

# Genome Analysis of Deep-Sea Thermophilic Phage D6E<sup>∇</sup>

Yiqian Wang<sup>2,3</sup> and Xiaobo Zhang<sup>1\*</sup>

Key Laboratory of Conservation Biology for Endangered Wildlife of the Ministry of Education and College of Life Sciences, Zhejiang University, Hangzhou 310058, People's Republic of China<sup>1</sup>; Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, State Oceanic Administration, Xiamen 361005, People's Republic of China<sup>2</sup>; and National Institute for Communicable Disease Control and Prevention, China CDC, Changping, Beijing 102206, People's Republic of China<sup>3</sup>

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**In deep-sea hydrothermal vent communities, viruses play very important roles. However vent thermophilic bacteriophages remain largely unexplored. In this investigation, a novel vent *Geobacillus* bacteriophage, D6E, was characterized. Based on comparative genomics and proteomics analyses, the results showed an extensive mosaicism of D6E genome with other mesophilic or thermophilic phages.**

Bacteriophages are the most abundant life forms in the biosphere: they can be detected in almost every biological niche and represent a huge source of biodiversity (2, 3, 8, 11). Therefore, phages are thought to play very important roles in the ecological balance of microbial life and in microbial diversity (10, 25). This view has gained strong support from the work on viruses in extreme ecosystems, especially the deep-sea hydrothermal vents (5, 7, 14, 23). The isolation and characterization of viruses often lead to new insights into virus relationships and to a more detailed understanding of the biochemical environment of their host cells (15, 19, 21). Generally our understanding of the deep-sea thermophilic bacteriophages is far behind our knowledge of the terrestrial, mesophilic bacteriophages (31). The recent discoveries of many novel thermophilic bacteriophages, especially among members isolated from deep-sea hydrothermal vents, are likely to lead to a more complete understanding of not only thermophiles, but also the biochemical adaptations required for the life in extreme environments, and to new insights into both host and virus evolution (14, 22, 24, 26–28, 30). Comparative viral genomics creates a wealth of information that has made it possible to construct gene and genome phylogenies as well as to observe complex relationships among virus genomes. However, bacteriophages that infect thermophilic eubacteria have remained largely unexplored (29). At present, only several genomes of thermophilic bacteriophages are available (14, 17, 18). Among them, only one phage from a deep-sea hydrothermal vent, i.e., GVE2, has been characterized at the molecular level (14). In this study, a deep-sea thermophilic bacteriophage, D6E, was isolated. The comparative analysis of the genome sequences of D6E and GVE2 revealed extensive genetic mosaics.

During the cultivation of *Geobacillus* sp. strain E263 isolated from a deep-sea hydrothermal field in the east Pacific (12°42'29"N, 104°02'01"W), many phage plaques were observed. The bacteriophage particles were purified from the

host cultured at 65°C as described previously (14). The phage DNA was extracted and subjected to DNA sequencing as described previously (14). An initial set of open reading frames (ORFs) likely to encode proteins (coding sequences [CDSs]) was identified with the program Glimmer (6). All CDSs were compared to a nonredundant amino acid database. The BLAST algorithms were used for similarity searches in the databases available through the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov>) (1). tRNAscan-SE was used to search for tRNAs (16). The rho-independent transcription terminators were detected with TransTerm (9). Multiple alignments were generated with the DNAMAN program (Lynnon Biosoft), using the ClustalW algorithm. The purified virions were subjected to mass spectrometry as described before (14). All peptide mass fingerprintings (PMFs) were analyzed with the protein search engine Mascot (Matrix Science, United Kingdom) against the genome sequence of the phage obtained in this study.

The results showed that D6E was a typical myovirus with an icosahedral capsid (60 nm in diameter), a contractile tail (16 nm in width and 60 nm in length), and a tail fiber (4 nm in width and 60 nm in length) (Fig. 1A). Based on these proper-

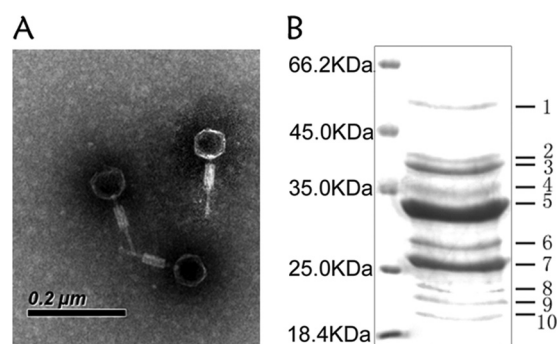


FIG. 1. Characterizations of deep-sea thermophilic bacteriophage D6E. (A) Electron micrograph of D6E virions. Scale bar, 200 nm. (B) SDS-PAGE of proteins from purified D6E virions, followed by staining with Coomassie brilliant blue R250. The numbers indicate the excised bands for mass spectrometric analysis. The left lane contains the protein molecular mass marker (kDa).

\* Corresponding author. Mailing address: Key Laboratory of Conservation Biology for Endangered Wildlife of the Ministry of Education and College of Life Sciences, Zhejiang University, Hangzhou 310058, People's Republic of China. Phone and fax: 86-571-88981129. E-mail: zxb0812@zju.edu.cn.

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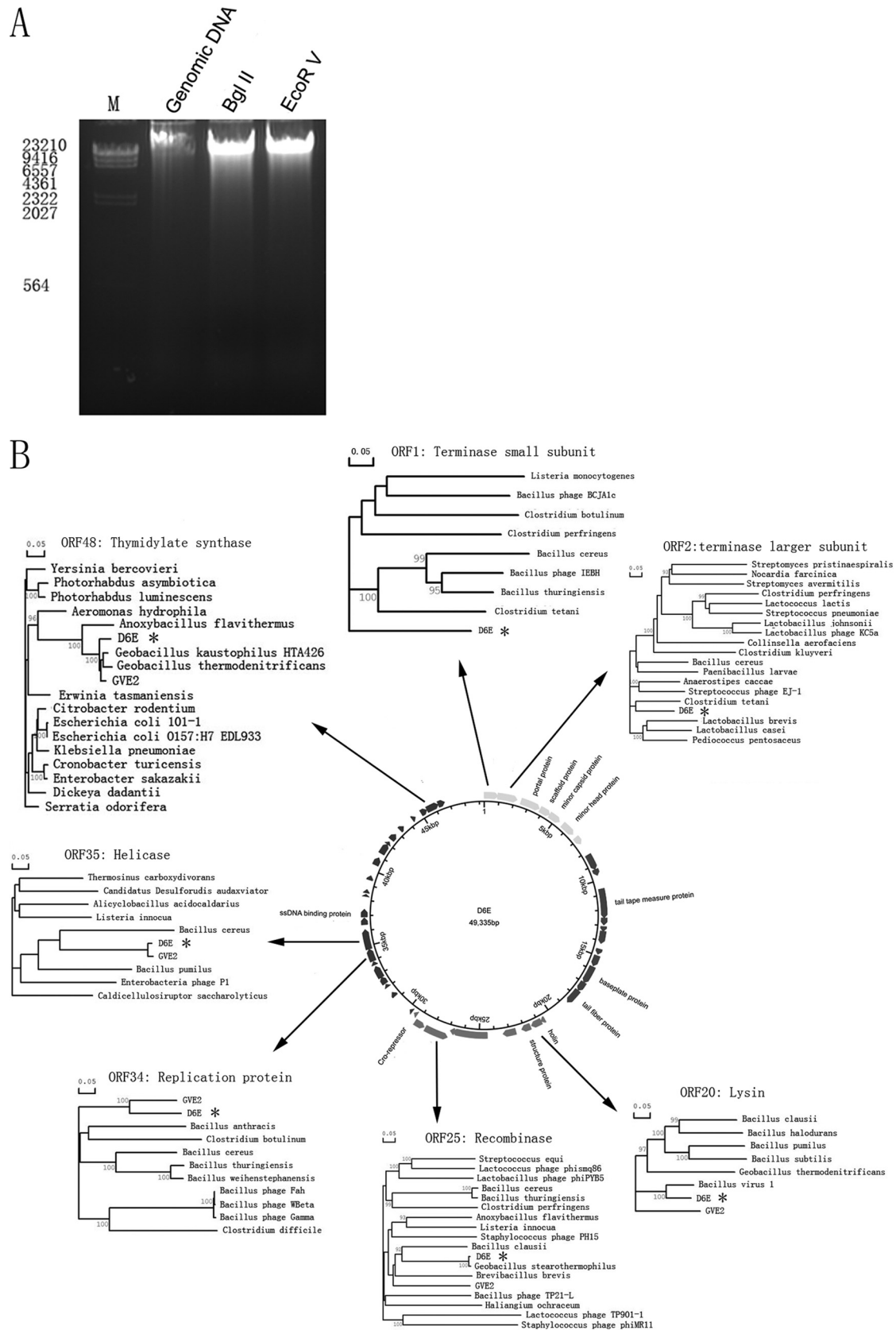


FIG. 2. Genome of D6E. (A) The viral genomic DNA of D6E. Native DNA was digested by BglI or HindII prior to electrophoresis. M, DNA marker. (B) Circular representation of the D6E genome map. The outer circle represents the D6E ORFs. The predicted protein coding sequences are indicated according to their corresponding homologues. Dendrograms are displayed for the predicted protein coding sequences with highly conserved homologues.

TABLE 1. ORFs of deep-sea thermophilic bacteriophage D6E

ORF	Position in the genome (amino acid length)	Closest homologue in GenBank (accession no., E value, % identity)	Predicted function/feature	Identification by mass spectrometry	
				Band	Sequence coverage (%)
1	1–900 (299)	Phage terminase small subunit, <i>Bacillus thuringiensis</i> strain Al Hakam (YP_891149.1, 5e–75, 52)	Phage terminase small subunit		
2	887–2170 (427)	Phage terminase, large subunit, pbsx family, <i>Bacillus cereus</i> W (ZP_03104491.1, 6e–172, 68)	Phage terminase, large subunit		
3	2466–3734 (422)	Phage portal protein, SPP1 family, <i>Bacillus cereus</i> AH1134 (ZP_03232437.1, 5e–85, 41)	Phage portal protein	1	42
4	3816–4520 (235)	Hypothetical protein CTC01553, <i>Clostridium tetani</i> E88 (NP_782165.1, 5e–24, 35)	Phage scaffold protein	7	50
5	4535–5304 (289)	Hypothetical protein Haur_0657, <i>Herpetosiphon aurantiacus</i> ATCC 23779 (YP_001543433.1, 2e–107, 65)	Phage minor capsid protein	5 6 8 10	82 64 64 67
6	5605–6510 (301)	Phage-related minor head protein, <i>Bacillus licheniformis</i> ATCC 14580 (YP_078649.1, 2e–33, 34)	Phage-related minor head protein		
7	6809–7315 (168)	Hypothetical protein Amet_0423, <i>Alkaliphilus metalliredigens</i> QYMF (YP_001318310.1, 7e–22, 31)			
8	8196–9246 (349)	Hypothetical protein Amet_0426, <i>Alkaliphilus metalliredigens</i> QYMF (YP_001318313.1, 3e–58, 37)			
9	9259–9663 (134)	Hypothetical protein Amet_0427, <i>Alkaliphilus metalliredigens</i> QYMF (YP_001318314.1, 9e–21, 42)	Phage conserved protein	3	64
10	10584–12494 (636)	Putative phage membrane protein, <i>Clostridium botulinum</i> C strain Eklund (ZP_02620637.1, 2e–26, 29)	Phage tail tape measure protein		
11	12494–12961 (156)	Hypothetical protein lin1715, <i>Listeria innocua</i> Clip11262 (NP_471051.1, 3e–11, 33)			
12	13063–13380 (105)	Hypothetical protein EF1475, <i>Enterococcus faecalis</i> V583 (NP_815196.1, 3e–19, 47)			
13	13370–14191 (274)	Hypothetical protein lin1713, <i>Listeria innocua</i> Clip11262 (NP_471049.1, 8e–65, 48)			
14	14567–14920 (117)	Hypothetical protein Amet_0435, <i>Alkaliphilus metalliredigens</i> QYMF (YP_001318322.1, 2e–12, 37)	Phage-related protein		
15	15025–15735 (239)	HNH endonuclease family protein, <i>Gramella forsetii</i> KT0803 (YP_862450.1, 2e–29, 45)			
16	15804–16979 (391)	Hypothetical protein Amet_0436, <i>Alkaliphilus metalliredigens</i> QYMF (YP_001318323.1, 1e–90, 45)	Baseplate protein		
17	16976–17617 (214)	Hypothetical protein Amet_0437, <i>Alkaliphilus metalliredigens</i> QYMF (YP_001318324.1, 8e–46, 47)			
18	17630–18661 (343)	Hypothetical protein GK0545, <i>Geobacillus kaustophilus</i> HTA426 (YP_146398.1, 4e–23, 84)	Tail fiber protein		
19	20539–20802 (87)	Hypothetical protein Aftv_0679, <i>Anoxybacillus flavithermus</i> WK1 (YP_002315044.1, 4e–35, 89)	Holin		
20	20802–21485 (227)	Cell wall hydrolase/autolysin, <i>Geobacillus</i> sp. strain Y412MC61 (ZP_03558146.1, 1e–107, 85)	Lysin		
21	21584–22162 (192)	Hypothetical protein BH3064, <i>Bacillus halodurans</i> C-125 (NP_243930.1, 5e–47, 48)	Structure protein	9	54
22	22587–23423 (278)	Hypothetical protein Nther_0358, <i>Natranaerobius thermophilus</i> JW/NM-WN-LF (YP_001916543.1, 5e–61, 47)			
23	24473–26731 (752)	Lipid A export ATP-binding/permease protein MsbA, <i>Clostridium botulinum</i> Bf (ZP_02619785.1, 1e–78, 30)			
24	26655–26909 (84)	Hypothetical protein BV1_gp42, <i>Bacillus virus</i> 1 (YP_001425622.1, 9e–16, 74)			
25	28508–27069 (479)	Putative recombinase, <i>Geobacillus stearothermophilus</i> (CAQ19390.1, 0.0, 98)	Putative recombinase		
26	29188–28568 (205)	Transcriptional regulator, XRE family, <i>Paenibacillus</i> sp. strain JDR-2 (ZP_02846182.1, 5e–58, 57)	Transcriptional regulator		
27	29346–29549 (67)	Transcriptional regulator, XRE family, <i>Paenibacillus</i> sp. strain JDR-2 (ZP_02846181.1, 3e–8, 46)	Cro repressor		
28	29721–29900 (59)	Hypothetical protein GBVE2_gp030, <i>Geobacillus virus</i> E2 (YP_001285836.1, 1e–22, 89)			
29	31242–31532 (96)	Hypothetical protein GBVE2_gp064, <i>Geobacillus virus</i> E2 (YP_001522900.1, 2e–17, 48)	Conserved hypothetical protein		
30	32002–32199 (65)	Hypothetical protein GBVE2_gp034, <i>Geobacillus virus</i> E2 (YP_001285840.1, 6e–29, 92)			

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TABLE 1—Continued

ORF	Position in the genome (amino acid length)	Closest homologue in GenBank (accession no., E value, % identity)	Predicted function/feature	Identification by mass spectrometry	
				Band	Sequence coverage (%)
31	32278–32757 (159)	Siphovirus Gp157-like protein, <i>Geobacillus</i> virus E2 (YP_001285841.1, 3e–78, 78)	Siphovirus Gp157-like protein		
32	32754–33551 (265)	Hypothetical protein GBVE2_gp036, <i>Geobacillus</i> virus E2 (YP_001285842.1, 8e–115, 97)			
33	33678–33893 (71)	Hypothetical protein GBVE2_gp066, <i>Geobacillus</i> virus E2 (YP_001522902.1, 7e–30, 90)			
34	33946–34759 (271)	Putative replication protein, <i>Geobacillus</i> virus E2 (YP_001285845.1, 7e–89, 63)	Phage replication protein		
35	34770–36065 (431)	Putative DnaB-like helicase, <i>Geobacillus</i> virus E2 (YP_001285846.1, 2e–170, 97)	Putative DnaB-like helicase		
36	36403–36837 (144)	Hypothetical protein GBVE2_gp043, <i>Geobacillus</i> virus E2 (YP_001285849.1, 1e–57, 65)	HNH endonuclease		
37	36902–37396 (164)	Putative ssDNA <sup>a</sup> binding protein, <i>Geobacillus</i> virus E2 (YP_001285850.1, 1e–21, 80)	ssDNA binding protein		
38	38251–38388 (46)	Hypothetical protein GBVE2_gp068, <i>Geobacillus</i> virus E2 (YP_001522904.1, 9e–12, 83)			
39	38487–38699 (70)	Hypothetical protein EFP_gp176, <i>Enterococcus</i> phage phiEF24C (YP_001504285.1, 5e–11, 46)			
40	39359–39637 (92)	Hypothetical protein GBVE2_gp047, <i>Geobacillus</i> virus E2 (YP_001285853.1, 5e–12, 37)			
41	40412–40921 (169)	Hypothetical protein GBVE2_gp049, <i>Geobacillus</i> virus E2 (YP_001285855.1, 3e–48, 64)	dUTPase		
42	41255–41974 (239)	Conserved hypothetical protein, <i>Bacillus cereus</i> 03BB108 (ZP_03115270.1, 1e–54, 49)	Conserved hypothetical protein		
43	41992–42171 (59)	Hypothetical protein GBVE2_gp050, <i>Geobacillus</i> virus E2 (YP_001285856.1, 1e–17, 90)			
44	42376–42807 (143)	Hypothetical protein GBVE2_gp051, <i>Geobacillus</i> virus E2 (YP_001285857.1, 9e–12, 35)	Possible sensor protein		
45	43233–43535 (99)	Conserved hypothetical protein, <i>Geobacillus</i> sp. strain Y412MC61 (ZP_03558114.1, 3e–41, 84)	Group-specific protein		
46	44296–44529 (77)	Hypothetical protein GBVE2_gp054, <i>Geobacillus</i> virus E2 (YP_001285860.1, 4e–31, 89)	Conserved hypothetical protein		
47	44999–45409 (136)	Hypothetical protein BC03BB108_E0069, <i>Bacillus cereus</i> 03BB108 (ZP_03115598.1, 1e–31, 51)			
48	45437–46231 (264)	Putative thymidylate synthase, <i>Geobacillus</i> virus E2 (YP_001285862.1, 4e–149, 95)	Phage thymidylate synthase		
49	46247–46678 (143)	RinA transcriptional activator-like protein, <i>Geobacillus</i> virus E2 (YP_001522905, 2e–58, 75)	RinA transcriptional activator-like protein		

<sup>a</sup> ssDNA, single-stranded DNA.

ties, the phage D6E belonged to the family of *Myoviridae*. The SDS-PAGE data indicated that the viral particle was composed of 10 proteins (Fig. 1B).

The sequencing results, more than 16-fold redundant, indicated that the phage contained a 49,335-bp double-stranded genomic DNA (dsDNA). Based on sequence analysis, there were only two restriction sites (one *Bgl*II and one *Eco*RV) in the D6E genome. After digestion of D6E genomic DNA with *Bgl*II or *Eco*RV, only one band was observed. The results suggested that the D6E genome might be circular (Fig. 2A). The PCR amplification data supported this finding.

The genome of D6E contained 49 putative ORFs (Table 1 and Fig. 2B). As determined by tRNAscan analysis, the D6E genome did not carry any tRNA genes. Based on BLAST results, the D6E genome could be split into four clusters of genes with related functions. These clusters were supposed to support DNA packaging and capsid assembly, tail assembly, lysis, and lysogeny, as well as DNA replication and transcription (Fig. 2B and Table 1).

The mass spectrometric identification of 10 proteins of D6E virions presented the unambiguous identification of five unique proteins, covering 42 to 82% of amino acid sequences (Table 1). The results showed that one of the major protein bands was the product of ORF 5 (major capsid protein) (Fig. 1B and Table 1). This protein was also found in the bands 6, 8, and 10. The proteomics approach proved that some ORFs were indeed protein-coding sequences, although the proteins had no functionally assigned homologs in the database: e.g., the protein encoded by ORF 21 (band 9).

Although the conservation of the gene order in the D6E genome was remarkable to other bacteriophages, the D6E genome sequence shared low similarity to the known phages. Based on sequence analysis, the D6E genome displayed extensive mosaicism of genes with other species (Fig. 3). The D6E genes belonging to the tail assembly gene cluster showed high similarity to those of three mesophilic bacteriophages (*Listeria innocua* Clip 11262, *Enterococcus faecalis* V583, and *Alkaliphilus metalliredigens* QYMF), and the D6E genes from the DNA

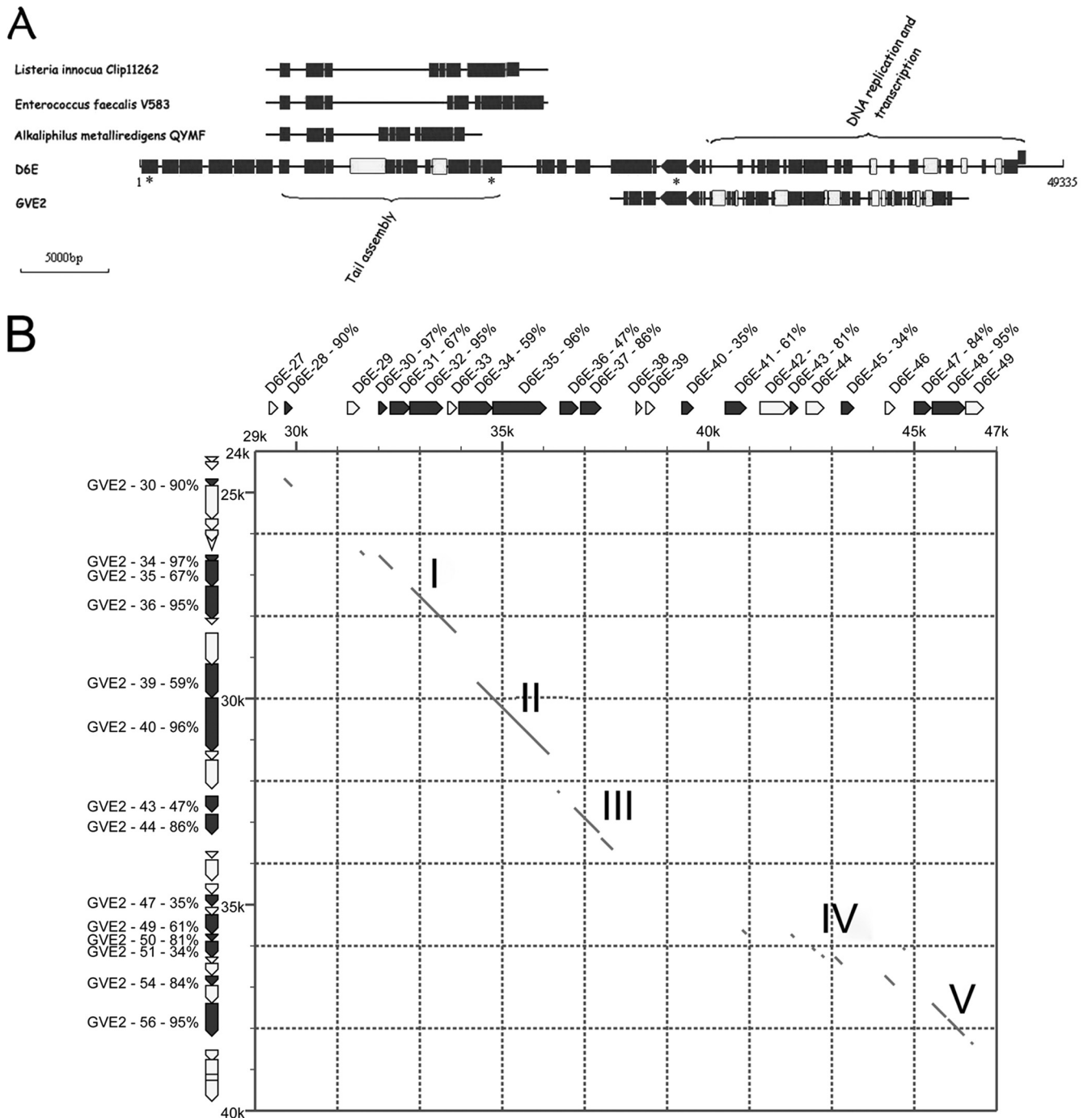


FIG. 3. Mosaicism in bacteriophage D6E. Multiple alignments were generated with DNAMAN program (Lynnon Biosoft) using the ClustalW algorithm. (A) Comparative genomics among D6E, three mesophilic bacteriophages (*Listeria innocua* Clip 11262, *Enterococcus faecalis* V583, and *Alkaliphilus metalliredigens* QYMF), and deep-sea thermophilic bacteriophage GVE2. The full-length D6E genome is displayed, while the partial genomes of three mesophilic phages and GVE2 with similarities to D6E are shown. Black boxes represent genes with high similarities, and white boxes show genes with low similarities. (B) Comparison of DNA replication and transcription gene cluster between D6E and GVE2. The regions of D6E and GVE2 were compared by a dot-matrix-dot method. Gene positions are indicated in thousands. The diagonal lines show regions where the nucleotide and protein sequence similarity extended five segments over the DNA transcription and replication region I~V. The percentages show amino acid sequence similarities between ORFs from bacteriophages D6E and GVE2.

replication and transcription gene cluster were very highly similar to those of another deep-sea thermophilic bacteriophage, GVE2 (Fig. 3A), suggesting the existence of gene or gene fragment transfers among bacteria and/or bacteriophages be-

longing to different species or genera through recombinant events. The analysis showed that D6E, a member of the *Myoviridae*, shared low sequence similarity with thermophilic bacteriophage GVE2 (*Siphoviridae*). However, both phages

showed high identities in the DNA replication and transcription gene cluster (Fig. 3A and B). Considering that D6E and GVE2 were bacteriophages infecting thermophiles, the results suggested that the DNA replication and transcription gene cluster played very important roles in response to high temperature.

Up to date, mosaic genomes of bacteriophages have been from mesophilic phages (4, 12, 13, 20). In this investigation, our study provided the first report on the mosaicism of viral genome in a deep-sea vent community. Based on D6E genome structure, it could be suggested that at least three recombination events (the overall gene organization, recombination between gene boundaries, and mutation in gene sequences) that appeared to have arisen by illegitimate recombination occurred sufficiently. The mosaic nature of D6E highlighted that mobile DNA elements like bacteriophages contributed substantial amounts of foreign DNAs to deep-sea vent community genomes in evolution. Therefore, our findings open up a realm of opportunities for studying horizontal gene transfer and for investigating the evolution of bacterial communities in deep-sea vents.

**Nucleotide sequence accession number.** The DNA sequences reported in this article may be found in the GenBank database under accession no. GU568037.

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