Gene 479 (2011) 1–9

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Proteins responsible for lysogeny of deep-sea thermophilic bacteriophage GVE2 at high temperature

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ARTICLE INFO

Article history:
Accepted 20 January 2011
Available online 16 February 2011

Received by J.G. Zhang

Keywords:
Thermophilic bacteriophage GVE2
Lysogeny
CI
Site-specific recombinase

ABSTRACT

The lytic and lysogenic life cycle switch of bacteriophages plays very important roles in virus–host interactions. However, the lysogeny of thermophilic bacteriophage infecting thermophile at high temperatures has not been addressed. In this study, two lysogeny-related genes encoding the CI protein and recombinase of GVE2, a thermophilic bacteriophage obtained from a deep-sea hydrothermal vent, were characterized. Temporal analyses showed that the two genes were expressed at early stages of GVE2 infection. Based on chromatin immunoprecipitation (ChIP) assay and electrophoretic mobility shift assay (EMSA), the GVE2 CI protein was bound with only one DNA fragment located at 24264–24036 bp in the GVE2 genome. This location might be the original transcription site and the lysis–lysogeny switch site, which was very different from mesophilic bacteriophages. The GVE2 CI and recombinase proteins could function only at high temperatures. Therefore our study improved our understanding of the lysogeny process of bacteriophages at high temperatures.

1. Introduction

With the exploration for marine biological resources, more and more attention has been given to the deep-sea hydrothermal vent ecosystems in which thermophilic viruses, including bacteriophages, play very important roles in carbon cycling, community composition and evolution. Therefore, understanding of the process involved in the genetic switch between the lytic and lysogenic life cycles of thermophilic bacteriophages infecting thermophiles has become increasingly important. Currently, a limited number of thermophilic bacteriophages have been isolated from the deep-sea hydrothermal vents. In our previous study, a thermophilic *Geobacillus* bacteriophage GVE2 belonging to *Siphoviridae* was obtained from a deep-sea hydrothermal vent. Its host strain *Geobacillus sp.* E263 grew within a temperature range of 45–80 °C with an optimum at 60–65 °C (Liu et al., 2006). Comparative analysis of the GVE2 genome with other bacteriophages' genomes revealed that GVE2 was strikingly similar to temperate phages of lactic acid bacteria (LAB) from dairy environments. Furthermore, LAB belonged to the *Firmicutes* that were defined to be of a core group of the low-GC content Gram-positive bacteria (Lucchini et al., 1999a, 1999b).

With the development of large-scale sequencing techniques, some lactic acid bacteria (LAB) bacteriophages' genomes have been sequenced, such as *Lactococcus* bacteriophages sk1 (Chandry et al., 1997), c2 (Lubbers et al., 1995), Tuc2009 (Seegers et al., 2004), *Lactobacillus* bacteriophage phi adh (Altermann et al., 1999), and *Streptococcus thermophilus* bacteriophages DT1 (Tremblay and Moineau, 1999), Sfi19 and Sfi21 (Lucchini et al., 1999a, 1999b). These phages can be divided into two groups according to their life cycle: lytic phages and temperate phages. The lytic phage can result in the destruction of the infected host, and the temperate phage experiences the lysogenic process. However the lytic and lysogenic life cycles can be switched. After infecting a host cell, the temperate phage makes a decision to follow either a lytic or a lysogenic pathway. Some phages can remain as episomes in the host. When DNA damage occurs, some infected bacteria enter an abortive lytic cycle by losing the prophage and becoming nonlysogens (Oppenheim et al., 2005). The lysogenic state is maintained by the CI repressor. The CI proteins from phages of lactic acid bacteria are grouped into two classes. Class I repressors are proteins consisting of two domains. The N-terminal domain, with a helix-turn-helix motif, is believed to be the specific binding site for the target DNA. The C-terminal domain is assumed to be involved in oligomerization and contains Ala/Gly motif that are necessary for RecA-mediated cleavage of the hinge region between the N- and C-

Abbreviations: ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; LAB, lactic acid bacteria; GST, glutathione S-transferase; *E. coli*, *Escherichia coli*; IPTG, isopropyl-β-D- thiogalactoside; NTA, NI-NTArilotriacetic acid; SDS–PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

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doi:10.1016/j.gene.2011.01.015

terminal domains (Little, 1993). Class II repressors are smaller proteins and contain HTH motif without RecA-mediated cleavage site. The function of CI repressors in phages of lactic acid bacteria have been studied in detail (Garcia et al., 1999).

Phage integrases or recombinases play an important role in integrating the genome of a phage into the host chromosome. In this course, the phage's integrases or recombinases mediate site-specific recombination between two DNA recognition sequences, the bacterial attachment site *attB* and the phage attachment site *attP*. Based on amino acid sequence homology and mechanistic relatedness, the site-specific integrases or recombinases are categorized mainly into two families, the tyrosine recombinase or λ integrase family and the serine recombinase or resolvase/invertase family. Integrases have been applied as tools in the movement of genetic elements such as transposons, plasmids, bacteriophages and integrons involved in prokaryotic and eukaryotic systems (Groth and Calos, 2004; Smith and Thorpe, 2002). The integrases from *lactococcal* phages BK5-T, Tuc2009, and rlt belong to the Int family of site-specific recombinases (Wood and Warner, 2003).

Bacteriophages have been speculated to play a significant role in controlling the communities of deep-sea hydrothermal vents by controlling genetic material exchanges among hosts. However the lysogeny of thermophilic bacteriophages from deep-sea hydrothermal vents remains largely unknown. In this study, two lysogeny-related genes encoding the CI-like protein and recombinase were further characterized. The results revealed in this study improved our understanding of the lysogeny process of thermophilic bacteriophages from deep-sea.

2. Materials and methods

2.1. Culture of deep-sea thermophilic bacteriophage GVE2

The thermophilic bacteriophage GVE2 was separated from its host *Geobacillus* sp. E263 (China General Microbiological Culture Collection Center accession no. CGMCC1.7046), which was isolated from a deep-sea hydrothermal field in the East Pacific (Liu et al., 2006). The *Geobacillus* sp. E263 was infected with GVE2 and grown at 60 °C with shaking (150 rpm) in the medium TTM consisting of 0.2% NaCl, 0.4% yeast extract, and 0.8% tryptone (pH 7.0).

2.2. Sequence analysis

The open reading frames of GVE2 were identified using GenMark (Borodovsky and McIninch, 1993). The sequence similarities and protein domains were respectively analyzed using Blast software at the National Center for Biotechnology Information with the search tool Pfam release 20.0 (Liu and Zhang, 2008).

2.3. Recombinant expressions and purifications of CI and recombinase of GVE2

The two proteins were individually expressed as a fusion protein with glutathione *S*-transferase (GST). The *cI* gene was amplified from the GVE2 genomic DNA using the forward primer (5′-GC*GGATCC*GGAGGAGGAGGGTCGGGGGGAGGAGGAA TAGAAGTGACAAAGGAAGAGAT-3′) and the reverse primer (5′-CG*GAATTC*TCA ATCTAATAACACGGCATAC-3′) containing recognition sequences for BamH I and EcoR I (italic), respectively. To easily remove the GST in the recombinant fusion protein, a linker was used in the forward primer (underlined). The amplicon was cloned into the pGEX-4T-2 vector and expressed in *Escherichia coli* BL21 (DE3). The recombinase gene was cloned into the Nde I and Not I (italic) sites of the pET28 vector using the forward primer (5′-GG*CATATG*AGAGTGGCCAT TTACGTGAG-3′) and the reverse primer (5′-CC*GCGGCCGC*TCATTTTAAAGTAT AGTCC-3′) and expressed in *E. coli* BL21 as a fusion protein with 6× His-tag. The recombinant plasmids were confirmed using sequencing analysis. The bacterial cultures were induced with 0.5 mM isopropyl-β-D- thiogalactoside (IPTG) for 5 h at 37 °C and then harvested. The recombinant CI protein was purified with glutathione–sepharose beads (Sigma, USA) as recommended by the manufacturer, and the purified fusion protein with beads was incubated with thrombin (Amersham, USA) in a reaction buffer of PBS at 22 °C for 8 h to remove GST. The recombinase was purified using NI-NTArilotriacetic acid (NTA) affinity chromatography according to the QIA expressionist handbook (Qiagen). Fractions of the proteins were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in a 12% gel. Proteins in the gel were stained using Coomassie Brilliant Blue G250.

2.4. Antibody preparation

The purified CI and recombinase proteins of GVE2 were individually used as antigens to immunize mice using intradermal injection once every 10 days. The antigens (50 μg) were mixed with an equal volume of Freund's complete adjuvant (Sigma, USA) for the first injection. The three subsequent injections were conducted using 50 μg antigens mixed with an equal volume of Freund's incomplete adjuvant (Sigma, USA). Four days after the last injection, the mice were exsanguinated and the antisera were collected. The immunoglobulin (IgG) fraction was purified using protein A-sepharose (Bio-Rad, America) and stored at −80 °C until use. The titers of the purified IgG were 1:20,000 as determined using the enzyme-linked immunosorbent assay (ELISA). The antigens were replaced using PBS in negative control assays. The specificity of the antibodies was confirmed using Western blot with the recombinant proteins.

2.5. Western blot analysis

The proteins were separated using SDS–PAGE and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) in an electroblotting buffer (25 mM Tris, 190 mM glycine, 20% methanol) at 70 V for 70 min. The membranes were immersed in a blocking buffer (0.1% skimmed milk in PBS) at room temperature for 2 h. Subsequently they were incubated with the CI-specific an recombinase-specific antibodies for 1.5 h, respectively, followed by incubation in alkaline phosphate-conjugated goat anti-mouse IgG (Pierce Biotechnology, USA) for 1 h. Detection was performed with a substrate solution (4.8×10^{-4} mol/l 5-bromo-4-chloro-3-indolyl phosphate/5.1×10^{-4} mol/l nitroblue tetrazolium chloride) (Sigma).

2.6. Northern blot assay

Total RNAs were extracted from noninfected *Geobacillus* sp. E263 and from GVE2-infected bacteria at various postinfection times using Trizol reagent (Invitrogen) according to the manufacturer's instructions. After treatment with RNase-free DNase I (TakaRa, Japan) for 30 min at 37 °C, the total RNAs were separated using electrophoresis in a 1.2% agarose gel in 1× TBE buffer (90 mM Tris-boric acid, 2 mM EDTA, pH 8.0) and transferred to a nylon membrane (Amersham Biosciences, America). The blots were probed with the DIG-labeled CI and recombinase gene, respectively. The DNA labeling, hybridization, and detection were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II according to the manufacturer's manual (Roche, Germany).

2.7. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using the EZ ChIP™ kit (Upstate, America). The bacteriophage GVE2-infected *Geobacillus* sp. E263 cells were harvested when OD_{600} reached 0.8 using centrifugation at 5000*g* and resuspended with ice-cold phosphate-buffered saline (PBS, pH 7.4) containing the protease inhibitor Cocktail II. Subsequently 37% formaldehyde was added to crosslink the in vivo GVE2 protein and