Roles of bacteriophage GVE2 endolysin in host lysis at high temperatures

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The holin–endolysin system is used by double-stranded DNA phages to lyse their bacterial hosts at the terminal stage of the phage reproduction cycle. Endolysins are proteins with one of several muraletic activities able to digest the bacterial cell wall for phage progeny release. However, the functions of thermophilic bacteriophage endolysin in host lysis have not been extensively investigated. In this study, the roles of the endolysin of a thermophilic bacteriophage, GVE2, from a deep-sea hydrothermal vent, which could infect Geobacillus sp. E263 at high temperatures, were characterized. The results showed that GVE2 could lead to lysis of host cells. The confocal microscopy data showed that GFP–endolysin aggregated in GVE2–infected Geobacillus sp. E263 cells, showing the involvement of endolysin in the lysis process at high temperatures. The results revealed that the GVE2 endolysin and holin interacted directly. It was found that the endolysin could interact with the host protein ABC transporter, suggesting that host proteins might participate in the regulation of the lysis process. Therefore, our study presents a novel insight into the mechanism of the lysis process of a thermophilic bacterium by its phage at high temperatures, which should be helpful in revealing the roles of thermophilic bacteriophages in the biosphere of deep-sea hydrothermal vents.

INTRODUCTION

Bacteriophages with double-stranded nucleic acids achieve lysis of their hosts with a holin–endolysin system (Young, 1992; Young et al., 2000). For the most extensively studied lambda phage and T4 phage, endolysins accumulate in the cytosol harmlessly in a fully folded and active form during the vegetative cycle. At a genetically determined time point, holins aggregate to form oligomers and disrupt the inner membrane, thus allowing endolysins to be released and to degrade the cell wall of infected cells (Tran et al., 2007; Young et al., 2000). It has been reported that the holes formed by lambda holin are irregular and of unprecedented size (>300 nm) (Dewey et al., 2010; White et al., 2011), which is large enough to allow escape of fully folded tetrameric R-β-galactosidase chimeras (>450 kDa) (Wang et al., 2003). But in the case of lambda 21 and P1 phages, endolysins are exported by the host sec system using an N-terminal signal anchor-release (SAR) sequence. Endolysins accumulate in the periplasm tethering to the membrane in an enzymically inactive form until the membrane is disrupted by the holin. They are then released from the membrane to the cell wall and convert into an enzymically active form (Xu et al., 2004, 2005). The regulation of endolysins bearing the SAR domain from the membrane-tethered inactive form to the active form has been reported (Baase et al., 2010; Kuty et al., 2010; Sun et al., 2009).

Evidence has accumulated that lysis is a precisely scheduled process during phage infection. The timing is genetically programmed in the holin structure (Chang et al., 1995; Wang et al., 2000). holin genes of many phages have a novel translational initiation region with dual start codons, resulting in the production of two protein products: the lysis effectors (holins) and their inhibitors (antiholins). The antiholins are identical to the holins except for two or more additional amino acids present on the N terminus of antiholins. Antiholins can regulate the hole forming by inhibiting holin oligomerization (Pang et al., 2010; Park et al., 2006).

So far, considerable advances have been made in revealing the lysis process of host bacteria by double-stranded DNA phages. However, most of these studies have been performed in mesophilic bacteria. Little is known about the lysis process of thermophilic bacteria in a high temperature environment, and particularly of thermophilic bacteria isolated from deep-sea hydrothermal vents. In deep-sea vent ecosystems, thermophilic viruses are a major cause of vent thermophiles’ mortality (Wei & Zhang, 2010). They have a significant impact on the community composition of deep-sea ecosystems (Reysenbach & Shock, 2002). Thus, it is necessary to characterize the lysis process
of thermophilic bacteria by their phages in order to elucidate the effect of bacteriophages on the biosphere in deep-sea hydrothermal vents.

To address this issue, GVE2, a deep-sea thermophilic bacteriophage isolated from its host Geobacillus sp. E263, was characterized in this study. Our previous studies revealed that GVE2 was a virulent tailed Siphoviridae bacteriophage, which contained a 40,863 bp linear double-stranded genomic DNA (GenBank accession no. DQ453159) (Liu & Zhang, 2008). The muralytic activities of GVE2 endolysin were characterized previously (Ye & Zhang, 2008). In this investigation, the results showed that the GVE2 endolysin played important roles in the lysis process of Geobacillus sp. E263, providing us with a novel insight into the mechanism of the lysis process of a host bacterium by its phage at high temperatures.

METHODS

Strain, phage and growth conditions. The thermophilic Geobacillus sp. E263 strain was isolated from a deep-sea hydrothermal field in the east Pacific (12° 42' 29" N, 104° 02' 01" W) (Liu et al., 2006). Phage plaques were observed during the culturing of Geobacillus sp. E263, which was grown at 60 °C with shaking (150 r.p.m.) in TTM medium (0.2% NaCl, 0.4% yeast extract, 0.8% tryptone, pH 7.0). The thermophilic GVE2 bacteriophage was purified from virus-infected Geobacillus sp. E263 by CsCl gradient centrifugation (Liu et al., 2006). In brief, an overnight Geobacillus sp. E263 culture was infected with GVE2 at 55 °C for 30 min to ensure the most effective GVE2 infection, then diluted 100-fold with fresh TTM medium, and subsequently subjected to shaking at 60 °C for 4–6 h until lysis was complete. After removing the bacterial cells and debris by centrifugation at 6000 g for 30 min, the supernatant was supplemented with PEG6000 at a concentration of 10% and then incubated at 4 °C overnight to precipitate the virus particles. The precipitated virus particles were spun down by centrifugation at 40 000 g for 1 h and were subsequently suspended in SM buffer (100 mM NaCl, 8 mM MgSO4·7H2O, 50 mM Tris/HCl, pH 7.5). The suspension was subjected to CsCl gradient centrifugation at 150 000 g for 24 h. The phage band was collected and dialysed against SM buffer twice for 1 h.

Turbidity assay. A turbidity assay was conducted to monitor the growth of the GVE2-infected or non-infected Geobacillus sp. E263 by measuring the optical density of the bacteria. When the OD600 of the bacteria reached 0.3, the purified GVE2 at an m.o.i. of 5.

Quantitative real-time PCR. To quantify the GVE2 genome copies, the real-time PCR was carried out using the GVE2-specific TaqMan fluorogenic probe (5′-FAM-CCGTCTTTGCCTGTGCTCTGC-Eclipse-3′) and GVE2-specific primers (5′-ATCGGTTGTAGTAACCTTAC-3′ and 5′-GCTGTCTGTATTTCCCTAT-3′). GVE2-infected Geobacillus sp. E263 cells were harvested by centrifugation at 7000 g for 10 min and lysed at 99 °C for 20 min to obtain the total DNA of the virus-infected Geobacillus sp. E263. The real-time PCR mixture (10 μl) included 1 mM TaqMan probe, 1 mM each primer, 1× Premix ExTaq reaction buffer (Takara) and the total DNA of the virus-infected Geobacillus sp. E263. The procedure used to amplify the GVE2 genome was as follows: 95 °C for 30 s, followed by 5 s at 95 °C and 30 s at 60 °C for 45 cycles.

Bioinformatic analysis. The sequence similarities and protein domains of GVE2 endolysin and holin were analysed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The N terminus of GVE2 endolysin was characterized to reveal the N-terminal SAR sequence using SignalP (http://www.cbs.dtu.dk/services/SignalP-2.0/). The TMHMM server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict the trans-membrane domain of GVE2 holin.

DNA manipulations. Restriction enzymes were purchased from Takara (Japan), and restriction of DNA was performed according to the manufacturer’s instructions. A spin miniprep kit (Qiagen) was used according to the manufacturer’s recommendations to obtain plasmid DNA. Gel-purified DNA fragments for cloning were excised from agarose gels and purified with a gel extraction kit (Omega). Ligation of DNA was performed using a DNA ligation kit (Takara).

The endolysin gene was amplified from the GVE2 genomic DNA with a forward primer (5′-GGGAGCTCAAGGGAGAGTCGTTTTGTTGCTC-3′) and a reverse primer (5′-GGGATCCCTGTTGCTTCATCCCTACG-3′), which contained the recognition sequences for BamHI and EcoRI (indicated by italic text). The full-length holin gene was amplified from GVE2 genomic DNA using a forward primer (5′-GCGATCCATGGAAGCAATCTTATC-3′) and a reverse primer (5′-GGGGAGGAGGAGGGTCGTTTTGTTGCTC-3′), which contained the restriction sites for BamHI and EcoRI (indicated by italic text). The forward primer (5′-AATTGATCAGGGAGGAGGAGGC-3′) and a reverse primer (5′-AATTCGAGATCGATCCATGGAAGCAATCTTATC-3′) were used to amplify the periplasmic domain (217–261 bp) of the holin gene. Then the three amplicons were cloned into pGEX-4T-2 (Invitrogen).

To make the pNW33N-sgE Endolysin-GFP construct, the thermosensitive sgE promoter (Novotny et al., 2008), the GVE2 endolysin gene, an intervening linker encoding 9 amino acids (GGGGSGGGG) and the thermostable GFP gene were cloned into the NAP3 vector (from the Bacillus Genetic Stock Center, Columbus, Ohio, USA). The linker was placed between the endolysin and GFP. The sgE promoter was amplified from Geobacillus sp. E263 genomic DNA with a forward primer (5′-GCGAAGCTTTTATGGAAGCAATCTTATC-3′) and a reverse primer (5′-GATGATCAGGGAGGAGGAGGGTCGTTTTGTTGCTC-3′), which contained the recognition sites for HindIII and BamHI (indicated by italic text). The endolysin gene with a linker sequence (5′-GGGAGGAGGAGGGTCGTTTTGTTGCTC-3′) was amplified from GVE2 genomic DNA using a forward primer (5′-GGCAACGGATCCCACTTATC-3′) and a reverse primer (5′-GGATGATCAGGGAGGAGGAGGGTCGTTTTGTTGCTC-3′), which contained the recognition sequences for BamHI and PstI (indicated by underlined text) and EcoRI (indicated by italic text), which were used to amplify the periplasmic domain (217–261 bp) of the holin gene. Then the three amplicons were cloned into pGEX-4T-2 (Invitrogen).

Recombinant expression in Escherichia coli, protein purification and antibody preparation. For overexpression and purification of glutathione S-transferase (GST)-tagged endolysin, holin and the periplasmic domain of holin, the recombinant plasmids were transformed into E. coli (BL21 DE3) and induced with 0.5 mM
IPTG for 15 h at 16 °C. The bacteria were collected and sonicated for 15 min at a pulse frequency of 5 s/s. After centrifugation for 15 min at 15 000 g, the supernatant was collected. Then the recombinant proteins were purified with glutathione-Sepharose beads (Sigma) according to the manufacturer’s instructions. The purified recombinant proteins were incubated with thrombin to remove the GST. The purified proteins without GST were used as antigens to raise antibodies. Antibodies were incubated with thrombin to remove the GST.

GVE2-proteins were purified with glutathione-Sepharose beads (Sigma) at 15 000 rpm for 15 min at a pulse frequency of 5 s/5 s. After centrifugation for 15 min, the supernatant was collected. Then the recombinant proteins were purified with glutathione-Sepharose beads (Sigma). The slides were examined under a Leica TCS SP5 confocal microscope. LAS AF version 2.0.0 software was used to analyse and deconvolve the acquired digital images.

**Northern blotting.** An overnight Geobacillus sp. E263 culture was infected by purified GVE2 (m.o.i.=5) at 55 °C for 15 min, then subjected to shaking at 60 °C after 100-fold dilution with fresh TTM medium. An equal amount of GVE2-infected Geobacillus sp. E263 was harvested at various times (0, 1, 2, 3, 4, 5 and 6 h post-infection). After treatment with 5 mg lysozyme ml⁻¹ at 37 °C for 30 min, total RNAs were extracted from the collected GVE2-infected Geobacillus sp. E263 at different times using 1 ml TRzol reagent (Promega) according to the manufacturer’s instructions. Then the total RNAs were treated with RNase-free DNano I (Takara) for 30 min at 37 °C, separated by electrophoresis on a 1.2 % agarose gel in 1 × TBE buffer (90 mM Tris-boroc acid, 2 mM EDTA, pH 8.0), and then transferred to a nylon membrane (Amersham Biosciences). The blots were probed with DIG-labelled holin probe (5'-DIG-GAAACAAGTG-GGGGGCCGATTTGATTGAT-3') or DIG-labelled endolysin probe (5'-DIG-ACGGCATATGTTGGCGGTTGCTTGCT-3'). In vitro RNA labelling, hybridization and signal detection were performed with a DIG high prime DNA labelling and detection starter kit II according to the manufacturer’s instructions (Roche).

**SDS-PAGE and Western blotting.** Protein samples were routinely analysed by Tris/glycine SDS-PAGE. Tris/Tricine 16 % or 10 % SDS-PAGE was also used routinely according to the method of Schagger and Von Jagow (Schagger & Von Jagow, 1987). Proteins were visualized with Coomassie blue. For analysis of GVE2 endolysin or holin gene expression by Western blotting over time, GVE2-infected Geobacillus sp. E263 cells collected at different times (0, 1, 2, 3, 4, 5 and 6 h post-infection) were boiled at 99 °C for 15 min upon mixing with equal volume of 2 × SDS loading buffer (20 mM Tris/HCl, pH 8.0, 100 mM DTT, 2 % SDS, 0.016 % bromophenol blue). The obtained protein samples and prestained molecular mass standards were resolved on Tris/glycine SDS-PAGE, then transferred to a nitrocellulose membrane under 70 V for 70 min. The membrane containing the transferred proteins was probed with anti-endolysin or anti-holin antibody after blocking with 2 % skimmed milk in PBS buffer. Then the membrane was incubated with alkaline phosphatase (AP)-coupled goat anti-mouse IgG. After several washes with water, the membrane was incubated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) for colorimetric detection.

**Fluorescence microscopy.** In order to localize the viral endolysin during the lysis of host bacterium at high temperature, the pNW33N-sgsE-endolysin-GFP construct was used. The Geobacillus sp. E263 was transformed with the recombinant plasmid by electroporation using a Bio-Rad electroporator at 15 kV cm⁻¹ for 2.5 ms. The plasmid containing sgsE-promoter-controlled GFP was also included in the transformation as a control. Overnight culture of Geobacillus sp. E263 harbouring endolysin–GFP was diluted with TTM medium by 100-fold, and incubation continued at 55 °C with shaking. When the OD₆₀₀ reached 0.6, the bacteria were infected with purified GVE2 at an m.o.i. of 5. As controls, the bacteria containing endolysin–GFP were treated with the SM buffer that was used to suspend the GVE2 virions. For imaging, Geobacillus sp. E263 cells collected at different times (0, 15, 30, 60, and 120 min post-infection) were washed with PBS buffer several times, followed by immobilization on 1 % agarose-covered slides (Sigma), and further incubated for 2 h at 50 °C. The slides were examined under a Leica TCS SP5 confocal microscope. LAS AF version 2.0.0 software was used to analyse and deconvolve the acquired digital images.

**Far-Western assay.** The purified GST–holin, GST and endolysin were resolved by 15 % Tris/glycine SDS-PAGE, then transferred to a nitrocellulose membrane in electrophoresing buffer (25 mM Tris, 190 mM glycine, 20 % methanol) at 70 V for 70 min. The nitrocellulose membrane containing the transferred proteins was blocked with purified endolysin (0.1 mg ml⁻¹) in blocking buffer (2 % BSA, 20 mM Tris, 150 mM NaCl, 0.1 % Tween 20, pH 7.5) at room temperature for 2 h. The membrane was incubated with antibody against endolysin for 1 h, washed with water several times, and then incubated with AP-coupled goat anti-mouse IgG for 2 h. Finally the membrane was treated with NBT and BCIP for colorimetric detection.

**Bacterial two-hybrid assay.** Bacterial two-hybrid assays were performed using the BacterialMatch two-hybrid system kit II (Stratagene) according to the manufacturer’s manual. Protein–protein interactions were detected based on transcriptional activation of the His3 reporter gene, which allows cells to grow in the selective medium supplemented with 3-amino-1, 4-triazole, a competitive inhibitor of His3 enzyme. A second reporter gene, aadA, which conferred streptomycin resistance, was used to verify the positives. The testing fragments of pair-wise combinations of genes encoding whole proteins or different predicted protein domains were cloned into vector pBT and pTRG (Stratagene), respectively, followed by co-Transformation of BacterialMatch two-hybrid system reporter strain competent cells (Stratagene) with the two recombinated vectors. The transformants were grown on both non-selective plates (supplemented with 25 µg chloramphenicol ml⁻¹, 12.5 µg tetracycline ml⁻¹) and selective plates (supplemented with 25 µg chloramphenicol ml⁻¹, 12.5 µg tetracycline ml⁻¹ and 5 mM 3-amino-1,2,4-triazole) at 37 °C for subsequent screening for resistance to 3-amino-1,2,4-triazole. The positive clones were further verified on the streptomycin-complemented media.

**GST pull-down assays.** The purified GST and GST–endolysin proteins were incubated with glutathione beads for 2 h at 4 °C. Cells from an overnight culture of Geobacillus sp. E263 were collected by centrifugation at 6000 g for 15 min, followed by resuspension with 10 ml GST binding buffer [200 mM NaCl, 20 mM Tris/HCl, 1 mM EDTA (pH 7.6) and 1 mM PMSF]. The suspension was sonicated for 15 min at the pulse frequency of 5 s/s, and subsequently subjected to centrifugation at 15 000 g for 15 min. The obtained supernatant was incubated with GST–endolysin or GST-coupled glutathione beads for 4 h at 4 °C with gentle rotation. Non-specific binding proteins were removed by five successive washes with GST binding buffer. Then the bound proteins were eluted with elution buffer (10 mM glutathione, 50 mM Tris/HCl, pH 8.0) and resolved by SDS-PAGE.

**MS.** The visible protein bands of interest from the SDS-PAGE gel were excised and subjected to MS analysis as previously described (Wei & Zhang, 2010). Briefly, after digestion with trypsin, the obtained peptides were analysed with a matrix-assisted laser desorption ionization (MALDI) mass spectrometer equipped with a time-of-flight (TOF) mass analyser (Bruker Daltonics). Bruker FlexControl 2.4 operation software (Bruker Daltonics) was used to process the obtained sample spectra in a default mode with an MS tolerance of 0.2 Da and a tandem MS tolerance of 0.6 Da. Mascot database of HTA426 in a local database. The database was searched the results against the National Center for Biotechnology Information non-redundant protein sequences database and the ORF database of Geobacillus kaustophilus HTA426 in a local database.
RESULTS

Lysis of Geobacillus sp. E263 by its bacteriophage GVE2

To characterize the role of GVE2 infection on the growth of Geobacillus sp. E263, the host cells were infected with purified GVE2 virions at 55°C when the optical densities (OD600) of the bacteria reached 0.3 (at 3.3 h of culture of Geobacillus sp. E263). The results of turbidity assays showed that the growth rate of GVE2-infected Geobacillus sp. E263 was significantly decreased compared with that of non-infected host cells, indicating the host lysis by GVE2 (Fig. 1a). The time-course detection of GVE2 genome copies revealed that the copies of GVE2 genomic DNA were increased during the virus infection process (Fig. 1b). The lysis of host cells by GVE2 occurred at 0.5–1 h post-infection. The above data are indicative that the infection of Geobacillus sp. E263 by GVE2 led to the lysis of host cells.

Temporal analyses of endolysin and holin gene expressions in vivo at high temperatures

Based on sequence analysis, a lytic cassette encoding endolysin and holin was found in the genome of GVE2 (Fig. 2a). To analyse the expressions of endolysin and holin genes in host cells, the full-length endolysin gene and a fragment of holin gene (217–261 bp) that encoded the predicted periplasmic domain of holin were expressed in E. coli. The respective antibodies against endolysin or the holin protein fragment were prepared. Proteins extracted from GVE2-infected Geobacillus sp. E263 cultured at 60°C at various time points (0, 1, 2, 3, 4, 5 and 6 h post-infection) were analysed by Western blotting to investigate the expression of the endolysin and holin proteins in vivo at high temperatures. The results showed that the endolysin protein could be detected at 1 h post-infection and was maintained at almost the same level from 1 to 6 h post-infection (Fig. 2b). The holin was detected from 1 to 6 h post-infection (Fig. 2c).

In order to investigate the transcriptional levels of the endolysin and holin genes in vivo at high temperatures, Northern blotting was performed to detect endolysin- or holin-specific transcripts at various infection stages (0, 1, 2, 3, 4, 5 and 6 h post-infection). As a control, 16S rRNA-specific transcript was also detected. The results were consistent with the Western blot results (Fig. 2b, c).

Distribution of endolysin in host cells at high temperatures

Due to the expected importance of endolysin in the lysis process of host cells, the endolysin was further characterized. Toward this end, we constructed a GVE2 endolysin fusion to a thermostable GFP and analysed its subcellular distribution during infection of the host bacterium with GVE2. Here, Geobacillus sp. E263 was transformed with the endolysin–GFP fusion (under control of the thermosensitive sgsE promoter). Transformants were then analysed by fluorescence microscopy at the indicated time points after infection with GVE2. The results showed that the
endolysin–GFP could be seen well distributed throughout the cytoplasm of *Geobacillus* sp. E263 cells from 15 to 120 min post-infection (Fig. 3a). However, a number of brighter fluorescence foci were observed in the cells at division and polar positions at 30 min post-infection onward (Fig. 3a), but not in the cells without infection (Fig. 3b). These foci showed an accumulation of endolysin–GFP at those positions, suggesting the formation of complex between endolysin and host or viral proteins. As a control, the GFP alone under the control of the *sgsE* promoter was expressed in *Geobacillus* sp. E263 cells that were subsequently infected by GVE2. It revealed that the GFP was distributed throughout the cytoplasm of cells at 60 min after infection of GVE2 and no accumulated GFP was observed (Fig. 3c).

**Interaction between GVE2 endolysin and viral holin**

Considering the well-described roles for endolysins and holins in bacterial lysis, we predicted interactions between the GVE2 endolysin and holin. In this light, a far-Western assay was first performed to test the hypothesis. The purified endolysin, GST–holin and GST proteins were transferred to a nitrocellulose membrane, followed by incubation with endolysin and subsequent detection with endolysin-specific antibody. The results showed that a band of about 35 kDa corresponding to the size of GST–holin was present in the GST–holin lane (Fig. 4a, lane 1), while the band could not be detected in the GST sample (Fig. 4a, lane 2). These findings indicated that endolysin and holin could interact directly.

To characterize the endolysin–holin interaction, a bacterial two-hybrid system assay was performed. Fragments of the *endolysin* or *holin* genes encoding different predicted domains were cloned into vectors pBT and pTRG, respectively (Fig. 4b). Control clones, in which only one fusion construct was present, yielded no colonies on selective plates (data not shown). By contrast, colonies containing pair-wise combinations of *endolysin* and *holin* grew on selective plates, providing strong support that endolysin and holin could interact directly (Table 1). Some positive colonies were subjected to DNA sequencing to confirm the constructs. Further screening of the interactive domains between endolysin and holin by bacterial two-hybrid system revealed that the interactions occurred within the full-length holin and an 81–233-amino-acid region of endolysin (Table 1). The data suggested that the viral endolysin and holin directly interacted during the lysis process of the host at high temperatures.

**Interactions between GVE2 endolysin and host proteins**

Besides the interaction between GVE2 endolysin and holin, host proteins might also participate in the lysis process. So we focused on the identification of host proteins that potentially interact with viral endolysin using endolysin–GST pull-down assays. The GST pull-down results revealed
that a band of about 30 kDa was observed in the lane corresponding to the GST–endolysin (Fig. 5a, lane 2), but not in the lane of the GST control (Fig. 5a, lane 4). This band was further identified as the ABC transporter by MALDI-TOF MS.

The bacterial two-hybrid assay was used to test the interactions between GVE2 endolysin and the ABC transporter. In contrast to controls, in which only one fusion construct was present (data not shown), transformants containing pair-wise combinations of endolysin and ABC transporter genes grew on selective plates, confirming the interactions between endolysin and the ABC transporter (Fig. 5b). The results showed that the viral endolysin and host ABC transporter might form a complex during the lysis of host by bacteriophage at high temperatures.

**DISCUSSION**

In deep-sea hydrothermal vents, the foundation of the food chain is dependent on thermophilic chemosynthetic microbes, which exploit the vent chemicals to produce energy for their growth and support other organisms in the ecosystem (Karl, 1995). As reported elsewhere, a high diversity of viruses, including bacteriophages, has been found in deep-sea hydrothermal vents (Liu et al., 2006; Ortmann & Suttle, 2005). The bacteriophages have a significant effect on the vent community composition by causing mortality of the thermophilic bacteria and thus disrupting the food chain foundation of the vent ecosystem (Reysenbach & Shock, 2002). In this context, studies on the lysis process of thermophilic bacteria by their phages will reveal new insights of the effects of the thermophilic
bacteriophages on the biosphere in deep-sea hydrothermal vents. To date, however, the lysis mechanism of a thermophilic bacteriophage in a high-temperature environment has not been investigated. In this study, the endolysin and holin of GVE2, a thermophilic bacteriophage from a deep-sea vent (Wei & Zhang, 2010), were characterized. The results showed that the viral endolysin plays important roles in the lysis process of bacteria in a high-temperature environment. Our study presents what is believed to be the first report that has characterized in depth the endolysin of thermophilic bacteriophages in deep-sea hydrothermal vents. It provides us with a new point of view for understanding the lysis process of hosts by thermophilic bacteriophages at high temperatures.

The lysis strategies taken by mesophilic bacteriophages to lyse hosts have been investigated. For mesophilic bacteriophages, holin and endolysin are found to efficiently cause host lysis (Young et al., 2000). For mesophilic bacteriophages of Gram-negative bacteria, two overlapping phage genes encoding spinning complex are required when high concentrations of divalent cations are present (Summer et al., 2007). It has been reported that the endolysin and holin of mesophilic bacteriophages are non-specific partners, since in vivo complementation data have shown that any holin and any endolysin can cooperate, even holin and endolysin pairs from mesophilic phages of Gram-positive and Gram-negative hosts (Steiner et al., 1993). In this study, it was revealed that endolysin and holin from thermophilic bacteriophage GVE2 could interact with each other, and the interaction occurred between the full-length holin protein and a region of endolysin between amino acids 81 and 233. According to prediction, this region contains a cell wall-binding domain and part of a catalytic domain. Three conserved catalytic residues (Glu-90, Lys-157 and His-169) were present in the aa 81–233 region of the GVE2 endolysin, but not the lysin residue at position 29 (Ye & Zhang, 2008). The results of our study showed that the host ABC transporter could interact with GVE2 endolysin, suggesting that host proteins might participate in regulation of the lysis process. To date, however, no host proteins have been found to interact with mesophilic bacteriophage endolysin. ABC transporters are integral membrane proteins that can hydrolyse ATP to ADP to obtain energy and can actively transport molecules across the lipid membrane. It has been reported that a diverse range of molecules can be transported across membranes by ABC transporters, ranging from small compounds, such as ions and amino acids, to larger compounds, such as lipids, oligopeptides and drugs (Moussatova et al., 2008). In this study, the ABC transporter that interacted with the GVE2 endolysin belonged to the eukaryotic type ABC transporter type based on classification of ABC transporters (Igarashi et al., 2004). During the lysis process, the GVE2 endolysin might be exported with the help of ABC transporters.

The interactions between GVE2 endolysin and GVE2 holin or the host’s ABC transporters might be part of the mechanism by which the GVE2 endolysin could function at high temperatures. Several endolysins from mesophilic bacteriophages have been reported to possess the feature of extreme thermoresistance (up to 90 °C) (Walmagh et al., 2012). The thermoresistance of this group of endolysins...
may be due to rapid refolding of endolysins upon cooling, as suggested by Schmelcher and coworkers (Schmelcher et al., 2012). But no conserved motif was found between GVE2 endolysin and thermostable endolysin from mesophilic bacteriophages according to BLAST analysis.

The time-course of bacterial lysis is tightly regulated by phages for effective phage progeny production and release. For lambda phage and many other mesophilic bacteriophages, the sensitive regulation is partly due to a novel translational initiation region with dual start codons, resulting in two functionally opposite proteins: holins and antiholins (Chang et al., 1995; Tran et al., 2005). In thermophilic bacteriophage GVE2, by contrast, the holin gene did not have canonical dual start codons. Sequence analyses revealed that GVE2 endolysin did not bear an N-terminal SAR sequence. Therefore, unlike the endolysins from mesophilic bacteriophages lambda 21 and P1, the GVE2 endolysin might not be exported by the host’s sec system. Taken all together, it is possible that GVE2 might use a novel lysis mechanism. Further investigation is merited to test the hypothesis.

GFP from Aequoria victoria has been used as a conventional approach to monitor gene expression and protein localization in different live cells (Chalfie et al., 1994; Tsien, 1998). However, the application of GFP in monitoring gene expression and protein localization in a high-temperature environment is compromised, largely due to the thermostability of the wild-type GFP. To examine the distribution of holin in host cells, in this study, a thermostable GFP was employed (Kiss et al., 2009), which was fused with the thermosensitive sgsE promoter of thermophilic Geobacillus sp. E263 and the target. Although a dual-colour reporter assay cannot be performed due to

![Fig. 5. Interactions between GVE2 endolysin and ABC transporter. (a) SDS-PAGE results of GST pull-down of GVE2 endolysin. Lanes: 1, purified GST–endolysin; 2, GST pull-down of GST–endolysin; 3, purified GST; 4, GST pull-down of GST; M, protein size marker. The arrow indicates the position of the ABC transporter (30 kDa). (b) Validation of interaction between endolysin and ABC transporter by bacterial two-hybrid assay. Transformants grown on both non-selective and selective plates are shown. See Methods for procedural details.](image-url)
the lack of other thermostable fluorescent proteins of different colours, the results presented here are indicative that the GFP cassette established in our study was an efficient approach for the protein localization in live cells of thermophiles at high temperatures.

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