Proteomic Analysis of Interactions between a Deep-Sea Thermophilic Bacteriophage and Its Host at High Temperature

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The virus-host interaction is essential to understanding the role that viruses play in ecological and geochemical processes in deep-sea vent ecosystems. Virus-induced changes in cellular gene expression and host physiology have been studied extensively. However, the molecular mechanism of interaction between a bacteriophage and its host at high temperature remains poorly understood. In the present study, the virus-induced gene expression profile of Geobacillus sp. E263, a thermophile isolated from a deep-sea hydrothermal ecosystem, was characterized. Based on proteomic analysis and random arbitrarily primed PCR (RAP-PCR) of Geobacillus sp. E263 cultured under non-bacteriophage GVE2 infection and GVE2 infection conditions, there were two types of protein/gene profiles in response to GVE2 infection. Twenty differentially expressed genes and proteins were revealed that could be grouped into 3 different categories based on cellular function, suggesting a coordinated response to infection. These differentially expressed genes and proteins were further confirmed by Northern blot analysis. To characterize the host proteins in response to virus infection, aspartate aminotransferase (AST) was inactivated to construct the AST mutant of Geobacillus sp. E263. The results showed that the AST protein was essential in virus infection. Thus, transcriptional and proteomic analyses and functional analysis revealed previously unknown host responses to deep-sea thermophilic virus infection.

Bacteriophages, one of the most common biological agents in the sea, can undergo rapid decay and replenishment and influence many biogeochemical and ecological processes (7). It is now realized that bacteriophages play important quantitative and qualitative roles in controlling marine bacterial populations and microbial ecosystems, including the evolution of bacterial genomes, bacterial biodiversity and species distributions, and genetic transfer (3, 7). Moreover, the genetic diversity and composition of marine microbial communities are related to the interaction between bacteriophages and their host organisms, which provides a tool for understanding the interaction itself (25). It is believed that the study of marine bacteriophage-host interactions is essential to understand the role that bacteriophages play in the marine environment. Recently, many viruses have been documented from deep-sea hydrothermal vents (12). In these ecosystems, thermophilic microbes exploit the vent chemicals to obtain energy for their growth. Living on the energy harnessed by the thermophiles, the vent animals, such as tubeworms, huge clams, crabs, and several species of fish, populate the sulfide mounds. In this context, thermophiles comprise the basis of the food chain in the deep-sea hydrothermal vent ecosystem, the source of the energy to power the biological communities at these remote locations. However, the most significant players in nutrient and energy cycling are thermophilic viruses, including archaeal viruses and bacteriophages, which are major causes of vent thermophile mortality (21, 22). To date, the interactions between virus and host in deep-sea hydrothermal vents are still not explored.

In mesophiles, during virus infection, bacteriophages are known to regulate host macromolecular synthesis by modifying host transcription and translation machinery and making the hosts serve the requirements of viruses (15). Therefore, host proteins that are involved in virus infection play essential roles in the replication, packaging, and proliferation of bacteriophages. There is an increasing body of knowledge regarding bacteriophage-host interactions, as well as molecular aspects of bacteriophages, in particular, the host stress response proteins and the proteins induced by bacteriophages. With the accumulated data, it was found that the process of bacteriophage-host interaction requires the actions of the most highly induced genes encoding chaperones and other stress-inducible proteins (19, 20). Previous studies have revealed that bacteriophage infection is actually dependent on several cellular chaperonins, proteases, ABC transporters, and other heat shock regulons (17, 19). The upregulated host gene expression may constitute a direct stress response to bacteriophage infection or may have been facilitated by bacteriophage factors injected into the host cell or expressed from a bacteriophage expression cluster (11). The bacteriophage-host interaction regulatory network is composed of host factors consisting of host stress response genes, many of which are located in clusters and interact with each other. Proteomics approaches have become increasingly popular in studies to reveal the effects of viral infection on the cellular proteome (1, 27). Comparative proteomic approaches coupling two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) are widely used to analyze host responses in animals, humans, and plants during virus infection (18, 19, 20). The approaches effectively facilitate investigations of
the molecular profiles of virus-infected cells (27). Therefore, the proteomics strategies provide an overall understanding of the cellular factors involved in various stages of infection and give insight into the alteration of signaling pathways, allowing us to further understand viral pathogenesis (19, 20).

Although it is generally known that phage, host, and environmental factors contribute to phage infection, the molecular mechanism of interactions between the bacteriophage and its host remains poorly understood. In the present investigation, the response of the deep-sea thermophile Geobacillus sp. E263 to infection by its bacteriophage, GVE2, was analyzed using the proteomics approach. As demonstrated in our previous study (12), GVE2, a virulent tailed *Siphoviridae* bacteriophage, was isolated from the deep-sea thermophilic Geobacillus sp. E263 in the eastern Pacific Ocean. The strain Geobacillus sp. E263 is very closely related to *Geobacillus kaustophilus* HTA426 isolated from the deep-sea sediment of the Mariana Trench. The genome of *G. kaustophilus* HTA426 has been sequenced, which facilitates the proteomic analysis of Geobacillus sp. E263. In this study, the results showed that a total of 20 host proteins were involved in virus infection. Our study provided the first insight into the molecular mechanism of bacteriophage-host interactions at high temperature.

**MATERIALS AND METHODS**

### Infection of Geobacillus sp. E263 by its thermophilic bacteriophage, GVE2.

The deep-sea thermophile *Geobacillus sp.* E263 (China General Microbiological Culture Collection Center accession no. CGMCC1.7046) was cultured at 60°C with shaking (150 rpm) in TTM medium consisting of 0.2% NaCl, 0.4% yeast extract, and 0.8% tryptone (pH 7.0). The host strain was infected with its thermophilic bacteriophage GVE2 as described previously (12). Briefly, cultures of *Geobacillus* sp. E263 grown overnight were inoculated into 200 ml fresh TTM medium at 1:100. The strain was incubated and infected by GVE2 at 60°C. The cultures in late log phase were collected for protein electrophoresis. At approximately 5% TMM medium at 1:100. The strain was incubated and infected by GVE2 at 60°C. The virus-free thermophilic *Geobacillus* sp. E263 grown overnight were inoculated into 200 ml fresh TTM medium at 1:100. The strain was incubated and infected by GVE2 at 60°C. The bacterial cell pellets were resuspended in 7 ml TE buffer (50 mM Tris-HCl, 10% glycerol, 2% SDS, 1% β-mercaptoethanol [β-ME], pH 6.8) as samples for SDS-PAGE.

### Sample preparations for protein electrophoresis.

The GVE2-infected and noninfected *Geobacillus* sp. E263 cells were harvested by centrifugation at 5,000 × g for 5 min. After three washes with sterile phosphate-buffered saline (PBS) (pH 7.4), the bacterial pellets were resuspended in 1× lysis buffer (50 mM Tris-HCl, 10% glycerol, 2% SDS, 1% β-mercaptoethanol [β-ME], pH 6.8) as samples for SDS-PAGE.

### SDS-PAGE.

For these sample preparations, the bacterial-cell pellets were resuspended in 7 ml TE buffer (50 mM Tris-HCl, 10% glycerol, 2% SDS, 1% β-mercaptoethanol [β-ME], pH 6.8) as samples for SDS-PAGE.

**2-DE and image analysis.** The first-dimension electrophoresis of the protein sample (about 300 μg) was conducted in immobilized pH gradient gel (IPG) strips (pH 4 to 7; 13 cm; Amersham Biosciences) with IPGPhore 3 (Amersham Biosciences). The IPG strip, rehydrated at 50 V for 12 h at 20°C, was focused using immobilized pH gradient strips (500 V, 8000 V, 1000 V for 1 h with rapid ramping, and 8000 V for 3 h with rapid ramping until 16,000 to 20,000 V· h was reached). Subsequently, the IPG strip was incubated in equilibration buffer (6 M urea, 50 mM Tris-HCl, 2% SDS, 30% glycerol, 0.002% bromophenol blue, pH 8.8) containing 1% (wt/vol) DTT for 15 min before being washed for a further 15 min in equilibration buffer containing 2.5% (wt/vol) iodoacetamide (Sigma). The second-dimension separation was carried out on 12% SDS-PAGE at 100 V for 30 min and then 200 V until the dye front reached the bottom of the gels. After electrophoresis, the gels were stained with Coomassie blue R-250 and scanned using an ImagingScanner III (GE Healthcare). Spot detection, spot matching, and quantification analysis were performed using ImageMaster 6.0 2-D analysis software (GE Healthcare). 2-D analysis was biologically repeated three times. The gel images were normalized according to the total quantity in the analysis set. Student’s *t* test was adopted to evaluate the spots that were significantly different between GVE2-infected and noninfected groups.

### Digestive digestion.

The gel spots of samples were performed as described previously with minor modification (26). Protein bands or spots were excised and dehydrated several times with 50% acetonitrile (ACN). After being vacuum dried, the gels were incubated with 100 mM ammonium bicarbonate (ABC) buffer at 57°C for 60 min and subsequently with 55 mM iodoacetamide (Sigma) in 100 mM ABC buffer at room temperature for 60 min, followed by washing with 100 mM ABC buffer. After drying, the in-gel protein digests were conducted using sequencing-grade modified porcine trypsin (Promega, Madison, WI) in 50 mM ABC buffer at 37°C for 16 h. Then, the digests were centrifuged at 6,000 × *g*. The supernatants were separated, and the gel pieces were extracted further, first with 50% ACN, 5% formic acid and then with ACN. The extracts were combined with the original digesting supernatants, vacuum dried, and redissolved in 0.5% trifluoroacetic acid (TFA) and 50% ACN.

Electrophoresis was carried out as described previously (23) with some modifications. RAP-PCR samples were heated at 95°C for 3 min after addition of loading dye (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue). Subsequently, samples were loaded on a 6% denaturing polyacrylamide gel containing 7 M urea and electrophoresed in a standard sequencing apparatus (Bio-Rad). After silver staining, individual bands representing differentially expressed products were excised from the gel (13). Twenty microliters of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4°C for at least 2 h and subsequently centrifuged for 10 min (15,000 × *g*) at 4°C. The bacterial pellets were lyophilized and resuspended in 2-DE sample buffer (8 M urea, 4% CHAPS [3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate), 100 mM DTT, 2% 3/10 carrier ampholine [Amersham Biosciences]). The concentrations of the protein samples were determined using a Bradford Protein Assay Kit (Bio-Rad).

**SDS-PAGE**. Samples were heated for 5 min in boiling water, followed by protein separation by 12% SDS-PAGE. The protein bands were visualized by staining them with Coomassie blue R-250. SDS-PAGE separation of proteins from *Geobacillus* sp. E263 was biologically repeated three times.

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MALDI-TOF MS. A 1.5-μl aliquot was spotted onto a matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) sample plate with an equal volume of matrix. The matrix used was a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma) in 0.1% TFA and 50% ACN. The AnchorChip plate with protein samples was loaded onto a Bruker AutoFlex MALDI-TOF mass spectrometer (Bruker Daltonics). The mass spectrometer was operated under 19 kV accelerating voltage in the reflection mode with an m/z range of 800 to 4000 Da to reach a typical mass measurement accuracy of 100 ppm. MALDI-TOF spectra of the peptides were obtained with a time-of-flight delayed-extraction MALDI mass spectrometer (Bruker Autoflex). A nitrogen laser (337 nm) was used to irradiate the sample; the energy of the laser was controlled at about 20%, and the baseline of spectra less than 100 in intensity was accepted. All peptide mass fingerprints (PMFs) were externally calibrated. DNA sequencing was performed using standard peptide mixtures and internally calibrated using the mass of trypsin autolysis products (m/z = 842.509 and m/z = 2211.105) to reach a typical mass measurement accuracy of 100 ppm. The following parameters were used for database searches: one missed tryptic cleavage per peptide, carbamidomethylation of cysteine as a fixed modification, and oxidation of methionine and N-terminal pyroglutamyl (peptide) as variable modifications. All acquired spectra of samples were processed using Bruker Flexcontrol 2.4 software (Bruker Daltonics) in a default mode with an MS tolerance of 0.2 Da and a tandem MS (MS-MS) tolerance of 0.6 Da. Based upon mass signals, protein identification was performed using Mascot software (version 2.1; Matrix Science, London, United Kingdom) and GPS Explorer software (version 3.6; Applied Biosystems) against the NCBI NR database (release date, 31 March 2008) and the open reading frame (ORF) database of Geobacillus kaustophilus HTA426 on a local database, which was generated by a shotgun approach. The total ESTs of Geobacillus sp. E263 were formatted in FASTA, and a specific protein database of Geobacillus kaustophilus strain HTA426 was constructed. The mass signals were also taken for a search against the NCBI NR database with bacteria as the taxonomy. To eliminate the redundancy of proteins that appeared in the database under different names and accession numbers, the single protein member belonging to the species Geobacillus kaustophilus HTA426 or else with the highest protein score (top rank) was singled out from the multiprotein family. Northern blotting. Total RNAs were isolated from thermophilic Geobacillus sp. E263 using Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Takara, Japan) for 30 min at 37°C. After electrophoresis on a 1.2% agarose gel in 1× Tris-borate-EDTA (TBE) buffer, the RNAs were transferred to a nylon membrane (Amersham Biosciences). The blots were probed with the digoxigenin (DIG)-labeled gene of interest (see Table S1 in the supplemental material). In vitro RNA labeling, hybridization, and signal detection were carried out according to the manufacturer’s instructions for the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany).

Construction of a Geobacillus sp. E263 aspartate aminotransferase mutant (ΔAST). To understand the roles of genes in GVE2 infection, the Geobacillus sp. E263 AST gene, selected as the target gene, was inactivated by inserting a kanamycin nucleotidytranferase gene (kan) into their coding regions to construct the mutant. The insertion of a kan gene encoding a thermostable protein resistant to kanamycin facilitated screening for mutants. The kan gene was amplified from the E. coli-Geobacillus sp. E263 shuttle vector pMK18 with primers 5′-GGATCCCCTTCAGATATTAC-3′ and 5′-GGATCCTCTAGAGTCGAC-3′ (4) and inserted into the middle site of the target (AST) gene. Subsequently, the recombinant DNA fragment containing the disrupted target AST genes and the kan gene was obtained in quantity by PCR. The recombinant DNA fragment was transformed into Geobacillus sp. E263 cells by natural transformation at 60°C. The transformants were screened on TMM containing 40 μg/ml kanamycin at 60°C to obtain mutants. The mutants were further confirmed by Southern blotting, DNA sequencing, Northern blotting, and Western blotting.

Southern blotting. The genomic DNAs of Geobacillus sp. E263 and mutant strains were extracted with an S9 Tissue DNA Kit (Omega) (12). After partial digestion by EcoRI, the genomic DNAs were transferred onto a nylon membrane (GE Healthcare). Subsequently, the membrane was probed with a DIG-labeled kan fragment. The DIG labeling and detection were conducted according to the recommended protocol for the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany).

Protein recombinant expression in E. coli and antibody preparation. The AST gene of Geobacillus sp. E263 was cloned into the pET-28a vector (Novagen, Germany) and expressed in E. coli BL21 (DE3) as a 6-His-tagged fusion protein. The recombinant bacteria, confirmed by DNA sequencing, were induced by isopropyl-β-D-thiogalactoside (IPTG) when the optical density at 600 nm (OD_{600}) reached 0.5. After further incubation for 6 h at 37°C, the induced cells were harvested by centrifugation at 4,000 × g for 5 min. The recombinant protein was purified by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) resins under native conditions according to the recommended protocol (Qiagen).

The purified recombinant fusion protein was used as an antigen to immunize mice according to a standard procedure (8). The immunoglobulin (IgG) fraction of the antisera was purified with protein A-Sepharose (Bio-Rad) and stored at ~8°C until it was used. As determined by enzyme-linked immunosorbent assay (ELISA), the titer of the antisera was 1:10,000. The specificity of the antibody was confirmed by Western blotting with the recombinant protein.

Western blotting. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Bio-Rad) in electroblotting buffer (25 mM Tris, 190 mM glycine, 20% methanol) for 70 min. After being incubated in the blocking buffer (0.1% skim milk, 20 mM Tris, 0.9% NaCl, 0.1% Tween 20, pH 7.2) at 4°C overnight, the membrane was further incubated with polyclonal mouse anti-AST IgG for 3 h. Subsequently, the membrane was incubated in alkaline phosphate-conjugated goat anti-mouse IgG (Pierce Biotechnology, Inc.) for 1 h and detected with nitroblue tetrazolium chloride (NBT) and BCIP (5-bromo-4-chloro-3-indolylphosphate) solutions (Amresco Inc.).

Statistical analysis. The numerical data from biologically independent experiments were analyzed by one-way analysis of variance (ANOVA) to calculate the mean and standard deviation of repeated assays. Comparison of differences among the groups was carried out by Student’s t test. Significance was defined as P < 0.05.

RESULTS

Identification of differentially expressed Geobacillus sp. E263 proteins and genes involved in GVE2 infection. The overnight-grown cultures of Geobacillus sp. E263 were infected with GVE2 and incubated at 60°C (Fig. 1A). The cultures in late log phase were collected for protein electrophoresis. After GVE2 infection, the alterations of protein expression patterns of Geobacillus sp. E263 represented the bacterial responses to virus infection. To evaluate the bacterial proteins involved in virus infection, the proteins from GVE2-infected and virus-free Geobacillus sp. E263 cultures were subjected to SDS-PAGE analysis. The results showed that there were two kinds of protein profiles (Fig. 1B), one of which came from GVE2-infected culture and the other from GVE2-free culture. In order to reveal more differentially expressed proteins, the proteins of GVE2-infected and noninfected Geobacillus sp. E263 were analyzed by 2-DE. After protein separation, up to 400 polypeptide spots were detected by Coomassie staining. Quantitative intensity analyses revealed that 21 proteins of GVE2-infected Geobacillus sp. E263 were significantly upregulated or downregulated, which indicated a statistically significant difference (P < 0.05) in comparison with those of noninfected Geobacillus sp. E263 (Fig. 1C).

The major differential protein bands and spots were excised and subjected to mass spectrometric analysis. PMF was obtained by tryptic in-gel digestion and MALDI-TOF MS analysis. The PMF data from 5 different protein bands from SDS-PAGE and 21 different protein spots from 2-DE were searched in the database. This analysis presented the unambiguous identification of 17 unique proteins, covering 27 to 88% of amino acid sequences (Table 1). Among the 17 differentially expressed proteins, 11 proteins were significantly upregulated while 6 proteins were downregulated after GVE2 infection. The 17 differentially expressed proteins in response to GVE2 infection were proteins of interest related to virus infection.

To identify transcriptionally regulated cellular genes potentially involved in virus infection, the mRNA differential display patterns of GVE2-infected and noninfected Geobacillus sp. E263 were compared. By using 14 different arbitrary primers in the RAP-PCR analysis, a total of 5 unique gene fragments,
which were differentially regulated, were obtained from 27 RAP-PCR products sequenced, representing 5 differentially expressed cDNAs of *Geobacillus* sp. E263 involved in GVE2 infection (Fig. 1D). The sequences of all the RAP-PCR products shared no homology with rRNA genes. This information was helpful in avoiding artifacts for identifying differentially expressed genes efficiently.

**Transcription analyses of Geobacillus sp. E263 genes involved in GVE2 infection.** In an attempt to confirm the mass spectrometric and RAP-PCR data, the transcription analyses of genes differentially expressed between the GVE2-infected and noninfected *Geobacillus* sp. E263 were conducted by Northern blot analysis. After being labeled with DIG, the 17 genes encoding the differentially expressed proteins and 5 differentially expressed cDNAs were hybridized with the total RNAs extracted from GVE2-infected or noninfected *Geobacillus* sp. E263 cultured at different times. To facilitate quantitative analysis, the 16S rRNA gene was included in the hybridizations as a reference gene (2, 14, 16, 28).

As assayed by Northern blot analysis, it was found that, among 5 cDNAs obtained with RAP-PCR, 3 cDNAs were confirmed to be differentially expressed at the transcriptional level (Fig. 2 and Table 1). For the 17 differentially expressed proteins obtained with SDS-PAGE and 2-DE, the transcriptional analyses of their corresponding genes yielded results similar to those of protein electrophoresis in response to GVE2 infection (Fig. 2 and Table 1).

Among the 20 differentially expressed genes, 13 genes were confirmed to be upregulated and 7 genes were downregulated in response to GVE2 infection, while the transcription level of the 16S rRNA gene remained constant (Fig. 2 and Table 1), yielding results similar to those of protein electrophoresis and RAP-PCR. Therefore, the gene transcription analyses suggested that the 20 differential genes were involved in GVE2 infection.
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<th>Protein separation/mRNA differential display</th>
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<th>Size/mass (aa)</th>
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<th>Sequence coverage (%)</th>
<th>Intensity</th>
<th>Mowse score</th>
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*Note: The predicted molecular masses (in kilodaltons) and pIs of the sequence. aa, amino acids.*
FIG. 2. Transcription analyses of *Geobacillus* sp. E263 genes involved in GVE2 infection by Northern blotting. The total RNAs extracted from virus-free *Geobacillus* sp. E263 or GVE2-infected *Geobacillus* sp. E263 at different times (2, 4, 6, 8, and 10 h postinfection) were probed by 20 DIG-labeled genes in response to virus challenge. Each bar represents the mean of triplicate biological assays with ±1% standard deviation. The times are hours postinfection by GVE2. (A) Virus-free *Geobacillus* sp. E263. (B) GVE2-infected *Geobacillus* sp. E263.
infection, which was identical with those from protein electrophoresis and RAP-PCR.

Categorization of bacterial proteins in response to virus infection at high temperature. Based on homology searches using BLAST and PROSITE analyses in GenBank, 19 of the 20 proteins involved in GVE2 infection shared homology with known proteins, except for a hypothetical protein revealed in spot 20 (Table 1). The 19 proteins could be classified into 3 functional categories based on their functional significance. The most abundant category was related to energy metabolism, including proteins involved in the tricarboxylic acid cycle. They were aspartate aminotransferase (GE1), pyruvate kinase (GE5), δ-adenosynmethionine synthetase (GE8), phosphatase acetyltransferase (GE9), succinyl-coenzyme A (CoA) synthetase (GE11), dehydrogenase E1 component (GE12), glyceraldehyde-3-phosphate dehydrogenase (GE14), triose-phosphate isomerase (GE16), tetrahydrodipicolinate succinylase (GE17), peroxiredoxin (GE18) (also involved in the OxyR oxidative stress response), and intracellular proteinase (GE20). It was found that the two-component response regulator (GE2), 30S ribosomal protein (GE4), chaperonin GroEL (GE6), elongation factor Tu (GE10), and elongation factor Ts (GE15) formed the second category, which was concerned with replication. The third category was the proteins concerned with matter and energy transportation, such as the ferrichrome ABC transporter (GE3), maltose transport system (GE7), and ferric ion ABC transporter (GE15). Interestingly, the three transportation proteins were all downregulated in response to GVE2 infection.

Function of aspartate aminotransferase in GVE2 infection. To further characterize these proteins in response to virus challenge, AST was selected as the target protein for this investigation. The gene encoding AST, a key enzyme in metabolism involved in the transfer of an amino group, was upregulated in response to GVE2 infection.

To understand the role of AST in virus infection, mutant analysis was conducted. The AST gene of Geobacillus sp. E263 was inactivated by inserting a kanamycin resistance gene (kan) in its coding region to construct an AST mutant of Geobacillus sp. E263 (ΔAST). After being screened on TTM broth medium containing kanamycin three times at 60°C, many AST mutants were obtained. To confirm the ΔAST mutant, its genomic DNA was probe by a DIG-labeled kan gene. Only one positive band was observed by Southern blot analysis (Fig. 3A), indicating that the kan gene was inserted into the Geobacillus sp. E263 genome. The ΔAST strain was further confirmed by DNA sequencing, showing the correct insertion of the kan gene, as expected. In order to detect the transcription and expression of the AST gene in the ΔAST strain, Northern and Western blots were performed. Northern blot analysis detected the mRNA of the AST gene in wild-type Geobacillus sp. E263, but not in the ΔAST mutant (Fig. 3B), indicating that the AST gene was inactivated in ΔAST. As demonstrated by Western blot analysis, the protein encoded by the AST gene was detectable only in the wild-type strain (Fig. 3C), showing that there was no expression of the AST protein in the ΔAST mutant. Under the conditions of AST gene inactivation revealed above, the wild-type and ΔAST Geobacillus sp. E263 were infected with GVE2 at 60°C. Subsequently, quantitative real-time RT-PCR analyses were used to detect the copies of GVE2 in the wild-type and ΔAST strains at various infection stages (1, 2, 3, 4, 5, 6, 7, 8, and 9 h postinfection). The results showed that the GVE2 copies were significantly decreased in the ΔAST strain in response to virus challenge by comparison with those in the wild-type strain (Fig. 3D). These data demonstrated that the AST protein played a very important role in GVE2 infection of Geobacillus sp. E263 at high temperature.

To evaluate the effect of the AST protein on the ΔAST strain in response to GVE2 infection, the AST gene of Geobacillus sp. E263 was expressed in E. coli as a fusion protein with a 6-His tag. After induction with IPTG, the recombinant AST protein was purified (Fig. 3E) and the medium was supplemented with it at a final concentration of 0.25 μg/ml for culturing the ΔAST Geobacillus sp. E263. Then, the wild-type and ΔAST Geobacillus sp. E263 strains were infected with GVE2 at 60°C. Based on the quantitative real-time RT-PCR results, it was found that the GVE2 of the ΔAST strain slowly recovered its replication and infection abilities when the medium was supplemented with the recombinant AST protein (Fig. 3F). These data clearly showed that AST was essential for virus infection. Therefore, it could be inferred that the AST protein plays an important role in the course of virus infection at high temperature.

DISCUSSION

Deep-sea hydrothermal vents are believed to play important roles in the ocean’s temperature, chemistry, and circulation patterns. In the deep-sea ecosystems, thermophilic chemosynthetic prokaryotes exploit the vent chemicals to obtain energy for their growth, and other organisms in the environment are supported by on-site production by chemosynthetic thermophiles (10, 21). As important agents for thermophile mortality, the thermophilic viruses infecting thermophiles are considered to be the major players in ecological and geochemical processes in the deep-sea vents. Viruses can shape microbial community structure and function, while hosts can integrate short stretches of phase-derived sequences (spacers) within the clustered regularly interspaced short palindromic repeat (CRISPR) loci to become virus resistant (1, 5). In this context, the interaction between thermophilic microbial inhabitants and viruses in deep-sea hydrothermal vents is of great importance (21, 22). To date, however, this concern has not been addressed. Essential to the analysis of virus-host interaction is the identification of the proteins/genes involved in virus infection. In the present study, based on a comparison of protein/gene expression profiles of GVE2-infected and noninfected Geobacillus sp. E263 bacteria, 20 Geobacillus genes were revealed to be responsible for virus infection at high temperature. Therefore, our study provides a clue to understanding host-virus interaction in the deep-sea vent ecosystems.

The 20 Geobacillus genes with significant regulation had diverse metabolic functions at high temperature. GVE2 might either harness the host cell for its own reproduction by efficient takeover and reprogramming of the host physiology or replicate within the cell without affecting major biosynthetic pathways during the period of progeny production. The latter strategy might ensure virus propagation without the induction of host defense mechanisms, while the first strategy likely activates host responses. The induction of host defense systems would probably have an adverse effect on phage progeny pro-
duction and decrease phage fitness (19). It could be inferred that the direct stress response to virus infection might be ensured by regulating the expression of key proteins relevant to energy metabolism, regulatory adaptive and transportation proteins involved in the tricarboxylic acid cycle (TCA).

In the present study, AST was selected as a target protein for its importance in protein processing (9). Its role in virus infection of thermophiles at high temperature is not documented. Our study demonstrated that AST was crucial in GVE2 infection of Geobacillus sp. E263. AST might function to catalyze a reversible transamination reaction between the dicarboxylic R-amino and R-keto acids. Interestingly, it was evidenced that the chaperonin GroEL and the elongation factor EF-Ts were upregulated in response to virus challenge. Molecular chaperones, including the heat shock proteins, are a ubiquitous feature of cells, in which these proteins cope with stress-induced denaturation of other proteins (19, 20). In this report, a molecular chaperonin, GroEL, was revealed to be involved in GVE2 infection. Upregulation of the chaperonin GroEL in the host could be due to the presence of misfolded or aggregated proteins under stress conditions. The elongation factors EF-Tu and EF-Ts have been found to bind tightly to the viral RNA-dependent RNA polymerase and have a major function in the delivery of aminoacyl-tRNA (aa-tRNA) to the A site on the ribosome during the translation of mRNA into protein (18). In our study, the upregulation of EF-Ts in the GVE2-infected Geobacillus sp. E263 suggested that EF-Ts plays an important role in the translation of GVE2 viral mRNA. Considering the very limited data on mesophilic bacteriophage-host interaction, our study of bacteriophage-bacterium interaction at high temperature has contributed novel information to understand the molecular events in the host in response to virus challenge. To delineate the virus-host interaction, future studies need to focus on the pathways of virus infection using molecular approaches, for example, protein interaction strategies. In addition, subsequent experiments need to examine the differential expression of host proteins at different developmental stages to identify more genes involved in GVE2 infection.
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REFERENCES