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Spatial Distribution of Viruses Associated with Planktonic and Attached Microbial Communities in Hydrothermal Environments

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Viruses play important roles in marine surface ecosystems, but little is known about viral ecology and virus-mediated processes in deep-sea hydrothermal microbial communities. In this study, we examined virus-like particle (VLP) abundances in planktonic and attached microbial communities, which occur in physical and chemical gradients in both deep and shallow submarine hydrothermal environments (mixing waters between hydrothermal fluids and ambient seawater and dense microbial communities attached to chimney surface areas or macrofaunal bodies and colonies). We found that viruses were widely distributed in a variety of hydrothermal microbial habitats, with the exception of the interior parts of hydrothermal chimney structures. The VLP abundance and VLP-to-prokaryote ratio (VPR) in the planktonic habitats increased as the ratio of hydrothermal fluid to mixing water increased. On the other hand, the VLP abundance in attached microbial communities was significantly and positively correlated with the whole prokaryotic abundance; however, the VPRs were always much lower than those for the surrounding hydrothermal waters. This is the first report to show VLP abundance in the attached microbial communities of submarine hydrothermal environments, which presented VPR values significantly lower than those in planktonic microbial communities reported before. These results suggested that viral lifestyles (e.g., lysogenic prevalence) and virus interactions with prokaryotes are significantly different among the planktonic and attached microbial communities that are developing in the submarine hydrothermal environments.

In deep-sea and shallow submarine hydrothermal environments, a diversity of microbial communities associated with steep physical and chemical gradients has been intensively investigated using both culture-dependent and culture-independent microbiological techniques (3, 27, 42, 44, 46, 51, 63). These chemosynthesis-dominated ecosystems are primarily sustained by chemolithoautotrophic and methanotrophic prokaryotes that are planktonic, attached to vent surfaces, or symbiotically associated with macrofauna endemic to the vent (28, 40).

Viruses are now recognized to be significant components of marine surface ecosystems (58, 75). It has been suggested that they regulate microbial cellular and functional abundances and, consequently, affect global nutrient and energy cycles (13). Viruses can also mediate lateral gene transfers and drive the coevolution between viruses and hosts (55, 72). However, in contrast to the extensively studied marine surface environments, viral functions and ecology in deep-sea hydrothermal environments remain poorly characterized. To our knowledge, only a few studies on viral abundance and distribution in deep-sea hydrothermal vents have been reported (19, 29, 48, 76). Additionally, several viruses have been isolated from prokaryotic hosts obtained from deep-sea vents, and their molecular biological and genomic traits have been characterized (17, 18, 31, 32, 69, 71, 77). Viral abundance and virus-host interactions in shallow marine hydrothermal environments are not as well understood as those in deep-sea vents (35, 37).

Based on the abundances of viruses in the hydrothermal vent fluids, diffusing-flow fluids, plume waters, and vent waters where macrofaunal colonies are endemic (29, 48, 76), Ortmann and Suttle (48) hypothesized the potentially high, virus-mediated mortality of the microbial populations in the vicinity of hydrothermal vent fluids and the low viral production in the plume water. However, using induction assays, Williamson et al. (74) demonstrated that lysogenic virus-host interactions dominated in the diffusing-flow fluids rather than in the ambient seawater. Additionally, a number of novel genes were identified in the inducible prophage communities using a metagenomic analysis. Although these studies examined the distribution of viral abundance in the various planktonic microbial habitats in deep-sea hydrothermal environments, the relationships among viral abundance, the host prokaryotic community composition, and geochemical conditions remain unclear. Additionally, viral abundance and production in attached microbial communities have not yet been explored. These communities potentially represent those with the

Received 9 August 2011 **Accepted** 12 December 2011
Published ahead of print 30 December 2011
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Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.06491-11

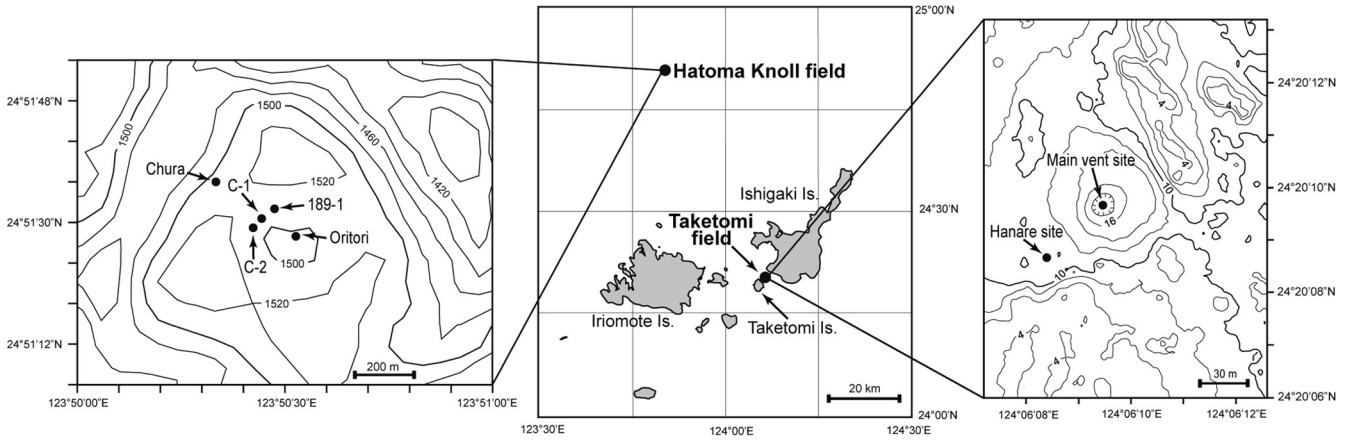

FIG 1 Location of the deep-sea hydrothermal field of the Hatoma Knoll and the shallow submarine hydrothermal vent field off Taketomi Island.

highest biomass and productivity of the chemosynthetic prokaryotic communities in deep-sea hydrothermal environments.

In this study, the viral and prokaryotic cellular abundances in various planktonic and attached microbial habitats of deep-sea and shallow submarine hydrothermal environments were investigated. The geochemical properties and prokaryotic community compositions were characterized by fluid chemistry analysis and 16S rRNA gene clone analysis, respectively. The ratio between the abundances of virus-like particles (VLPs) and prokaryotic cells (the VLP-to-prokaryote ratio [VPR]) provides important insight into viral productivity and the various ecological roles of viruses in the different microbial habitats of submarine hydrothermal environments.

MATERIALS AND METHODS

Study area. The deep-sea hydrothermal field of the Hatoma Knoll is located at the southern Okinawa Trough, Japan (24°51′30″N, 123°50′30″E, at a depth of 1,457 m) (20). Of the many hydrothermal vent sites discovered in this field, five hydrothermal sites named the C-1, C-2, 189-1, Oritori, and Chura vent sites were the focus of our studies (Fig. 1). The C-1, C-2, and 189-1 sites are located on the north side of the crater on top of the Hatoma Knoll, and the Oritori site is located at the center of the crater. Hydrothermal fluids with high temperatures of approximately 300°C were venting from active chimneys in these areas. At the Chura site, which is located on the northwest of the crater, hydrothermal fluids were characterized by a moderate temperature of less than 200°C.

The shallow submarine hydrothermal vent field is located off Taketomi Island, Okinawa, Japan (24°20′09″N, 124°06′10″E, at depths of 13 to 20 m) (Fig. 1) (16, 43). The main vent site is located in the deepest part of the basin (at a depth of 20 m). The seafloor around the main vent site was covered with dense white microbial mats (microbial mat site). The Sunachi hydrothermal vent is located adjacent to the main vent site. The Hanare vent on the coral reef seafloor is approximately 50 m southwest from the main vent (approximately 10 m of water depth), and abundant gas bubbles constantly spout from this vent.

Sample collection and subsampling. In the Hatoma Knoll field, samples were obtained using the *Hyper-Dolphin* remotely operated vehicle (ROV) during the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) NT08-13 (July 2008) and NT09-11 (July and August 2009) cruises on the R/V *Natsushima*. Water that was overlying macrofaunal colonies (colony water samples) was collected using both a gas-tight fluid sampler, the water hydrothermal-fluid *Atsuryoku* tight sampler II (WHATS II) (50), and a plastic bag sampler, as described previously (47). The *in situ* temperatures of the hydrothermal fluids and the mixing water were measured with a self-recording thermometer on the WHATS II sampler. The hydrothermal fluid and seawater samples were obtained in duplicate and placed into two WHATS II gas-tight bottles (150 ml in volume). One of the bottles was used for analysis of the gas components, and the other was used for analysis of soluble chemical components and microbiological investigations. The bottle devoted to gas chemistry was processed onboard within a few hours after recovery using a high-vacuum line. The chimney structures were divided into surface layers and interior structures, as described previously (61). The ventral setae of galatheid crabs (*Shinkaia crosnieri*), which are covered with epibiotic microorganisms, were removed with scissors (70). The nests of annelid polychaetes were collected from the exterior of the chimney structures.

In the Taketomi field, samples were collected in February 2010 by scuba diving, as described previously (23). Water samples were obtained using gas-tight acrylic syringes. Background seawater (nonhydrothermally influenced) was collected from a coral reef site (at a distance of 5.6 km south from the hydrothermal site; 24°17.93′N, 124°06.02′E, at a depth of 10 m). Microbial mats at the main vent site were collected gently with gloved hands and immediately transferred to a plastic zipper bag while in the water. The *in situ* temperatures of the sampling sites were measured with a self-recording thermometer.

For prokaryotic cell and VLP counts, the subsamples from chimney structures, galatheid setae, and polychaete nests (microbial mats) were stored at −80°C, after fixation with 3.7% formaldehyde in filtered (pore size, 0.02 μm) seawater or modified SM buffer (50 mM Tris-HCl, 10 mM $MgSO_4 \cdot 7H_2O$, 0.01% gelatin) supplemented with 3% (wt/vol) NaCl. The water samples for the VLP counts were filtered through a 0.22-μm-pore-size Millex-GP filter (Millipore, Bedford, MA) and stored at −80°C, after they were frozen in liquid nitrogen. The water samples for the prokaryotic cell analysis were fixed by adding formaldehyde (final concentration, 3.7%) and stored at −80°C. The subsamples for DNA extraction were stored at −80°C. The prokaryotic cells in the water samples were collected on 0.22-μm-pore-size cellulose acetate filters and stored at −80°C. The samples used in this study are summarized in Table 2.

Chemical characterization of water and gas components. Immediately after sample recovery, the concentrations of SiO_2 and NH_4^+ in the sample fluids were analyzed. This procedure was performed onboard for the Hatoma Knoll samples or in the onshore laboratory near the port for the shallow submarine hydrothermal vent samples. SiO_2 levels were measured by spectrophotometry using the silicomolybdate complex method. NH_4^+ concentrations were measured by phenol blue colorimetry. The analytical precision for both SiO_2 and NH_4^+ was estimated to be within 7%.

The concentrations of H_2, CO_2, CH_4, and H_2S were determined using the extracted gas components by gas chromatography using a pulse ion-

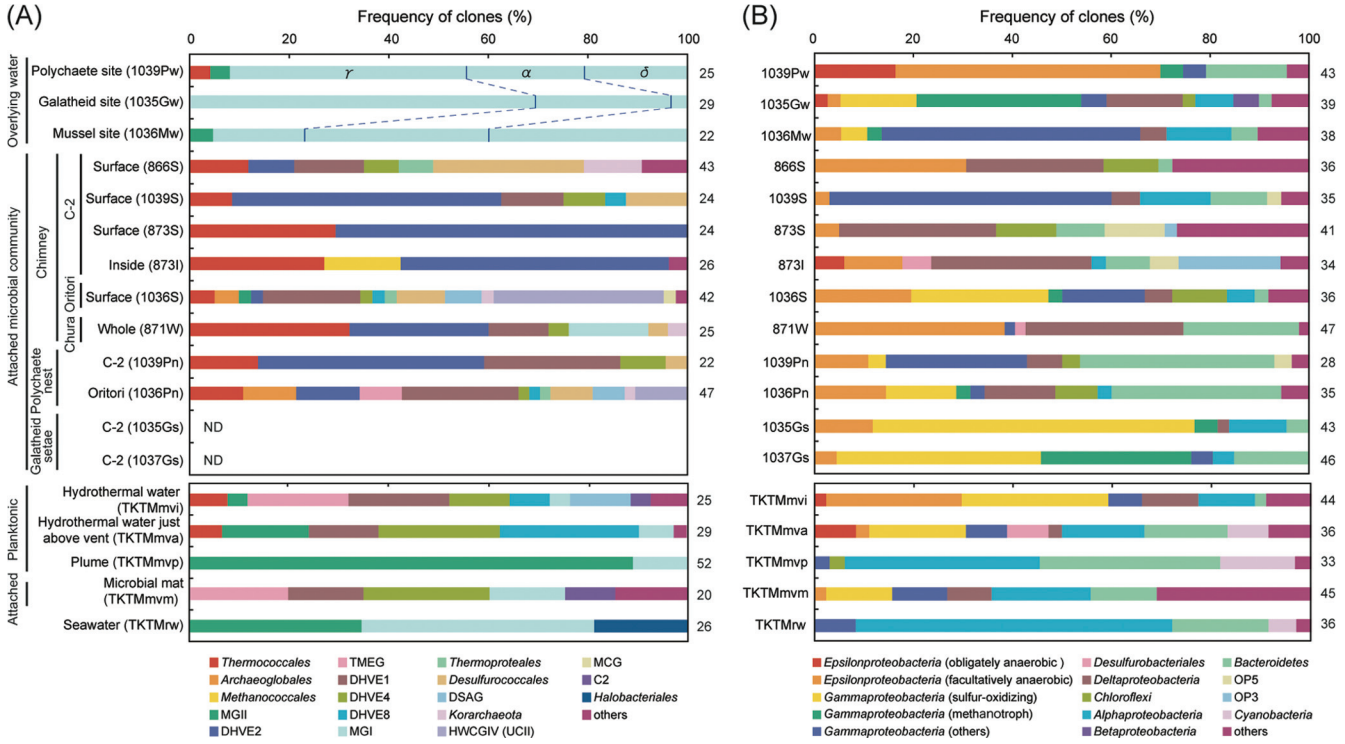

FIG 2 Microbial community structures based on the archaeal (A) and bacterial (B) 16S rRNA gene clone sequences of the Hatoma Knoll (upper) and the Taketomi (lower) hydrothermal systems. The numbers on the right of each row show the sequenced numbers. ND, not determined; DSAG, deep-sea archaeal group; MCG, miscellaneous crenarchaeotic group. In the macrofaunal colony waters of the Hatoma Knoll field, the MGI subgroups (alpha, gamma, and delta) are shown using Greek letters.

ization detector (PID; GL Science, Tokyo, Japan). The concentrations were determined within a 5% error margin.

Prokaryotic cell and VLP abundances. Detachment of cells and VLPs from chimney structures, microbial mats, and macrofaunal tissues was performed as follows: subsample suspensions fixed in seawater or modified SM buffer were shaken at maximum speed using a ShakeMaster BMS-A15 apparatus (Bio Medical Science, Tokyo, Japan) for 1 min and centrifuged for 1 min at 800 × *g*. The supernatants were then used for prokaryote and virus counts.

For the prokaryotic cell counts, each sample was filtered through a 0.2-μm-pore-size Isopore membrane filter (Millipore, Bedford, MA), after staining with 4′,6-diamidino-2-phenylindole (DAPI). VLP counts were performed as described previously (66). One milliliter of each environmental sample was stained with 25 μl of 100× SYBR gold (Invitrogen, Carlsbad, CA) for 15 min and filtered onto a 0.02-μm-pore-size Anodisc filter (GE Healthcare, Piscataway, NJ). The cells and VLPs on these filters were counted with an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan) at a magnification of ×1,500. At least 400 viral particles per sample were counted in more than 20 randomly chosen fields in triplicate for both the prokaryotic cell and VLP counts.

DNA extraction. Microbial DNA was extracted from chimney structures, polychaete nests, galatheid setae, microbial mats, and microbial cells filtered from the water samples using an UltraClean MegaPrep soil DNA isolation kit or UltraClean soil DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions.

Quantitative PCR for small-subunit (SSU) rRNA genes. Quantification of the archaeal and whole prokaryotic 16S rRNA genes using primer and probe sets Arch349F-Arch806R-Arch516F and Uni340F-Uni806R-Uni516F, respectively, was performed according to a previously published method (60) with some modifications, as follows. As described previously, we used a prepared mixture of qPCR Quick GoldStar Mastermix Plus (Eurogentec, Seraing, Belgium), and concentrations for primers and probes were modified following the manufacturer's instructions (45). These primers have been applied in many studies targeting hydrothermal environments as well as sedimentary habitats (41, 46, 47).

Construction of 16S rRNA clone libraries and sequence analysis. Bacterial and archaeal 16S rRNA genes were amplified by LA *Taq* polymerase (Takara Bio, Otsu, Japan) with primer sets Bac27F-Bac927R (30) and Arch21F-Arch958R (11) for the bacterial and archaeal SSU rRNA genes, respectively. For the bacterial 16S rRNA gene amplification, the PCR program consisted of an initial preheating step for 1 min at 96°C, followed by 25 to 35 cycles of 96°C for 25 s, 53°C for 45 s, and 72°C for 90 s and a final extension at 72°C for 7 min. For the archaeal 16S rRNA gene amplification, 25 to 45 cycles of PCR amplification, after preheating for 1 min at 96°C, were performed using the following conditions: 96°C for 25 s, 50°C for 45 s, and 72°C for 90 s and a final extension at 72°C for 7 min. The amplified PCR products were cloned into the pCR2.1 vector using the TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The inserts in the vectors were directly sequenced using M13 primers with a BigDye (version 3.1) sequencing kit (Applied Biosystems, Carlsbad, CA). Approximately 900 bp of 16S rRNA gene fragments was assembled and analyzed using Genetyx-MAC/ATSQ software (Genetyx Co. Ltd., Tokyo, Japan). The number of sequenced 16S rRNA clones per sample is shown in Fig. 2. Sequences showing ≥97% identity were assigned to the same phylogenetic clone type (phylotype). Each of the 16S rRNA gene phylotypes was inserted into the reference tree using the parsimony insertion algorithm in ARB software (34) and phylogenetically classified into specific taxonomic units using Hugenholtz's small-subunit rRNA sequence database and phylogenetic classification, with minor modifications (24). To assess similarities and differences among 16S rRNA gene communities, we used Jackknife environment cluster analysis in the UniFrac program (http://bmf.colorado.edu/unifrac/) (33). The phylogenetic tree constructed from the representative phylotypes in the clone libraries using the neighbor-joining method with the ARB soft-

TABLE 1 Physicochemical properties of the waters in the Hatoma Knoll field and the Taketomi field

Hydrothermal field	Sample	Sample description	Sample identifier	Temp (°C)	Concn					
					SiO_2 (μM)	H_2 (μM)	CH_4 (μM)	CO_2 (mM)	NH_4^+ (μM)	H_2S (μM)
Hatoma Knoll	Hydrothermal fluid	C-2 vent site	1035W1	320	6,996	198	14,300	1,770	6,621.9	38,250
	Overlying water	Polychaete site	1039Pw	8	205	0.306	40.9	1.95	78.9	34.7
	Overlying water	Galatheid site	1035Gw	7	184	0.028	5.2	0.351	14.9	0.4
	Overlying water	Mussel site	1036Mw	3.7	130	<0.01	3.4	0.202	4.2	—[a]
	Seawater	1,000-m depth	1035N1	4	93	ND[b]	ND	ND	5.6	—
Taketomi	Hydrothermal water	Main vent (inside)	TKTMmvi	42	393.55	ND	ND	ND	87.2	ND
	Hydrothermal water	Main vent (just above)	TKTMmva	34.2	276.4	ND	ND	ND	71.34	ND
	Hydrothermal water	Main vent (1 m above)	TKTMmvp	23.1	—	ND	ND	ND	—	ND
	Hydrothermal water	Sunachi vent (inside)	TKTMsvi	22.8	463.55	ND	ND	ND	93.1	ND
	Hydrothermal water	Mat site (just above)	TKTMmsa	22.8	28.8	ND	ND	ND	2.12	ND
	Hydrothermal water	Hanare vent (inside)	TKTMhvi	51	828.6	ND	ND	ND	209.4	ND
	Seawater	Reference site	TKTMrw	ND	—	ND	ND	ND	—	ND

[a] —, not detected.
[b] ND, not determined.

ware was applied to the UniFrac analyses. Jackknife values were estimated using 100 permutations and are shown in the nodes of the unweighted-pair group method using average linkages (UPGMA) tree.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences obtained in this study were deposited into the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB611047 to AB611479 for the sequences from the deep-sea hydrothermal vents at the Hatoma Knoll hydrothermal field and numbers AB611480 to AB611684 for the sequences from the shallow hydrothermal field off Taketomi Island.

RESULTS

Geographical and geochemical variation of microbial habitats. Zonation of different chemosynthetic macrofaunal colonies with an increasing distance from the hydrothermal vent emissions was evident in the Hatoma Knoll hydrothermal field. The polychaetes (*Paralvinella hessleri*) colonized the surface zones of the hydrothermal deposits adjacent to the high-temperature vent emissions. The galatheid crabs (*Shinkaia crosnieri*) dwelled on the hydrothermal deposits in the vicinity of the polychaete colonies surrounding the hydrothermal emissions. Colonies of deep-sea mussels (*Bathymodiolus platifrons*) were located at the foot of the hydrothermal deposits in the most distant hydrothermal mixing zones (68). The distribution of these macrofaunal colonies is shown in Fig. S1 in the supplemental material. The physical and chemical properties of the mixing water in these macrofaunal colonies are shown in Table 1. Both the highest concentrations of SiO_2, H_2, CH_4, CO_2, H_2S, and NH_4^+ and the highest temperatures were observed in the water near the polychaete colonies, and all of the concentrations and temperatures became lower in the water samples near the galatheid and mussel colonies. These results indicated that the zonation of the macrofaunal colonies was associated with the chemical inputs of the hydrothermal vent emissions.

In the shallow submarine hydrothermal field off Taketomi Island, a high-temperature fluid emission (approximately 42°C) with gas bubbles occurred from a fissure in the bedrock (main vent site) (Table 1). The concentrations of NH_4^+ and SiO_2 and the temperature of the water samples decreased as the distance from the hydrothermal emissions increased. The highest temperature (80°C) was recorded at the subseafloor of a microbial mat that was created without any visible evidence of fluid outflow (microbial mat site).

Prokaryotic cell abundance. In the Hatoma Knoll field, the abundance of prokaryotic cells ranged from 1.0×10^5 to 1.1×10^5 cells ml^{-1} in the water habitats, from 3.2×10^5 to 3.6×10^7 cells g^{-1} in the chimney habitats, and from 7.4×10^8 to 1.7×10^{10} cells g^{-1} in the macrofauna-associated habitats. However, the prokaryotic cell counts in some of the interior structures were below the detection limit (1.0×10^3 cells g^{-1}) (Table 2), probably due to exposure to the high temperatures that may exceed the upper limit of microbiologically habitable ranges. The highest cell abundance was found in the epibiotic microbial community in the galatheid setae. The attached microbial communities showed much higher prokaryotic cell abundances than the planktonic microbial communities.

In the shallow hydrothermal vent field off Taketomi Island, the prokaryotic cell abundance ranged from 1.3×10^5 to 7.1×10^5 cells ml^{-1} in the water samples and 2.1×10^8 cells g^{-1} in the microbial mat community.

Quantitative PCR of prokaryotic and archaeal 16S rRNA gene population. In most of the samples, the copy numbers of the whole prokaryotic 16S rRNA gene determined using quantitative PCR were about 10 times more than the prokaryotic cell numbers determined by direct counts (Table 2). The copy numbers of the whole prokaryotic 16S rRNA gene were significantly correlated with the cell numbers ($r = 0.78$, $P < 0.001$, $n = 19$ for $\log_{10}$-transformed variables). The proportion of the archaeal 16S rRNA gene abundance in the whole prokaryotic 16S rRNA gene community was less than 9.4% in the samples from the Hatoma Knoll and 16.6% in the microbial communities from the Taketomi field (Table 2).

TABLE 2 Prokaryote and viral abundances and VPRs in the hydrothermal fields

Hydrothermal field	Sample	Vent site	Sample identifier	Sample description	No. of prokaryote cells ml^{-1} or g^{-1}[a]	No. of VLPs ml^{-1} or g^{-1}	VPR	No. of rRNA gene copies ml^{-1} or g^{-1}[b]		Archaeal population ratio[c]
								Prokaryotic, universal	Archaeal	
Hatoma Knoll	Overlying water	C-2	1039Pw	Polychaete site	1.5×10^5 (2.6×10^4)[d]	2.9×10^6 (3.9×10^5)	19.1	3.9×10^6 (2.4×10^6)	1.2×10^5 (2.9×10^4)	3.1
	Overlying water	C-2	1035Gw	Galatheid site	1.0×10^5 (1.6×10^4)	7.5×10^5 (1.3×10^5)	7.4	3.5×10^6 (6.1×10^5)	3.5×10^4 (9.6×10^3)	1.0
	Overlying water	Oritori	1036Mw	Mussel site	1.1×10^5 (2.4×10^4)	5.0×10^5 (1.0×10^5)	4.6	1.8×10^6 (7.6×10^4)	4.7×10^4 (1.0×10^3)	2.7
	Chimney	C-2	866S	Surface layer	8.9×10^6 (2.5×10^6)	2.7×10^4 (2.2×10^3)	0.003	7.6×10^7 (5.0×10^6)	5.0×10^5 (2.3×10^3)	0.7
	Chimney	C-2	866I	Inside layer	—[e]	—	—	1.1×10^4 (3.7×10^3)	1.1×10^2 (2.0×10^1)	1.0
	Chimney	C-2	1035S	Surface layer	3.6×10^7 (3.7×10^6)	1.5×10^6 (4.2×10^5)	0.04	ND[f]	ND	ND
	Chimney	C-2	1039S	Surface layer	3.8×10^6 (4.6×10^5)	2.6×10^5 (2.4×10^4)	0.07	8.5×10^7 (8.6×10^6)	8.0×10^6 (6.3×10^5)	9.4
	Chimney	C-2	873S	Surface layer	2.6×10^7 (1.9×10^6)	3.3×10^6 (3.2×10^5)	0.13	3.9×10^9 (1.0×10^7)	3.5×10^8 (2.1×10^7)	8.9
	Chimney	C-2	873I	Inside layer	8.9×10^5 (1.53×10^5)	—	—	1.9×10^7 (1.5×10^6)	5.1×10^5 (7.9×10^3)	2.7
	Chimney	189-1	872S	Surface layer	3.2×10^5 (2.4×10^4)	1.8×10^4 (2.1×10^3)	0.05	4.3×10^6 (1.7×10^5)	4.6×10^3 (1.1×10^3)	0.1
	Chimney	189-1	872I	Inside layer	—	—	—	1.2×10^4 (7.5×10^2)	4.5×10^2 (1.7×10^1)	3.8
	Chimney	C-1	1037S	Surface layer	3.6×10^5 (7.3×10^4)	3.1×10^5 (4.6×10^4)	0.86	ND	ND	ND
	Chimney	Oritori	1036S	Surface layer	1.4×10^7 (1.3×10^6)	2.0×10^6 (1.7×10^5)	0.14	2.3×10^8 (3.1×10^7)	3.6×10^6 (2.1×10^5)	1.6
	Chimney	Oritori	1036I	Inside layer	3.0×10^6 (9.9×10^5)	—	—	ND	ND	ND
	Chimney	Chura	871W	Whole layer	2.8×10^7 (3.6×10^6)	6.7×10^4 (5.6×10^3)	0.002	1.1×10^8 (1.2×10^6)	2.2×10^5 (8.4×10^4)	0.2
	Polychaete nest	C-2	1039Pn	Whole layer	7.4×10^8 (1.5×10^8)	6.7×10^7 (9.3×10^6)	0.09	1.3×10^{10} (1.7×10^9)	5.8×10^8 (5.0×10^7)	4.5
	Polychaete nest	Oritori	1036Pn	Whole layer	9.6×10^8 (1.8×10^8)	5.8×10^7 (2.1×10^6)	0.06	1.1×10^{10} (2.5×10^8)	5.1×10^8 (1.5×10^7)	4.6
	Galatheid setae	C-2	1035Gs	Whole layer	1.7×10^{10} (3.2×10^9)	4.5×10^9 (7.9×10^8)	0.26	1.8×10^{11} (1.7×10^{10})	—	ND
	Galatheid setae	C-2	1037Gs	Whole layer	9.0×10^9 (1.2×10^9)	2.7×10^9 (7.3×10^8)	0.3	3.7×10^{11} (3.7×10^{10})	—	ND
Taketomi	Hydrothermal water	Main vent	TKTMmvi	Inside vent	2.1×10^5 (7.0×10^4)	2.6×10^6 (1.3×10^6)	12.1	1.3×10^7 (1.2×10^6)	1.6×10^6 (5.6×10^5)	12.3
	Hydrothermal water	Main vent	TKTMmva	Just above vent	1.7×10^5 (1.9×10^4)	1.4×10^6 (8.2×10^5)	8.07	6.3×10^4 (6.3×10^3)	1.0×10^4 (2.7×10^2)	16.6
	Hydrothermal water	Main vent	TKTMmvs	Separated from mats	1.8×10^5 (5.2×10^4)	1.4×10^6 (7.3×10^5)	5.8	ND	ND	ND
	Hydrothermal water	Main vent	TKTMmvp	1 m above vent	1.3×10^5 (1.5×10^4)	1.0×10^6 (2.2×10^5)	7.83	1.1×10^5 (1.4×10^4)	1.4×10^4 (1.1×10^3)	12.9
	Hydrothermal water	Sunachi vent	TKTMsvi	Inside vent	7.1×10^5 (1.8×10^5)	1.3×10^6 (1.9×10^5)	1.86	ND	ND	ND
	Hydrothermal water	Mat site	TKTMmsa	Just above mats	4.7×10^5 (8.0×10^4)	4.9×10^5 (2.5×10^5)	1.04	ND	ND	ND
	Hydrothermal water	Hanare vent	TKTMhvi	Inside vent	2.3×10^5 (1.2×10^5)	2.4×10^5 (6.1×10^4)	1.06	ND	ND	ND
	Microbial mat	Main vent	TKTMmvm	Microbial mats	2.1×10^8 (9.5×10^7)	7.2×10^7 (8.7×10^6)	0.35	9.4×10^8 (1.1×10^8)	7.0×10^7 (4.7×10^6)	7.5
	Seawater	Coral leaf	TKTMrw	At 10-m depth	2.6×10^5 (8.3×10^4)	1.1×10^6 (7.8×10^5)	4.28	3.3×10^5 (5.0×10^4)	1.7×10^4 (7.0×10^2)	5.3

[a] The prokaryotic cell abundances were determined by direct counts.
[b] The copy numbers were determined by quantitative PCR.
[c] The ratios of archaeal population to the total microbial community (archaeal 16S rRNA gene numbers/prokaryotic 16S rRNA gene numbers) were determined by using quantitative PCR.
[d] Numbers in parentheses are standard deviations.
[e] —, not detected (detection limit = 1.0×10^3 cells g^{-1} or VLPs g^{-1}).
[f] ND, not determined.

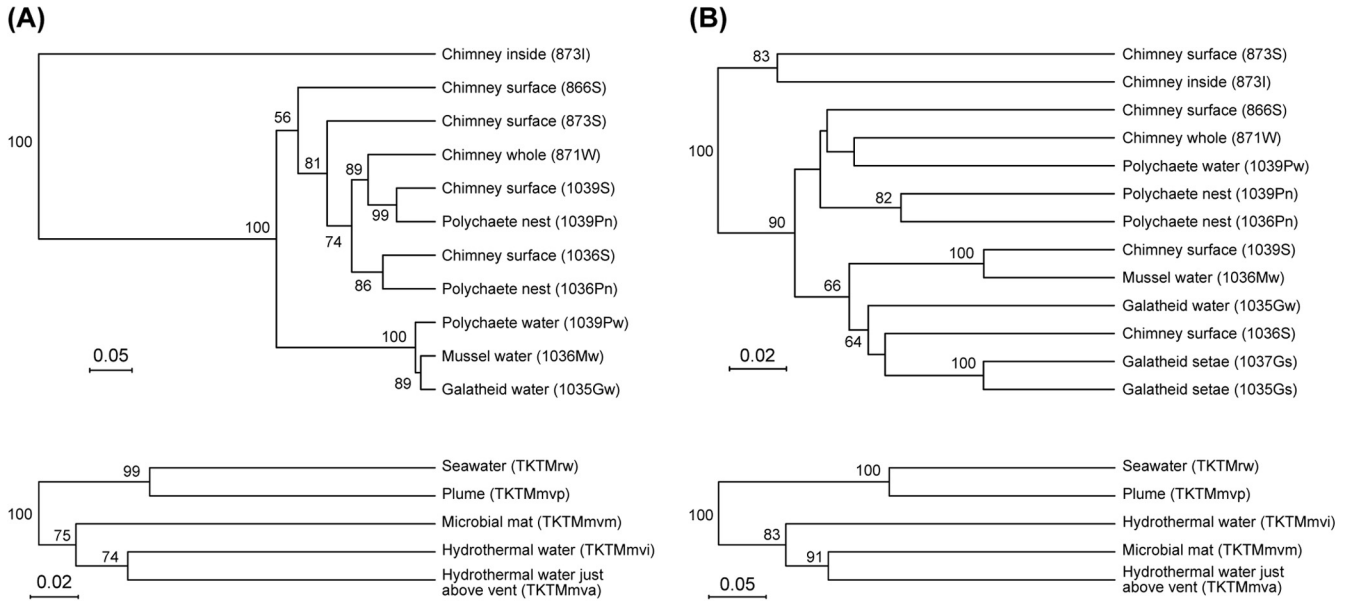

FIG 3 Cluster analysis of clone libraries. The trees were created with Jackknife environment clusters analysis in the UniFrac program. Jackknife with 100 permutations was performed. Jackknife values over 50 are given at corresponding branches. Comparison of archaeal (A) and bacterial (B) 16S rRNA gene clone libraries from the Hatoma Knoll (upper) and the Taketomi (lower) hydrothermal systems.

Prokaryotic 16S rRNA gene community structures. The 16S rRNA phylotype compositions of planktonic prokaryotes and the geographical variation were similar between the deep-sea and shallow submarine hydrothermal environments (Fig. 2). In the deep-sea macrofaunal colony waters, the archaeal phylotype compositions were similar to each other; the phylotypes of marine group I (MGI) archaea were dominant in the archaeal 16S rRNA gene population and were similar in composition in the MGI subgroups (Fig. 2A; see Fig. S2 in the supplemental material). In contrast, the dominant bacterial 16S rRNA gene compositions varied among the different macrofaunal colony waters. The most predominant bacterial phylotypes in the polychaete colony habitats were affiliated with the epsilonproteobacterial family *Thiovulgaceae*, which includes the facultatively anaerobic, mesophilic, and thiotrophic chemolithoautotrophs, represented by the genus *Sulfurovum* (group F) (4, 26). Thiotrophic and methanotrophic gammaproteobacterial groups (15) predominated in the galatheid colony habitats. Finally, the deep-sea-pervasive, heterotrophic gammaproteobacterial genera (1, 14) predominated in the mussel colony habitats (Fig. 2B).

In most of the chimney habitats and the polychaete nests, the archaeal 16S rRNA gene composition was characterized by a dominating clonal frequency of the *Thermococcales* and the deep-sea hydrothermal vent euryarchaeotic group 2 (DHVE2), which is represented by the genus *Aciduliprofundum* (49), and the occasional abundance of clones within the *Desulfurococcales*, *Archaeoglobales*, *Methanococcales*, the deep-sea hydrothermal vent euryarchaeotic group 1 (DHVE1), and hot water crenarchaeotic group IV (HWCGIV; also known as uncultured crenarchaea group II [UCII]) (25, 51) (Fig. 2A). Additionally, the bacterial 16S rRNA gene communities in the chimney habitats were always dominated by the phylotypes of *Deltaproteobacteria* and the *Thiovulgaceae*, which are members of the *Epsilonproteobacteria*. Some of these communities were also composed of the thiotrophic and methanotrophic gammaproteobacterial groups and the heterotrophic gammaproteobacterial and *Bacteroidetes* members (Fig. 2B). The prokaryotic 16S rRNA gene compositions in the galatheid setae were dominated mainly by thiotrophic and methanotrophic gammaproteobacterial groups (Fig. 2B).

In the planktonic microbial habitats of the shallow submarine hydrothermal field off Taketomi Island, the *Thiovulgaceae* and *Campylobacteraceae* phylotypes of the *Epsilonproteobacteria* and the thiotrophic phylotypes of the *Gammaproteobacteria* were predominant in the bacterial 16S rRNA gene compositions in the hydrothermal fluids (Fig. 2B). A similar but relatively more diverse bacterial 16S rRNA phylotype composition was obtained from the microbial mat communities that colonized the vent surfaces adjacent to the hydrothermal emissions (Fig. 2B). The archaeal 16S rRNA gene compositions in the hydrothermal fluids and the microbial mat were dominated not only by the DHVE1 and DHVE4 phylotypes but also by the terrestrial miscellaneous euryarchaeotal group (TMEG) (62, 67) (Fig. 2A). In the planktonic microbial communities of the mixed emissions and ambient seawater, the bacterial and archaeal rRNA phylotype compositions were considerably changed. In this area, the predominant phylotypes were affiliated with the *Alphaproteobacteria*, *Bacteroidetes*, and *Cyanobacteria* in the domain *Bacteria* and the MGI and marine group II (MGII) in the domain *Archaea* (Fig. 2). The obligately anaerobic *Epsilonproteobacteria*, *Desulfurobacteriales*, *Thermococcales*, and DHVE8 were found only in hydrothermal waters.

UniFrac cluster analysis showed that the planktonic archaeal communities were significantly distinct from the attached archaeal communities in the Hatoma Knoll (Fig. 3). On the other hand, the bacterial communities in the Hatoma Knoll were divided into three clusters. The classification of these groups coincided with the differences of the major components in each community as described above: bacterial communities in two clusters were dominated by facultatively anaerobic *Epsilonproteobacteria*, the *Deltaproteobacteria*, and the *Bacteroidetes* members (e.g.,

1036Pn and 871W), and those in the other cluster mainly consisted of the thiotrophic, methanotrophic, and heterotrophic *Gammaproteobacteria* groups (e.g., 1035Gs and 1039S). The result suggests that temperature and geochemical conditions but not attachment matrices (rocks or animal bodies) or lifestyles (attachment or planktonic) influence bacterial community structures in the Hatoma Knoll hydrothermal field. Similarly, both the archaeal and bacterial communities in the Taketomi field were divided into two groups, and their classifications were highly related to the major community components.

VLP abundance. The VLP abundance ranged from 5.0×10^5 to 2.9×10^6 and from 2.4×10^5 to 2.6×10^6 VLPs ml^{-1} in the planktonic microbial habitats of the Hatoma Knoll field and the Taketomi field, respectively (Table 2). Using both the prokaryotic cell and VLP abundances, the VPRs in the planktonic habitats were estimated to be 4.6 to 19 for the Hatoma Knoll field and 1.0 to 12.1 for the Taketomi field (Table 2). There were no correlations between the $\log_{10}$-transformed VLP abundances and prokaryotic cell abundances (Pearson correlation; $r = 0.155$, $P > 0.05$, $n = 9$).

The VLP abundance was highly variable among the deep-sea vent microbial communities attached to the hydrothermal deposits, animal colonies, and tissues, ranging from 1.8×10^4 to 4.5×10^9 VLPs g^{-1} (Table 2). The VLPs were undetectable in the interior parts of some chimney structures (below the detection limit of 1.0×10^3 VLPs g^{-1}) (Table 2). The maximum VLP abundance was found in the epibiotic microbial communities of the galatheid setae. In the Taketomi field, the VLP abundance was examined only in the microbial mat at the main vent site and was determined to be 7.2×10^7 VLPs g^{-1}. The VPRs in the attached habitats were estimated to be 0.001 to 0.86 for the Hatoma Knoll field and 0.35 for the Taketomi field. The VLP abundance in the attached microbial communities of both deep-sea and shallow submarine hydrothermal environments was significantly and positively correlated with the whole prokaryotic cell abundance ($r = 0.889$, $P < 0.001$, $n = 13$ for $\log_{10}$-transformed variables) (Fig. 4).

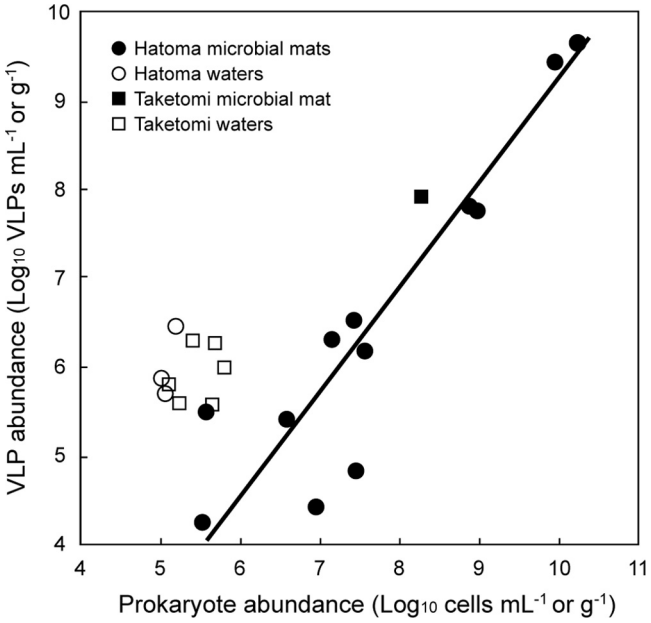

FIG 4 The relationship between the prokaryotic and VLP abundances in the Hatoma Knoll and Taketomi hydrothermal systems was assessed using model II (reduced major axis) regression. Only the reduced major axis regression of the attached habitats was shown: $y = a\,(x) + b$, where x is the $\log_{10}$-transformed prokaryotic abundance, y is the $\log_{10}$-transformed VLP abundance, a is 1.19 ± 0.15, and b is -2.65 ± 1.17 ($r^2 = 0.83$, $n = 13$).

DISCUSSION

Prokaryotic 16S rRNA gene community structures. The variability of the planktonic prokaryotic phylotype composition in the Hatoma Knoll field was similar to the patterns previously characterized in other deep-sea hydrothermal environments (42, 65). Similarly, the prokaryotic 16S rRNA gene compositions of the attached habitats in the Hatoma field were very similar to the compositions that were previously characterized and reported in the Iheya North field (chimneys and polychaete nests) (42), in the Yonaguni Knoll IV field (chimneys) (47), and in the Hatoma Knoll (galatheid setae) (70).

UniFrac cluster analysis showed that the planktonic prokaryotic communities were similar to the attached prokaryotic communities, with the exception of archaeal communities in the Hatoma Knoll. This suggests that some planktonic compositions were leaked from the attached microbial communities. However, the 16S rRNA gene clone analyses and the quantitative PCR analysis showed that the detailed prokaryotic microbial community composition was variable between the planktonic and attached habitats (Fig. 2 and Table 2). For example, not only was there a clear distinction between the archaeal communities, when comparing the prokaryotic 16S rRNA phylotype composition between the planktonic and attached habitats associated with the same macrofaunal colony in the Hatoma Knoll (e.g., 1039Pw and 1039Pn), but also the obligately anaerobic *Epsilonproteobacteria* and the methanotrophic *Gammaproteobacteria* were detected only in the planktonic habitats (1039Pw), while the thiotrophic *Gammaproteobacteria* and *Deltaproteobacteria* were found only in the attached habitats (1039Pn). In addition, the facultatively aerobic *Epsilonproteobacteria*, the heterotrophic *Gammaproteobacteria*, and the *Bacteroidetes* were detected in both the planktonic and attached habitats; however, their proportions in each community were largely different. Similarly, in the Taketomi field, the specific phylotypes (e.g., the *Thermococcales* and obligately anaerobic *Epsilonproteobacteria*) were found only in hydrothermal waters, and the proportions of common phylotypes were different between the planktonic and attached communities. As discussed in previous studies (23, 42), the prokaryotic 16S rRNA gene composition analyses indicate that the prokaryotic communities in the deep-sea and shallow submarine hydrothermal environments can be characterized by their preferences for their particular habitats; the planktonic and attached microbial communities have indigenous and distinct phylotype compositions. Microbial habitats associated with the macrofauna endemic to the hydrothermal vent and their colonies harbor unique community development and compositions (42, 70). Thus, it seems likely that the planktonic microbial communities in the submarine hydrothermal environments are not significantly affected by the dissociating and swarming microbial components from the abundant attached communities.

VLP abundances in planktonic and attached microbial communities of submarine hydrothermal environments. The planktonic VLP abundances reported here were similar to those in macrofaunal colony water samples, a diffusing-flow fluid in the East Pacific Rise (76), and the hydrothermal plume waters of the Gorda Ridge (29). These abundances were more than several times

higher than what was measured in nonhydrothermal deep seawater (22, 29, 76). The VLP abundances in the deep-sea vent environments represented 1/100 to 1/10 of the abundances obtained from the hydrothermal vent, plume, or diffusing-flow waters of the Endeavor Ridge (48). Because the VLP enumeration techniques were different among these studies, the results may have certain deviations due to biases in the methodologies used.

We found that viruses were widely distributed in a variety of attached hydrothermal microbial habitats: chimney surface areas or macrofaunal bodies and colonies. This is the first report to show VLP abundance in the attached microbial communities of submarine hydrothermal environments. However, the VLP abundances and VPR values in the hydrothermal attached microbial communities were significantly lower than expected, and all VPRs were below 1.0. These VPRs were also much lower than the VPR level in nonhydrothermal deep-sea benthic microbial communities in the sediment (6, 10). The efficiency of separation of viruses from sediment samples has been reported to be significantly increased by chemical treatments with surfactants and by mechanical shaking (7, 9). Using some attached microbial samples, we observed no significant effects of these treatments on VLP abundance (data not shown). However, some attached microbial samples in this study were fixed with formaldehyde, which has been reported by several authors to cause rapid viral decay after fixation (7). Thus, the viral abundance obtained in this study might underestimate the actual viral abundance because of differences in methodological approaches.

The unexpected VLP abundances may be due to the possible rapid diffusion of VLPs from the attached microbial communities during sampling and processing. To test this possibility, multiple samplings and procedures were examined for the microbial mat and the surrounding water at the main vent site in the Taketomi field. The VLP abundances in the interstitial seawater of the *in situ* microbial mat, which was carefully obtained from the seafloor by a plastic syringe (sample TKTMmva), and those in the residual seawater, which was taken from a plastic bag after the microbial mat sample was transferred into another plastic bag onboard (sample TKTMmvs), were compared. The VLP abundance of sample TKTMmva indicates the naturally diffusing VLP abundance from the *in situ* microbial mat, and the VLP abundance of sample TKTMmvs represents the diffusing VLP abundance during sampling and recovery. The VLP abundances were very similar among the *in situ* interstitial seawater, the residual seawater in the sampling bag, and the experimentally extracted water (Table 2). Thus, the low VLP abundances and VPR values in the hydrothermal attached microbial communities, at least in the microbial mat in the Taketomi field, were not highly affected by sampling biases.

VLP distribution in planktonic microbial communities of submarine hydrothermal environments. Although the statistically significant correlations between the prokaryotic cell and VLP abundances were not evident in the planktonic microbial communities, there was a trend for the VLP abundance in the planktonic habitats to decrease with increasing distance from the hydrothermal fluid sources (Tables 1 and 2). For instance, the abundance was the highest in the polychaete colony waters and became lower in the galatheid and mussel colony waters in the Hatoma Knoll field. Similarly, at the main vent site in the Taketomi field, the VLP abundance was the highest in the hydrothermal fluid (sample TKTMmvi) and became lower in the mixing waters between hydrothermal fluids and ambient seawater (samples TKTMmvs and TKTMmvp) (Tables 1 and 2). Similar patterns of distribution in viral abundance were also revealed in other deep-sea hydrothermal environments (29, 36, 48). In the hydrothermal plume waters, the viral abundance increased from the periphery to the center of plumes and was positively correlated with the increasing input of hydrothermal fluids, such as temperature and chemical composition (29, 36). Another study showed that the VLP abundances in the high-temperature hydrothermal fluids and diffusing flows were higher than those in the plume waters (48). Thus, there is a tendency for the abundances of the prokaryotic biomass and the chemolithoautotrophic population to be enhanced in the planktonic habitats of the hydrothermal mixing zones with higher inputs of hydrothermal fluids (42, 56). The higher inputs of the reductive substances, such as H_2, H_2S, CH_4, Fe(II), and Mn(IV), from the hydrothermal fluids provide greater energy to the chemosynthetic microbial communities. Indeed, it has been theoretically and empirically verified that the productivity and the community composition of the microbial communities are regulated by the geochemical energy state of the habitats (38, 52, 64). Additionally, viral production and propagation are highly associated with the productivity and nutrition states of the host organisms (21, 39). Thus, the viral abundance and productivity in the planktonic microbial communities of the hydrothermal mixing zones are likely associated with the geochemical energy state of the habitats, which is primarily driven by the chemical input of the hydrothermal fluid.

When comparing the prokaryotic cell and VLP abundances in the hydrothermal fluids from various vents in the Taketomi field, neither the temperatures nor the chemical components of the hydrothermal fluids correlated with the sizes of the prokaryotic cell and VLP populations (Tables 1 and 2). This result may reveal that prokaryote-virus productivity and their interactions are influenced not only by the geochemical features of the hydrothermal waters that sustain chemosynthetic microbial community development but also by the physical aspects of hydrothermal fluid emissions, such as the flow rate and the physical properties of the subseafloor hydrothermal fluid passages that establish the habitat conditions for each microbial community.

VLP distribution in attached microbial communities of submarine hydrothermal environments. The formation of a biofilm by the microorganisms entails a dynamic change in the physical and chemical conditions of the habitats and in the physiological state of the constitutive microorganisms (54). Thus, virus-host interactions can be highly variable (57). However, a statistically significant positive relationship between the prokaryotic biomass and VLP abundance in the attached microbial communities was observed. This suggests that in the attached habitats there was a specific virus-host interaction associated with hydrothermal activity.

Because the VPR values were quite different between the planktonic and attached microbial communities in the submarine hydrothermal environments, it is suggested that the viral productivity and propagation associated with the host prokaryotic communities were also different. Previous study has shown that the ratios of virus to bacteria were always <1.0 in the deep-sea sediments, and the values were significantly related to a low bacterial growth rate (8). Thus, the low VPRs in the attached habitats might be caused by low prokaryotic productivity. Furthermore, lysogeny is proposed to be a strategy for virus propagation in prokaryotic hosts under unfavorable growth conditions (13, 72). Since the accessibility of the chemolithotrophic energy sources from the

hydrothermal fluids may be limited, similar to what has been suggested to occur in biofilm microbial communities (53, 54, 73), the lower VLP abundance and VPR values in the attached microbial communities than the planktonic communities may result from a shift to more lysogenic infections rather than lytic infections. The lower VLP abundances and VPRs can also be interpreted as follows: the frequency of virus-infected cells and/or viral production rate might be significantly lower in attached microbial communities than in planktonic communities (5, 12), or lytic viral production occurs only at the surface layer of attached microbial communities and viral progeny are soon released from attached microbial communities into planktonic communities. The inactivation of viruses by adsorption to mineral and organic particles (59), radioactivity, such as gamma rays, degradation by extracellular proteases (72), and the effects of grazing by the coexisting protozoa and animals (59) should also be considered possible factors that regulate VLP abundance and VPR values in the microbial communities. There is a possibility that the low VPR values might be caused by the differences of minor phylotype compositions between planktonic and attached habitats, although the whole prokaryotic microbial community compositions in the two habitats were similar to each other, as described above (Fig. 2 and 3).

Finally, in this study, we demonstrated for the first time the overall pattern of distribution of viral abundance in the planktonic and attached microbial habitats of deep-sea and shallow submarine hydrothermal environments. This study suggested that the virus-mediated microbial mortality was high in the planktonic microbial communities present in the hydrothermal mixing zones. Additionally, lysogenic infections may be predominant in the attached microbial communities. Based on the predominance of inducible lysogens, a previous study suggested that hydrothermal diffusing flows could be perceived to be potential hot spots for lateral gene exchange in deep-sea hydrothermal environments (74). Furthermore, evidence of the presence of lysogenic viruses in the hydrothermal chimney biofilm has been found (2), and thus, our results provide new insight into the viral lysogenic lifestyle in hydrothermal environments. A comparative investigation of the lysogenic and lytic states of viruses and the relationship between the virus and the host prokaryotic physiology and lifestyle is needed to clarify further the role of viral ecophysiology and evolution in submarine hydrothermal environments.

ACKNOWLEDGMENTS

We thank the captain and crew of the R/V *Natsushima* (JAMSTEC) for helping us to obtain deep-sea hydrothermal vent samples. We also thank the onboard scientists of the NT08-13 and NT09-11 cruises, Hisako Hirayama, Tomoo Watsuji, and Hiroko Makita (JAMSTEC), Satoshi Nakagawa (Hokkaido University), and Michinari Sunamura (University of Tokyo) for their help in collecting samples.

This work was partially supported by a Grant-in-Aid for Scientific Research on Innovative Areas (no. 20109003) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) and a Grant-in-Aid for Young Scientists (B) (no. 22770092) from the Japan Society for the Promotion of Science (JSPS).

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