, which has since been lost (39, 40). The genome of the *Vibrio parahaemolyticus* phage VP882 has not yet been published, and thus, no information about the host-phage system is available in the literature.

The presence of the protelomerase gene in the genomes of six phages from different hosts and environments indicates that linear plasmid-like prophages may represent a common strategy for lysogeny. However, when the ΦHAP-1 protelomerase was searched against the global ocean sequencing project metagenomic data from the CAMERA database and the additional metagenomes found in the SCUMS database, no significant alignments were found. This lack of similarity to the protelomerase gene could be a result of insufficient sampling or may indicate that protelomerases are not abundant in the environment.

The genome of the temperate *H. aquamarina* phage ΦHAP-1 is an important addition to the growing number of marine prophages that have been characterized to date. To our knowledge, ΦHAP-1 is the first marine temperate phage characterized to have behaved like a telomeric, linear plasmid-like phage. Combining traditional host-phage characterization with molecular studies in this system has provided insight into the phage's genome structure.

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