Vibrio harveyi siphophage 1 (VHS1) is a tailed phage with an icosahedral head of approximately 66 nm in diameter and an unornamented, flexible tail of approximately 153 nm in length. When Vibrio harveyi 1114GL is lysogenized with VHS1, its virulence for the black tiger shrimp (Penaeus monodon) increases by more than 100 times, and this coincides with production of a toxin(s) associated with shrimp hemocyte agglutination. Curiously, the lysogen does not show increased virulence for the whiteleg shrimp (Penaeus [Litopenaeus] vannamei). Here we present and annotate the complete, circular genome of VHS1 (81,509 kbp; GenBank accession number JF713456). By software analysis, the genome contains 125 putative open reading frames (ORFs), all of which appear to be located on the same DNA strand, similar to the case for many other bacteriophages. Most of the putative ORFs show no significant homology to known sequences in GenBank, but notable exceptions are ORFs for a putative DNA polymerase and putative phage structural proteins, including a portal protein, a phage tail tape measure protein, and a phage head protein. The last protein was identified as a component of the species-specific toxin mixture described above as being associated with agglutination of hemocytes from P. monodon.

Vibrio harveyi siphophage 1 (VHS1) is a tailed bacteriophage with an icosahedral head of approximately 66 nm in diameter and an unornamented tail of approximately 153 nm in length (26). It has a circular double-stranded DNA genome of approximately 80 kbp (26). It produces initially clear lytic plaques on lawns of Vibrio harveyi 1114GL. However, after several days of incubation, colonies of lysogens appear within the formerly clear plaques (14). These lysogens produce VHS1 spontaneously upon subculture, without the need for induction of a lytic cycle. Since the phage is carried in the lysogens as an episome, cured isolates can be obtained at high rates by isolation of single colonies upon subculture (14). Random clones representing approximately 25% of the VHS1 genome were previously sequenced and deposited in GenBank (26).

Vibrio harveyi 1114GL (VH0) that has been lysogenized by VHS1 (VH1) is >100 times more lethal to the black tiger shrimp than VH0 (13, 14). In addition, culture supernatant solutions from VH1 are highly toxic for the black tiger shrimp (Penaeus monodon) but not for the whiteleg shrimp (Penaeus [Litopenaeus] vannamei) (13). Comparison of mass spectrometry data from protein bands originating from semipurified fractions of these toxic supernatant solutions showed no significant homology to protein data (direct and deduced) in existing databases (13), particularly for Vibrio species. Since no significant homology was found in the Vibrio database, it was suggested that the toxins might have originated from the VHS1 genome. A number of other toxins are known to originate from phage genomes in lysogenized bacteria, including a toxin proposed to originate from a Vibrio harveyi Myoviridae-like (VHML) phage reported to lysogenize a Vibrio harveyi isolate from Australia (9, 20, 22, 23). The complete genome sequence of VHML (approximately 40 kbp) has been reported (22). Here we present the complete genome sequence of VHS1, the second known bacteriophage that enhances the virulence of Vibrio harveyi for giant tiger shrimp.

MATERIALS AND METHODS

VHS1 propagation and preparation. Vibrio harveyi 1114GL type 1 (VH1) infected with VHS1 spontaneously produced VHS1 in the supernatant culture medium after overnight incubation at 30°C with shaking at 250 rpm (26). Cultures were centrifuged to remove bacterial cells and cell debris. The supernatant solution was filtered sequentially through 0.45-µm and 0.2-µm disposable membrane filters (Sartorius), and the presence of viable VHS1 particles was confirmed by dot plaque assays on lawns of strain 1114GL. As previously described (14, 26), the solution was precipitated by addition of polyethylene glycol 6000 (PEG 6000) and then subjected to ultracentrifugation at 100,000 × g for 4 h to pellet VHS1. The pellet was resuspended in phosphate-buffered saline (PBS) and layered over a discontinuous Urografin gradient (10 to 40%), followed by centrifugation at 100,000 × g for 4 h at 4°C. VHS1 was located at the 20 to 30% interface. Purified intact VHS1 phage particles were treated with DNase I and RNase before washing and extraction of DNA with QiAamp DNA minikit (Qiagen, Hilden, Germany) in preparation for genome sequencing. Purified virions were negatively stained as previously described (26) and examined by transmission electron microscopy (TEM) using a Hitachi H-7100 electron microscope equipped with a Gatan ES500W Orius model 782 charge-coupled device (CCD) camera that had been calibrated by the installation engineer. Using this setup with negatively stained T7...
phage at 100 kV, the mean head diameter was 59 ± 3 nm, compared to that of approximately 60 nm given in the VIIIth Report of the ICTV (10).

**DNA sequencing.** DNA sequencing was carried out by Macrogen Inc. (Seoul, South Korea), using Roche 454 technology. The seven resulting contigs were joined by primer walking and PCR amplification with primers designed from the ends of the various contigs. All postcontig sequencing was done by Macrogen and was carried out on both strands of the submitted DNA fragments. In summary, all final sequences were based on complete agreement between sequences of cDNA strands. In cases of any disagreement between the two sequences or between new sequences and VHS1 sequences previously deposited in GenBank, additional sequencing reactions were carried out, again on both strands, to obtain the final consensus sequence (i.e., at least three of four sequences were identical).

After obtaining the full sequence, the sequence was subjected to analysis of predicted restriction enzyme digest fragments, using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/), for EcoRI relative to the lambda DNA-HindIII digest marker for comparison to actual agarose gels of digests obtained previously using this enzyme with the same marker (26).

**Sequence analysis.** (i) Putative ORFs. After gap closing and assembly using CAP3 software (12), the VHS1 sequence was annotated ab initio by three Web server predictors, specifically Zcurve (8), GeneMarkS (1), and EasyGene (17), and by three locally run predictors, specifically MetaGene (21), Genevise v. 2 (2), and Glimmer3 (7), using default parameters. All polypeptides from 2,427 viral genomes (downloaded on 14 June 2010 from GenBank) were used as the protein database for Genevise, while a minimal length of 90 bp and GenBank genetic code table 11 were used from GenBank) were used as the protein database for Genewise, while a minimal length of 90 bp and GenBank genetic code table 11 were used for Glimmer3. Glimmer3 also requires a probability model of coding bias of approximately 60 nm given in the VIIIth Report of the ICTV (10).

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The sizes of the VHS1 genome, head, and tail are consistent with those previously reported for 20 isometric-head marine siphophages of *Vibrio parahaemolyticus* (i.e., mean sizes for genomes, heads, and tails of 88 ± 36 kbp, 78 ± 16 nm, and 164 ± 26 nm, respectively) (5). The ratio of genome size (kbp) to head diameter (nm) ranged from 0.54 to 1.89 (mean = 1.01 ± 0.37) for these phages, with the lowest ratio for isolate LH3a (genome size of 120 kbp and head size of 65 nm). This range correlated with the ratio of 0.83 for VHS1, which is similar to those of five other phages (0.78 to 0.83) with genome sizes ranging from 120 to 122 kbp. In summary, the morphology of VHS1 is similar to that of other siphophages of marine *Vibrio* species and distinct from other *V. harveyi* siphophages recently reported from Thailand (28) and India (31, 32), based on the combination of morphology and genome size. The relationship of VHS1 to *V. harveyi* siphophages recently reported from Australia (6) is uncertain because their genome sizes were not given.

**VHS1 sequence.** Seven contigs were obtained by pyrosequencing (Macrogen). These were combined with previously existing records of VHS1 genome sequences (approximately 20 kbp) in GenBank (ac-
cession no. AF465603, AF480606, AF480607, AF480608, AF480609, AF480611, AY579218, AY579219, AY579221, and AY579222), using CAP3 software. The initial process yielded 79,780 bp from the estimated total VHS1 genome of approximately 80 kbp. The remaining gaps between contigs were filled by primer walking until a single long contig was obtained. Using primers designed from each end of this continuous fragment, a 2.5-kb fragment was amplified. After cloning and sequencing, it linked the two ends and closed the DNA circle, yielding a molecule of exactly 81,509 bp (GenBank accession no. JF713456). Except for the seven contigs obtained by pyrosequencing, all other segments were sequenced at least twice on both strands. When this sequence was subjected to NEBcutter V2.0 for prediction of restriction enzyme digest fragments obtained using EcoRI, good agreement was obtained between the predicted gel and the actual gel obtained (Fig. 2). This supported the correctness of the sequencing results. A complete list of the EcoRI digestion fragments and their locations is given in Table S1 in the supplemental material.

VHS1 nucleic acid sequence analysis. The whole nucleotide sequence of VHS1 was used for a BLASTn search of GenBank. This yielded 10 hits for existing VHS1 sequences in GenBank (see the introduction), 3 hits for bacterial pyruvate phosphate dikinase (e.g., \(E = 3 \times 10^{-11}\) for 132/186 identities for \(Bacteroides\ fragilis\ CR626927\), and 93 hits for RecA proteins from many sources (e.g., \(E = 6 \times 10^{-7}\) for 63/79 identities of \(Desulfovoccus\ oleovorans\ CP000859\). There were no other significant hits, even with VHML (GenBank accession no. AY133112), the only other fully sequenced bacteriophage reported for shrimp-pathogenic \(Vibrio\ harveyi\) (22).

RNA genes. Neither rRNA nor tRNA genes were detected using RNAmmer (16) and tRNAscan (18), respectively.

Origin of replication. In order to number the putative ORFs of VHS1 in a nonarbitrary manner, we identified the putative area of the origin of replication as indicated in Fig. 3. This was predicted based on the presence of an unusually high AT sequence bias across 350-bp frames, on predicted secondary structure formation similar to origins of replication for some other bacteriophages (predicted by the Vienna RNA Web server) (29), and on the lack of putative ORFs in that AT-rich area.

Protein-encoding ORFs. Altogether, the six predictors used gave a total of 147 unique predicted ORFs, but only 123 ORFs were supported by at least two predictors. Of the 24 single-predictor-supported ORFs, we found two additional ORFs (047 and 060) with EST support (Table 1). Also shown in Table 1 are seven ORFs encoding putative phage structural proteins or DNA-related enzymes (see Table 2). Sequence identity between EST and the VHS1 genome was 98 to 100%. The presence of EST that spanned more than one ORF suggested that VHS1 produces polycistronic mRNA. Altogether, we concluded that the total number of putative VHS1 ORFs was 125, all transcribed from the positive strand (Fig. 3; see Table S2 in the supplemental material). Among these 125 ORFs, 27 were supported by the presence of homologues in other genomes, and 16 ORFs were supported by the presence of Pfam domains (see Table S2). The start codons of 14 ORFs were moved to the nearest putative starting site to reduce the number of overlapping ORFs. The ORFs were numbered in order from 001 to 125, beginning with the first ORF after the putative origin of replication.

Comparison of codon usage between VHS1 and \(Vibrio\ harveyi\) ATCC BAA-1116. Since the full genome sequence of \(V.\ harveyi\) 1114GL is not known, we chose the full sequence of \(V.\ harveyi\) ATCC BA-1116 to make a codon usage comparison. The VHS1 genome had a G+C content of 46.87%, which was ~1.4% different from that of \(Vibrio\ harveyi\) ATCC BAA-116 (G+C content = 45.44%). Small differences in G+C content of other host–phage pairs have also been reported (30). Despite the small difference in G+C content between the two genomes, the majority of the preferred codons in the VHS1 genome had G or C at the third position (G/C ending), while the majority in the \(V.\ harveyi\) BAA genome had A or T at the third position (A/T ending). In addition, it was found that 8 of 18 amino acids (aa) (44%) (excluding nondegenerate codons for Met and Trp) were different between the two genomes. Although this suggests that VHS1 and its host may have quite different codon usage, it can be argued that codon usage for \(Vibrio\ harveyi\) ATCC BAA-1116 and \(V.\ harveyi\) 1114GL may not be similar. To counter this argument, a recent publication suggests that codon usage among \(Vibrio\) species is quite similar (33). Since it has been suggested that the similarity of codon usage patterns is relatively high between well-adapted phages and their \(Vibrio\) hosts and relatively lower for less-well-adapted phages (3, 30), our analysis suggested that \(V.\ harveyi\) 1114GL might be a relatively recently acquired host of VHS1.

Functional classification of putative ORFs. Of the total of 125 putative ORFs, only 27 gave significant homology to known protein sequences in GenBank, and 17 of these had homologues in at least one member of the order \(Caudovirales\) (\(E < 10^{-3}\)) (Table 2; see Table S1 in the supplemental material). Of these 27 ORFs, 12 encoded hypothetical proteins of unknown function. Of the re-

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\(a\) The ORFs shown include those encoding putative homologues for phage structural proteins (ORF 053, 061, 091, and 104) and DNA-related enzymes (ORFs 174, 077, and 103), as well as two ORFs (047 and 060 [marked with asterisk]) for which there was only a single software predictor. See Table 2 for further details on ORFs.

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remaining 15, there were 4 encoding putative phage structural proteins, including ORFs 053 (head protein), 061 (tail tape measure protein), 091 (terminase), and 104 (portal protein). Four similar proteins have been reported for VHML, another phage of *V. harveyi* that is virulent for shrimp (22, 24), but they shared no significant homology with their counterparts in VHS1. There were no hits for other structural proteins, such as tail proteins. The DNA polymerase (ORF 103) was identified previously (26) and was used for phylogenetic comparison among phages (25). Other putative proteins associated with nucleic acids were encoded by ORFs 107 (DNA methyltransferase [DAM]), 071 (nucleoside NTPase), and 082 (RecA). The methyltransferase may be associated with phage defense against the host DNA restriction enzyme system (15). A DAM has also been reported for VHML (24), but it was suggested that it might be associated with toxicity of its lysozyme (15). 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MASCOT search of mass spectrum data against VHS1 ORF 053
Sequence Coverage: 64%
1 MPQPKAENII QKADMGL QLQD INNGGTLTA QSDRFIEVDP DQPTLGNAR
51 TIAMNSFQOE INRIGFQOR LHAAPQDGDV LPDNQ5SKPK TSKIELSTE
101 VMAVE6FLPKVP VEDINMRGS INPPGDRPSG APTDLRILRM AERAATDLDE
151 LARQDGGAX DPYALATDGW LKRTTSHIVN HQNAD1STM FKNGVKTLP
201 RYHRNSAMR HFSVSAQVNE YSDKLSSSRE TALGDSKQTL DQNGYSGVP
251 RGVPLMSETQ GILANPQLI MGIQREISIE YEKINRAREF VIVLTARIDT
301 QIDEQANVQK YINIAS

FIG 4 Details of MASCOT results for mass spectrum data matches to two putative VHS1 ORFs. Peptides from the mass spectrometry analysis that match the deduced amino acid sequence of VHS1 are indicated in bold, gray, underlined type.

ACKNOWLEDGMENTS
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