Functional linkages between replication proteins of genes 1, 3 and 5 of *Bacillus subtilis* phage ϕ 29

Takahiro Tone, Ari Takeuchi and Osamu Makino*

Laboratory of genetics, Department of Material and Life Science, Faculty of Science and Technology,
Sophia University, 7-1 Kioi-cho, Chiyoda-ku, Tokyo 102-8554, Japan

(Received 24 July 2012, accepted 28 December 2012)

Gene 1 product (gp1) of *Bacillus subtilis* phage ϕ 29 has been shown to be involved in viral DNA replication *in vivo*, but the essential role is still unknown. As part of an ongoing effort to understand the role of gp1 in viral DNA replication, we investigated genetic interaction between gene 1 and other viral genes. Because ϕ 29 mutants which do not produce functional gp1 show temperature-sensitive growth, we isolated temperature-resistant phages from the ϕ 29 gene 1 mutants, and eventually, obtained nine extragenic suppressors. These suppressor mutations were located in two essential genes for ϕ 29 DNA replication *in vivo*: gene 3 encoding terminal/primer protein (gp3) or gene 5 encoding viral single-stranded DNA binding protein (gp5). Most of these mutations resulted in single amino acid substitutions in the products. By trans-complementation assay, we confirmed that the absence of gp1 at non-permissive temperature can be compensated by the suppressors which have the single amino acid substitution in either gp5 or gp3. These results indicate that gp1 has functional relationship to gp5 and gp3. From the positions of amino acid substitutions in gp3, we propose its new regulatory subdomain at which other molecules including gp1 would interact with and regulate functions of gp3.

Key words: bacteriophage ϕ 29, DNA replication, protein-priming, suppressor, temperature-sensitivity

INTRODUCTION

Bacillus subtilis phage ϕ 29 has a linear double-stranded DNA genome with terminal proteins covalently linked at each 5'-end (Hirokawa, 1972) (Fig. 1). Such protein-linked DNAs have also been found in other viruses (e.g., PRD1 and adenovirus), plasmids (e.g., pSKL1) and bacteria (e.g., *Streptomyces*). In the case of these viruses, DNA replication initiates via a protein-priming mechanism, and ϕ 29 has been studied as the most simple and typical case of the protein-priming replication (Meijer et al., 2001).

The genes of ϕ 29 DNA replication proteins including DNA polymerase, gp3 (acts as terminal protein and primer protein), gp5 (single-stranded DNA binding (SSB) protein) and DBP (double-stranded DNA binding protein) are clustered in the left end early region of the genome (Fig. 1), and their roles have been investigated by *in vitro* experiments (Meijer et al., 2001). Among these proteins, DNA polymerase and gp3 encoded by viral gene 2 and gene 3, respectively, were found to be minimal essential

components for *in vitro* duplication of ϕ 29 genome. At the initiation of protein-primed DNA replication, a heterodimer composed of DNA polymerase and gp3 binds to the two replication origins located at both ends of ϕ 29 linear genome, and the DNA polymerase covalently combines the first nucleotide (dAMP) with the hydroxyl group of Ser²³² provided by the gp3. This initiation step is greatly facilitated by DBP that is believed to help opening the termini of genomic DNA by forming a multimeric nucleoprotein complex at the replication origins. Subsequently, the same DNA polymerase catalyzes highly processive polymerization by strand displacement mechanism. Gp5 was reported to bind to the displaced single-stranded DNA (ssDNA) *in vitro* for protection from degradation by nucleases and for prevention from nonproductive binding of DNA polymerase to the ssDNA (Gutiérrez et al., 1991; Gascón et al., 2000). These roles of gp5 probably explain the stimulation of dNTP incorporation by ϕ 29 DNA polymerase *in vitro*.

According to the genetic studies using conditional lethal mutants, products of ϕ 29 gene 2, gene 3, gene 5 and gene 6 are essential for *in vivo* ϕ 29 DNA replication (Talavera et al., 1972; Carrascosa et al., 1976; Mellado et al., 1980). In addition to these proteins, viral gene 1 product

Edited by Fujio Kawamura

* Corresponding author. E-mail: o-makino@sophia.ac.jp

Fig. 1. Schematic representation of ϕ 29 genome. Filled circles represent covalently linked terminal proteins. The direction of transcription is indicated by arrows. Left end early region is magnified. The genes are represented as white boxes and their products are indicated. This diagram is modified from previous report (Meijer et al., 2000). SSB; single-stranded DNA binding protein. DBP; double-stranded DNA binding protein.

(gp1) is required for efficient ϕ 29 DNA replication *in vivo*. ϕ 29 sus1(629), an ochre mutant in gene 1, has a replacement of a CCA codon (Gln at position 6) by a nonsense TAA codon (Prieto et al., 1989). ϕ 29 which bears this sus1 mutation does grow at 30°C but fails to replicate its genome at 42°C, indicating that gp1 is essential at 42°C but dispensable at 30°C for the growth (Carrascosa et al., 1976; Bravo and Salas, 1998). Gp1 has been reported to exhibit multiple characteristics including self-association (Bravo and Salas, 1998; Bravo et al., 2001; Serrano-Heras et al., 2003; Hashiyama et al., 2005), cellular membrane localization (Bravo and Salas, 1997; Serrano-Heras et al., 2003), RNA binding (Takeuchi et al., 1998) and interaction with gp3 (Bravo et al., 2000). Despite the extensive studies, the role of gp1 in DNA replication is still unknown. Suspecting from the various characteristics, gp1 may play the roles in partnership with other viral replication proteins. To clarify the roles of gp1 in ϕ 29 DNA replication, we attempt to find relationships between gene 1 and other genes by suppressor analysis. We focused on the temperature-sensitive phenotype of ϕ 29 gene 1 mutants and started to isolate temperature-resistant (*Tr*) revertants from it. Up to now, nine *Tr* mutants bearing the original gene 1 mutations were identified. These *Tr* mutants have second-site mutations in gene 3 or gene 5 which involved in ϕ 29 DNA replication. The functional relationship between gp1, gp5 and gp3 will be discussed.

MATERIALS AND METHODS

Bacterial strains, plasmid, bacteriophages Bacterial and viral strains used in this study are listed in Table 1 including a series of newly constructed ϕ 29 nonsense mutants (ns1, ns3, ns5 and their double mutants: “ns”

stands for nonsense mutant and number shows the genes that have the nonsense mutation). Integration vector pSG1729 (Lewis and Marston, 1999) was obtained from Bacillus Genetic Stock Center. *Escherichia coli* DH5 α was used for cloning.

Construction of *B. subtilis* strains expressing ϕ 29 genes

The xylose-inducible expression vector, pSG1729, allows the integration of a gene at *amyE* locus of *B. subtilis* genome. At first, ϕ 29 gene 3 or gene 5 were PCR amplified from phage genomes using primers carrying *Avr*II and *Bam*HI restriction sites (Table 2). After digestion with *Avr*II and *Bam*HI, these PCR fragments and vector pSG1729 were ligated and cloned in *E. coli* DH5 α to give pSG1729-gene 3 and pSG1729-gene 5. Although vector pSG1729 was developed to express N-terminal GFP fusion protein (Lewis and Marston, 1999), the *gfpmut1* gene was unnecessary in this study. Thus, the *gfpmut1* gene was excised and replaced by each of ϕ 29 genes. To construct *B. subtilis* strains expressing both genes 5 and 1 or genes 3 and 1, gene 1 was PCR amplified from phage genome using primers carrying *Bam*HI and *Cla*I restriction sites (Table 2). The gene 1 fragment was ligated with pSG1729-gene 5 or pSG1729-gene 3 previously digested with *Bam*HI and *Cla*I. The ligation mixtures were then subjected to PCR using the Pori and Pbla primers (Table 2) to give pSG1729-gene 3/gene 1 and pSG1729-gene 5/gene 1 fragments. Using these vectors (pSG1729-gene 3 and pSG1729-gene 5) and PCR fragments (pSG1729-gene 3/gene 1 and pSG1729-gene 5/gene 1 fragments), *B. subtilis* 168 was transformed as described (Young and Spizizen, 1961), and transformants were selected on LB plates containing spectinomycin (50 μ g/mL). To confirm the insertion sites, *amyE* region was PCR amplified using *amyE*_5 and *amyE*_3 primers (Table 2) and sequenced. Since Spo0A protein encoded by host genome inhibits ϕ 29 DNA replication (Castilla-Llorente et al., 2006), the *spo0A* gene was disrupted by inserting neomycin resistant gene.

Constructions of ϕ 29 mutants

We introduced mutations into ϕ 29 genome by overlap extension PCR method using mutagenic oligonucleotides listed in Table 2. For example, ϕ 29 ns5 was constructed as follows. A set of primers 29end and mu-R (ns5) in PCR 1, and 5895R and mu-L (ns5) in PCR 2 were used with ϕ 29 genome DNA as template. The products of PCR 1 and PCR 2 were mixed and used as templates in PCR 3 which was primed by two primers 29end and 5895R. The products of PCR 3 bearing the nonsense mutation in gene 5 contain two *Fba*I sites at near the both ends. After *Fba*I digestion, the DNA fragments were purified from agarose gel. ϕ 29 genomic DNA containing terminal proteins (TP-DNA) was prepared by incubating ϕ 29 particles in the presence of 3 mM EDTA and 0.5% SDS for 30 minutes at 25°C.

Table 1. Bacterial and phage strains used in this study

Strain	Relevant characteristic(s)	Source/Reference
<i>B. subtilis</i>		
SR22	<i>trpC2 spo0A12</i>	Laboratory stock
FR32	<i>metB5 thr5 spo0A3 trnSLys3</i>	Laboratory stock
168	<i>trpC2</i>	Laboratory stock
TT225	<i>trpC2 amyE:: (Pxyl gfpmut1 spc) Δspo0A::neo</i>	This study
TT299	<i>trpC2 amyE:: (Pxyl gene 5 Wild type spc) Δspo0A::neo</i>	This study
TT303	<i>trpC2 amyE:: (Pxyl gene 5 A71T/A86V spc) Δspo0A::neo</i>	This study
TT305	<i>trpC2 amyE:: (Pxyl gene 5 S77Y spc) Δspo0A::neo</i>	This study
TT307	<i>trpC2 amyE:: (Pxyl gene 5 L16I spc) Δspo0A::neo</i>	This study
TT309	<i>trpC2 amyE:: (Pxyl gene 5 T82N spc) Δspo0A::neo</i>	This study
TT535	<i>trpC2 amyE:: (Pxyl gene 5 Wild type gene 1 Wild type spc) Δspo0A::neo</i>	This study
TT459	<i>trpC2 amyE:: (Pxyl gene 3 E78K spc) Δspo0A::neo</i>	This study
TT475	<i>trpC2 amyE:: (Pxyl gene 3 N69K spc) Δspo0A::neo</i>	This study
TT486	<i>trpC2 amyE:: (Pxyl gene 3 Wild type spc) Δspo0A::neo</i>	This study
TT559	<i>trpC2 amyE:: (Pxyl gene 3 E100K spc) Δspo0A::neo</i>	This study
TT541	<i>trpC2 amyE:: (Pxyl gene 3 Wild type gene 1 Wild type spc) Δspo0A::neo</i>	This study
ϕ 29		
sus1(629)	Gln ⁶ (CAA) to Ochre (TAA) in gene 1, and several mutations in the genome	Reilly et al., 1973
ns1	Gln ⁶ (CAA) to Ochre (TAA) in gene 1	Laboratory stock
		manuscript in preparation
mis1(L57S)	Leu ⁵⁷ (TTG) to Ser (TCG) in gene 1	Laboratory stock
		manuscript in preparation
ns5	Lys ²¹ (AAA) to Ochre (TAA) in gene 5	This study
ns3	Lys ¹⁴ (AAA) to Ochre (TAA) in gene 3	This study
ns1/ns5	Gln ⁶ (CAA) to Ochre (TAA) in gene 1, Lys ²¹ (AAA) to Ochre (TAA) in gene 5	This study
ns1/ns3	Gln ⁶ (CAA) to Ochre (TAA) in gene 1, Lys ¹⁴ (AAA) to Ochre (TAA) in gene 3	This study

Table 2. Oligonucleotide primers used in this study

Primer name	Sequence (5' to 3')	Purpose
29end	aaagtaagccccaccctcac	amplification of ϕ 29 left end region for sequencing and construction of ϕ 29 mutants
5895R	tcggcttctcagcatcgta	
AvrII-gene 3 ATG Fw	ccgctaggatggcgagaagtccacgtat	cloning of ϕ 29 gene 3
gene 3-BamHI-EcoRI Rv	ggaattccgcgatccgctagaaacccctttaagcttagatca	
AvrII-gene5 Fw	tgcacctaggatggaacacacaaacatcgta	cloning of ϕ 29 gene 5
gene5-BamHI Rv	cgggacacctataggatagttgtaagctaaagaat	
1Bam-sSD	cgcggatcctaaggaggaagcaggtatgggtaaaatcttcgatcaagagaagaga	cloning of ϕ 29 gene 1
1Rv ClaI	aagcttatcgattcattttgcacccatgtggagca	
Pori	agcgtcgattttgtgatgctcgtcagggggcgaggcct	amplification of pSG1729 vector (Meile et al., 2006)
Pbla	gctgttgagatccagttcgatgaaccactcgtgcacc	
amyE_5	tgaagaggaggaagcggaagaa	confirmation of integration into <i>amyE</i> locus
amyE_3	cggtaagtccgtctagccttg	
mu-R (ns1)	gtaatgggtaaaatcttcgattaagagaagagattagaag	construction of ϕ 29 ns1/ns5 mutant
mu-L (ns1)	cttctaattctctcttaatacgaagattttaccattac	
mu-R (ns3)	acgcattaaggataatgactaagccgaatacgtcgtgattg	construction of ϕ 29 ns3 and ns1/ns3 mutants
mu-L (ns3)	accaatcgcgctattcggcttagtcattatctcttaatgcgta	
mu-R (ns5)	actcttgaaggacaaatctaattttaatgctcagacaggcgg	construction of ϕ 29 ns5 mutant
mu-L (ns5)	ctgtctgagcattaaagatttagattgtcctcaagagtttctgtgtc	

Purified TP-DNA was digested with *FbaI*, and ligated with the PCR fragment containing ns5 mutations. The resultant TP-DNA was used to transfect *B. subtilis* TT299

expressing ϕ 29 gene 5. Protoplast cells of *B. subtilis* were prepared as described (Akamatsu and Taguchi, 2001) with minor modifications. *B. subtilis* cells cul-

tured at 37°C for 2.5 hours in LB medium containing 1% glucose were concentrated 5-fold in PPB buffer (0.5 M sucrose, 20 mM MgCl₂ and 20 mM Maleic acid pH 6.5) containing 0.1 mg/mL lysozyme. After incubation at 40°C for 15 minutes, the protoplast cells were collected in 1 mL of PPB buffer, and mixed with TP-DNA in the presence of 40% polyethylene glycol 4000 for 2 minutes at room temperature. The cells were resuspended in RTSB medium (3% Trypticase soy broth, 0.1 mg/mL L-glutamine, 0.3 M sodium succinate, 20 mM MgCl₂ and 1 mg/mL BSA), and incubated for 2 hours at 25°C to develop phage particles in the cells. The RTSB cultures were plated with indicator strain TT299 on LB plate containing 0.5% xylose and incubated at 30°C to obtain plaques of ϕ 29 mutants. After single plaque isolation, left end early regions were PCR amplified using primers 29end and 5895R (Table 2) and sequenced. Other ϕ 29 mutants used in this study were constructed by the same method using specific primers (Table 2). For the construction of ϕ 29 ns1/ns5 double mutant, ns5 phage was used as template for PCR 1 and 2. For the construction of ϕ 29 ns1/ns3 double mutant, ns1 phage was used as template DNA for PCR 1 and 2, respectively. *B. subtilis* strain TT486 expressing ϕ 29 gene 3 was used as host for the construction of ϕ 29 ns3. *B. subtilis* strain TT541 expressing ϕ 29 gene 3 and gene 1 was used as host for the construction of ns1/ns3 mutant. *B. subtilis* strain TT535 expressing ϕ 29 gene 5 and gene 1 was used as host for the construction of ϕ 29 ns1/ns5 mutant.

RESULTS

ϕ 29 gene 1 mutants as parental strain for suppressor hunting We used three ϕ 29 gene 1 mutants as parental strain for suppressor hunting: sus1(629), ns1 and mis1(L57S) (Table 1). ϕ 29 sus1(629) and ns1 have the same nonsense mutation at the sixth codon in gene 1 and do not produce any detectable gene 1 product (gp1) in the infected cells as examined by western blot analysis using anti-gp1 serum (data not shown). Although ϕ 29 sus1(629) strain has been used in several studies on gp1, we found that our stock had numerous additional mutations in the left end early region where most of the ϕ 29 DNA replication genes locate (Fig. 1). Therefore, to simplify the analysis, we selected a clone, ns1 which does not have such additional mutations at least in the left end region. In contrast, ϕ 29 mis1(L57S) have the missense mutation in gene 1 which results in an alteration of Leu⁵⁷ to Ser. The missense mutation was assumed to affect gp1's function since it substitutes one of the key amino acid in the putative coiled-coil region which is important for the self-association property of gp1 *in vitro* (Hashiyama et al., 2005). Western blot analysis showed that the amount of gp1-L57S was gradually decreased after infection while wild type gp1 accumulated with time,

suggesting that stability of the mutant protein is reduced (data not shown).

Table 3 shows plating efficiency of these ϕ 29 gene 1 mutants at 30°C and 42°C. Although all three strains could grow in our standard host strain SR22 at 30°C, the growth was greatly inhibited at 42°C. This growth inhibition at 42°C was almost completely restored when gp1-producing *B. subtilis* TT275 were infected with these phages. These results indicated that these ϕ 29 gene 1 mutants showed the temperature sensitive phenotype due to the absence of functional gp1.

Isolation of suppressors of gene 1 mutations To isolate suppressors from these gene 1 mutants, approximately 10⁵–10⁶ ϕ 29 phages were plated on *B. subtilis* SR22 at 42°C in order to select for temperature-resistant (*Tr*) revertants. After single plaque isolation of the appeared plaques, the left end early region of ϕ 29 genome (Fig. 1) was PCR amplified using a set of primers 29end and 5895R (Table 2), and their DNA sequences were determined.

Tr revertants were appeared at a frequency shown in Table 3. These revertants would have second-site, compensatory mutations (suppressor) or reverse mutations to wild type gene 1. To avoid picking up the revertants to wild type gene 1, we selected for slightly small plaques among the *Tr* revertants. Such precaution was taken on the assumption that suppressors might have intermediate phenotypes between the wild type and the parental phage strains. In the screen using sus1(629) and ns1, one or two *Tr* phages forming smaller plaque were isolated, respectively. In the screen using mis1(L57S), 13 *Tr* phages were isolated, and four were shown to be revertants to wild type gene 1.

As shown in Table 4, nine kinds of *Tr* phages retaining the original gene 1 mutations were obtained, and extragenic- and missense mutations were found in the

Table 3. Effect of temperature on plating efficiency of ϕ 29 gene 1 mutants

phage	30°C		42°C	
	TT275	SR22	TT275	SR22
ns1	1	0.44	0.98	9.2×10^{-6}
sus1(629)	1	0.25	0.81	1.5×10^{-5}
mis1(L57S)	1	0.44	0.96	9.8×10^{-6}
wild type	1	1.3	1.0	1.2

B. subtilis TT275 (producing gp1) and SR22 (not producing gp1) were mixed with each of ϕ 29 strains and top agar, and plated on LB plate containing 0.1% xylose. After incubation of the plates at 30°C or 42°C for about 13 hours, the plaques appeared were counted. The plating efficiency of each ϕ 29 mutant on *B. subtilis* TT275 at 30°C is expressed as 1, and the relative plating efficiencies were shown. These values were the average of three separate experiments.

Table 4. Temperature-resistant (*Tr*) strains isolated from ϕ 29 gene 1 mutants

<i>Tr</i> strains ^a	Second-site mutation			
	Affected gene	Codon change	Predicted product	no. of clones
sus1(629)- <i>Tr</i> 1 ^b	gene 5	GCT to ACT/ GCC to GTC	gp5-A71T/ A86V	1
ns1- <i>Tr</i> 1	gene 5	CTT to ATT	gp5-L16I	1
ns1- <i>Tr</i> 2	gene 2	GAT to CAT	gp2-D516H	1
ns1- <i>Tr</i> 2	gene 5	ACT to AAT	gp5-T82N	1
mis1(L57S)- <i>Tr</i> 1	gene 5	TCC to TAC	gp5-S77Y	1
mis1(L57S)- <i>Tr</i> 2	gene 3	AAC to AAG	gp3-N69K	1
mis1(L57S)- <i>Tr</i> 3a ^c	gene 3	GAA to AAA	gp3-E78K	2
mis1(L57S)- <i>Tr</i> 4a ^c	gene 3	GAG to AAG	gp3-E100K	2

^a All *Tr* strains have the original mutations in gene 1.

^b Although sus1(629)-*Tr*1 strain has another four mutations in the left end region, these are found in parental sus1(629) strain: gp5-A71T is one of such mutations which derived from parental sus1(629) strain.

^c Representative strains were shown.

From ϕ 29 mis1(L57S) mutant, we have recently obtained other three *Tr* strains in which second-site mutations were found in gene 5: two *Tr* strains have the mutation leading to gp5-S32T amino acid change, the other have the one leading to gp5-S74L amino acid change. At present, these two gene 5 alleles were not analyzed further.

three genes reported to be essential for ϕ 29 DNA replication: gene 2, gene 3 and gene 5. Most of the second site mutations were isolated only once (Table 4), suggesting that suppressor hunting was not saturating and further suppressors will be obtained.

Absence of gp1 can be compensated by the suppressors which have amino acid substitutions in gp5 (viral SSB protein) Four *Tr* phages have second-site mutations in gene 5 (Table 4). To verify whether these gene 5 alleles are suppressors of the gene 1 mutations, we conducted trans-complementation assay as follows. First, each of the gene 5 alleles was cloned into *amyE* locus of *B. subtilis* chromosome and expressed from the xylose inducible promoter (Table 1). Western blot analysis revealed that the levels of the synthesized gp5 were comparable with that between variants and wild type (data not shown). Then we examined whether the gp5 variants can support growth of the parental ϕ 29 strain ns1 at 42°C. To avoid production of wild type gp5 from infected ns1 phage, we introduced nonsense mutation into the gene 5 in ns1 phage genome, resulting in ϕ 29 ns1/ns5 double mutant (Table 1). Thus in this assay, ns1/ns5 phage cannot produce both gp1 and gp5, but gp5 is supplied *in trans* from the host genome infected. As shown in Fig. 2, wild type gp5 failed to support the growth of the ϕ 29 ns1/ns5 at 42°C. In striking contrast, four gp5 variants were able to support the growth of the

Fig. 2. The growth defects of ϕ 29 phage in the absence of gp1 can be compensated by the amino acid substitutions in gp5. *B. subtilis* strains TT299 producing wild type gp5 (open circle), TT303 producing gp5-A71T/A86V (filled triangle), TT305 producing gp5-S77Y (filled circle), TT307 producing gp5-L16I (open triangle) and TT309 producing gp5-T82N (open square) were grown in LB medium containing 5 mM MgSO₄ at 42°C. Before 60 minutes of infection, xylose was added at a final concentration of 1% to express gene 5 alleles, and these cells were infected with ϕ 29 ns1/ns5 mutant at a multiplicity of infection of 0.1. After 7 minutes of incubation with gentle shaking, the culture was diluted with the pre-warmed same medium to 10⁴-fold and incubated further at the same temperature. At indicated times plaque forming unit was measured by plating on indicator strain TT535.

ϕ 29 ns1/ns5. These results confirm that the identified gene 5 alleles are suppressors of nonsense mutation in gene 1, and indicate that the absence of gp1 at non permissive temperature of 42°C can be compensated by the amino acid substitutions in gp5. Although the gene 5-S77Y mutation was isolated from mis1(L57S) mutant (Table 4), it suppressed the nonsense mutation in gene 1 (Fig. 2). Therefore, the suppression by gene 5-S77Y mutation does not require gp1-L57S protein. The gene 2-D516H mutation was found in ns1-*Tr*1 in addition to gene 5-L16I mutation (Table 4). Although the effect of the gene 2-D516H mutation was not examined in this study, gp5-L16I alone was sufficient to suppress the nonsense mutation in gene 1 (Fig. 2).

Absence of gp1 can be compensated by the suppressors which have amino acid substitutions in gp3 (Terminal/Primer protein) Three *Tr* phages isolated from ϕ 29 mis1(L57S) mutant have second-site mutations in gene 3 (Table 4). By similar trans-complementation assay as described above, we verified whether the identified gene 3 alleles were also suppressors of the gene 1 mutation. When each of the gene 3 alleles was cloned into *B. subtilis* chromosome under xylose promoter (Table 1), the induced levels of the gp3 in the cells were comparable with that between variants and

wild type (data not shown). The ability of these gp3 variants to support growth of the $\phi 29$ gene 1 mutants at 42°C was examined with another $\phi 29$ strain, ns1/ns3 (Table 1). This strain was constructed from ns1 by inactivation of gene 3 with nonsense mutation so that the wild type gp3 would not be produced from the phage genome. Thus in this assay, the phage does not produce both gp1 and gp3, but gp3 was supplied *in trans* from the host genome infected. As shown in Fig. 3A, gp3-N69K and wild type gp3 were not able to support growth of $\phi 29$ ns1/ns3, while two gp3 variants, gp3-E78K and gp3-E100K, were able to support the growth of the mutant at 42°C. Although the two gene 3 alleles were isolated from $\phi 29$ mis1-L57S mutant (Table 4), gp3-E78K and gp3-E100K supported growth of the $\phi 29$ ns1/ns3. These results indicate that these two alleles of gene 3, gene 3-E78K and gene 3-E100K, can suppress the nonsense mutation in gene 1, and that the absence of gp1 can be compensated by the amino acid substitutions in gp3. Only gp3-N69K was not able to support the growth of $\phi 29$ ns1/ns3 (Fig. 3A). We confirmed that $\phi 29$ mis1(L57S)-*Tr*2, a *Tr* strain bearing the gene 3-N69K mutation (Table 4), formed smaller plaques at 42°C compared with other *Tr* strains isolated here (data not shown). Therefore, we suppose that the suppression by gp3-N69K is less effective than other suppressors.

As shown in Fig. 3A, wild type gp3 does not support the growth of $\phi 29$ ns1/ns3 mutant at 42°C. However, when pre-induced before infection, wild type gp3 did support the growth of the ns1/ns3 mutant (Fig. 3B).

The gp5 and gp3 variants retain their original activities As described above, amino acid substitutions in gp5 or gp3 compensated the absence of gp1 at 42°C. To examine whether these mutant proteins retain their original functions, we tested if the wild type gp5 or gp3 could be replaced by their variants. For this purpose, we constructed gp5 or gp3-deficient $\phi 29$ mutants, ns5 and ns3, which has a nonsense mutation in gene 5 and gene 3, respectively (Table 1). These phages could not direct synthesis of gp5 or gp3 as determined by western blot analysis using anti serum against gp5 or gp3 (data not shown).

First, each of gp3-producing cells described above was infected with $\phi 29$ ns3, and growth of the phage was monitored. As shown in Fig. 4A, all of gp3 variants supported growth of the $\phi 29$ ns3 as well as the wild type gp3 did, indicating that gp3 variants, gp3-N69K, gp3-E78K and gp3-E100K, retained their original activities.

Upon conducting the same experiment for gp5 variants, we unexpectedly found that $\phi 29$ ns5 did not grow at 45°C but grow at 42°C and lower temperatures, showing temperature-sensitive growth (Tone et al., 2012). Therefore, each of gene 5-producing cells was infected with $\phi 29$ ns5 at 45°C (Fig. 4B). Three gp5 variants, gp5-L16I, gp5-S77Y and gp5-A71T/A86V, supported the growth of ns5 mutant almost as well as the wild type gp5, whereas gp5-T82N did not support the growth. These results indicate that gp5's activity is retained in gp5-L16I, gp5-S77Y and gp5-A71T/A86V, but affected in gp5-T82N at least under this condition.

Fig. 3. The growth defects of $\phi 29$ phage in the absence of gp1 can be compensated by the amino acid substitutions in gp3. (A) Growth of $\phi 29$ ns1/ns3 double mutant at 42°C in various gp3-producing cells. Experimental conditions were essentially the same as described in Fig. 2. (B) *subtilis* strains TT486 producing wild type gp3 (open circle), TT475 producing gp3-N69K (filled circle), TT459 producing gp3-E78K (open triangle) and TT559 producing gp3-E100K (filled triangle) were infected with $\phi 29$ ns1/ns3 mutant. At the same time with infection, xylose was added at a final concentration of 0.1% to express gene 3 alleles. Plaque forming unit was measured by plating on indicator strain TT541. (B) Growth of $\phi 29$ ns1/ns3 double mutant at 42°C in wild type gp3-producing cells. Experimental conditions were the same as Fig. 3A. In *B. subtilis* strains TT486, wild type gp3 was induced simultaneously with infection (open circles) or before 60 minutes of infection (closed circles).

Fig. 4. Gp3 and gp5 with the amino acid substitutions retain their activities. (A) Growth of $\phi 29$ ns3 mutant at 42°C in various gp3-producing cells. Experimental conditions were the same as Fig. 3A. *B. subtilis* strains TT486 producing wild type gp3 (filled circle), TT475 producing gp3-N69K (open triangle), TT459 producing gp3-E78K (open square), TT559 producing gp3-E100K (filled triangle) and TT225 producing GFP as control (open circle) were infected with $\phi 29$ ns3 mutant. Plaque forming unit was measured by plating on indicator strain TT486. Note that growth of $\phi 29$ ns3 in *B. subtilis* strains TT486, TT475, TT459 and TT559 was almost identical, consequently the four lines of the growth curves overlapped. (B) Growth of $\phi 29$ ns5 mutant at 45°C in various gp5-producing cells. *B. subtilis* strains TT299 producing wild type gp5 (filled circle), TT303 producing gp5-A71T/A86V (open triangle), TT305 producing gp5-S77Y (open square), TT307 producing gp5-L16I (filled square), TT309 producing gp5-T82N (filled triangle) and TT225 producing GFP as control (open circle) were grown at 45°C and infected with $\phi 29$ ns5 mutant. At the same time, xylose was added at a final concentration of 2.5%. Plaque forming unit was measured by plating on indicator strain TT299.

DISCUSSION

Gene 1 product (gp1) is required for $\phi 29$ DNA replication at 42°C but dispensable at 30°C. Although several reports about gp1 have been published, its role in DNA replication is unclear. In this study, we identified gene 3 and gene 5 alleles as suppressor of gene 1 mutations, and showed that the absence of functional gp1 at 42°C was compensated by the amino acid substitutions in products of gene 5 (gp5) or gene 3 (gp3). These results demonstrate that gp1 has functional relationship with replication proteins gp5 and gp3. Because all gp5 or gp3 variants retained their original activities (Fig. 4), these suppressors are thought to be gain-of-function mutations. Only gp5-T82N did not support growth of gp5-deficient phage ns5 (Fig. 4B). This assay was performed at 45°C due to the phenotype of the ns5 mutant phage that can grow at 42°C in the absence of functional gp5 (Tone et al., 2012). We assumed that the gp5-T82N retained its original activity at temperature of 42°C where the suppression occurs.

Second-site mutations which suppress gene 1 defect were also found in other genes. Recently, we have isolated *Tr* phages from another $\phi 29$ gene 1 mutant, mis1(M53T). This mutant strain has missense mutation in gene 1 which results in an alteration of Met⁵³ to Thr and also showed temperature-sensitive growth: plating efficiency at 42°C was 51-fold lower than that at 30°C. Three *Tr* strains retaining the original gene 1-M53T

mutation were obtained. DNA sequencing revealed that two strains had second-site and missense mutation in gene 6, resulting in an alteration of Thr¹⁰ to Ser (T10S) or Glu⁶⁴ to Gly (E64G) in the product. The gene 6 product (double-stranded DNA binding protein; DBP) is also reported to be essential for $\phi 29$ DNA replication *in vivo* (Carrascosa et al., 1976), and initiation of $\phi 29$ DNA replication *in vitro* is greatly stimulated by DBP (Blanco et al., 1986). The other *Tr* strain has missense mutation in gene 1 leading to Ala⁸⁴ to Glu (A84E) substitution in addition to the original M53T mutation. It would be required to confirm that these gene 6 and gene 1 mutations are intergenic and intragenic suppressors of the gene 1 mutation, respectively. To perform the similar trans-complementation assay described in this study, $\phi 29$ double mutant which has mutations in gene 1 and in gene 6 is required. We have tried to construct the double mutant, but the trials are unsuccessful so far.

$\phi 29$ gp3 is a key protein for $\phi 29$ DNA replication: it acts as protein primer for initiation of DNA replication and is also the component of DNA replication origin as the "terminal protein" covalently linked to $\phi 29$ genome (Meijer et al., 2001). The crystal structure of gp3/ $\phi 29$ DNA polymerase heterodimer complex was determined (Kamtekar et al., 2006). As shown in Fig. 5A, gp3 is composed of three domains; the N-terminal domain (1-73 amino acid) that is important for the DNA binding (Zaballos and Salas, 1989; Muñoz-Espín et al., 2010), the intermediate domain (74-172 amino acid) that extensively interacts

Fig. 5. Structure of gp3-DNA polymerase complex and model for the role of gp1. (A) Ribbon representation of gp3/DNA polymerase heterodimer. This figure was made with the Swiss-PdbViewer software (<http://www.expasy.org/spdbv/>) using PDB code 2EX3. DNA polymerase is colored in blue. Gp3 N-terminal, intermediate, and priming domains are colored in pink, yellow, and green, respectively. The positions of the amino acid substitutions in gp3 are indicated by arrows and are colored in red. Note that PDB code 2EX3 does not contain data of several amino acids including Asn at position 69. Thus, the position of Asn⁶⁹ was predicted from the position of Ala⁷¹. (B) Schematic representation of gp3-DNA polymerase complex and a model for the role of gp1. Gp1 and other components which have been reported or suggested to interact with gp3 are shown (Bravo and Salas, 1997; Bravo et al., 2000; Serna-Rico et al., 2000, 2003). The intermediate domain of gp3 was divided into two subdomains, a DNA polymerase interaction subdomain and a proposed regulatory subdomain. In this model, gp1 regulates gp3's interactions by acting as a bridging molecule or as a competitive inhibitor, or by inducing conformational change of gp3.

with TPR-1 subdomain of the polymerase, and the priming domain (173-266 amino acid) which protrudes into the DNA template tunnel of the polymerase. In this study, we found that absence of gp1 at 42°C was compensated by the gp3 variants with single amino acid substitutions (N69K, E78K, and E100K). The positions of the amino acid substitutions in gp3 are also shown in Fig. 5A. All the substitutions are located in or near the intermediate domain that is reported to confer specificity to the interaction with the DNA polymerase (Pérez-Arnaiz et al., 2007). However, these substitutions are located in the opposite side of the interacting surface with the polymerase (Fig. 5A). In fact, biochemical analyses using purified gp3 indicated that gp3-DNA polymerase complex formation and DNA binding ability were not affected by these amino acid substitutions (data not shown). Here, we propose that the intermediate domain of gp3 would be divided into two subdomains, the DNA polymerase interaction subdomain and a suggested regulatory subdomain including amino acid residues N69, E78, and E100 where external molecules including gp1 would be accessible (Fig. 5B).

Gp3 is known to interact with versatile molecules other than the DNA polymerase or DNA: in addition to gp1, gp16.7 (viral gene 16.7 product), cellular membrane, and gp3 itself were reported to interact with gp3 (Bravo and Salas, 1997; Bravo et al., 2000; Serna-Rico et al., 2000,

2003). In the gp3 variants, interactions of gp3 with these components could be affected. Considering the functions of these components, three possibilities of the suppression mechanism could be argued. First, interaction between the protein primer (gp3 associated with the DNA polymerase) and the terminal protein (gp3 covalently bound to the ϕ 29 DNA) could be affected. This interaction is considered to be important for the initiation of ϕ 29 DNA replication (Serna-Rico et al., 2000). Alternatively, subcellular localization of gp3 variants could be affected. *In vivo* localization of gp3 was observed near the bacterial membrane or the nucleoid (Bravo and Salas, 1997; Muñoz-Espín et al., 2010), and the integral membrane protein gp16.7 and gp1 were proposed to help gp3 localize near the membrane (Bravo and Salas, 1997; Bravo et al., 2000; Meijer et al., 2000; Serna-Rico et al., 2003; Muñoz-Espín et al., 2009). Finally, although interaction with gp3 is unknown, DBP (gene 6 product) which has been reported to promote the initiation of viral DNA replication could be involved in the suppression mechanism. This possibility could be supported by the fact that gene 6 mutations were identified as candidates for suppressor of a gene 1 mutation (M53T), suggesting a functional linkage among gp1, gp3 and DBP. To clarify the suppression mechanism by the gp3 variants in the absence of gp1, above mentioned protein-protein interactions and its influences on viral DNA replication or

subcellular localization should be investigated. Also to fully understand the functional relationship between gp1 and gp3, involvement of gp1 in these interactions should be examined. Because physical interaction between gp1 and gp3 was reported (Bravo et al., 2000), we surmise that binding of gp1 influences gp3's interactions with other molecules by acting as a bridging molecule or as a competitive inhibitor, or by inducing conformational change of gp3 (Fig. 5B).

ϕ 29 gp5 is a viral SSB protein which has been shown to be essential for its DNA replication *in vivo* (Talavera et al., 1972; Carrascosa et al., 1976; Mellado et al., 1980) and greatly stimulated ϕ 29 DNA synthesis *in vitro* (Gutiérrez et al., 1991). In contrast to gp3 and DBP, gp5 has been reported to be involved in the elongation, but not in the initiation of viral DNA replication (Martín et al., 1989). Thus, the suppressor mechanism by gp5 variants might be different from that of the suppressors in gp3 and DBP. We found that ϕ 29 ns1/ns5 double mutant constructed in this study cannot grow at 30°C, although each single mutant can grow at this temperature, indicating that mutations in gene 5 and gene 1 cause synthetic lethal phenotype (to be published elsewhere). This suggests that gp1 and gp5 have redundant or mutually compensating functions. Considering the affinity of gp1 to RNA (Takeuchi et al., 1998), it would be interesting to test its ability to bind DNA as well, and investigate its role in stabilizing single-stranded DNA during the elongation of viral DNA replication. Furthermore, in recent years, SSB proteins have emerged as key components in recruiting and organizing the replication machinery via multiple protein-protein interactions (Lohman and Ferrari, 1994; Shereda et al., 2008). Such interaction partner has not been identified for gp5. Given the genetic relationship presented here, it would also be interesting to assess physical interaction between gp1 and gp5, and synergistic action of the interaction.

By isolating suppressors of gene 1 mutations, the presence of functional relationship between gp1 and gp3, gp5, or DBP is uncovered. Because gp1 is required for viral DNA replication at 42°C and higher temperatures but dispensable at 30°C, we assume that gp1 supports the function of other replication proteins, such as gp3, gp5 and DBP at 42°C. Therefore, it is important to investigate the physical interaction between these proteins, and the functions of the variants identified here. We are now examining biochemical properties of these gp3 and gp5 variants. Through investigation of the suppressor mechanism, we expect that the role of gp1 in ϕ 29 DNA replication will be clarified.

We would like to thank Dr. K. Asai, Dr. F. Kawamura and Dr. K. Matsumoto for useful discussions and advices.

REFERENCES

- Akamatsu, T., and Taguchi, H. (2001) Incorporation of the whole chromosomal DNA in protoplast lysates into competent cells of *Bacillus subtilis*. *Biosci. Biotechnol. Biochem.* **65**, 823–829.
- Blanco, L., Gutiérrez, J., Lázaro, J. M., Bernad, A., and Salas, M. (1986) Replication of phage ϕ 29 DNA *in vitro*: role of the viral protein p6 in initiation and elongation. *Nucleic Acids Res.* **14**, 4923–4937.
- Bravo, A., and Salas, M. (1997) Initiation of bacteriophage ϕ 29 DNA replication *in vivo*: assembly of a membrane-associated multiprotein complex. *J. Mol. Biol.* **269**, 102–112.
- Bravo, A., and Salas, M. (1998) Polymerization of bacteriophage ϕ 29 replication protein p1 into protofilament sheets. *EMBO J.* **17**, 6096–6105.
- Bravo, A., Illana, B., and Salas, M. (2000) Compartmentalization of phage ϕ 29 DNA replication: interaction between the primer terminal protein and the membrane-associated protein p1. *EMBO J.* **19**, 5575–5584.
- Bravo, A., Serrano-Heras, G., and Salas, M. (2001) A single amino acid substitution within a coiled-coil motif changes the assembly of a 53-amino acid protein from two-dimensional sheets to filamentous structures. *J. Biol. Chem.* **276**, 21250–21256.
- Carrascosa, J. L., Camacho, A., Moreno, F., Jiménez, F., Mellado, R. P., Viñuela, E., and Salas, M. (1976) *Bacillus subtilis* phage ϕ 29: characterization of gene products and functions. *Eur. J. Biochem.* **66**, 229–241.
- Castilla-Llorente, V., Muñoz-Espín, D., Villar, L., Salas, M., and Meijer, W. J. (2006) Spo0A, the key transcriptional regulator for entrance into sporulation, is an inhibitor of DNA replication. *EMBO J.* **25**, 3890–3899.
- Gascón, I., Lázaro, J. M., and Salas, M. (2000) Differential functional behavior of viral ϕ 29, Nf and GA-1 SSB proteins. *Nucleic Acids Res.* **28**, 2034–2042.
- Gutiérrez, C., Martín, G., Sogo, J. M., and Salas, M. (1991) Mechanism of stimulation of DNA replication by bacteriophage ϕ 29 single-stranded DNA-binding protein p5. *J. Biol. Chem.* **266**, 2104–2111.
- Hashiyama, K., Takeuchi, A., and Makino, O. (2005) Effects of single amino acid substitutions at the predicted coiled-coil or hydrophobic region on the self-assembly of ϕ 29 replication protein, gp1. *Biochem. Biophys. Res. Commun.* **331**, 1310–1316.
- Hirokawa, H. (1972) Transfecting deoxyribonucleic acid of *Bacillus* bacteriophage ϕ 29 that is protease sensitive. *Proc. Natl. Acad. Sci. USA* **69**, 1555–1559.
- Kamtekar, S., Berman, A. J., Wang, J., Lázaro, J. M., de Vega, M., Blanco, L., Salas, M., and Steitz, T. A. (2006) The ϕ 29 DNA polymerase: protein-primer structure suggests a model for the initiation to elongation transition. *EMBO J.* **25**, 1335–1343.
- Lewis, P. J., and Marston, A. L. (1999) GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. *Gene* **227**, 101–110.
- Lohman, T., and Ferrari, M. (1994) *Escherichia coli* single-stranded DNA-binding protein: multiple DNA-binding modes and cooperativities. *Annu. Rev. Biochem.* **63**, 527–570.
- Martín, G., Lázaro, J. M., Méndez, E., and Salas, M. (1989) Characterization of the phage ϕ 29 protein p5 as a single-stranded DNA binding protein. Function in ϕ 29 DNA-protein p3 replication. *Nucleic Acids Res.* **17**, 3663–3672.
- Meijer, W. J., Lewis, P. J., Errington, J., and Salas, M. (2000)

- Dynamic relocation of phage ϕ 29 DNA during replication and the role of the viral protein p16.7. *EMBO J.* **19**, 4182–4190.
- Meijer, W. J., Horcajadas, J. A., and Salas, M. (2001) ϕ 29 family of phages. *Microbiol. Mol. Biol. Rev.* **65**, 261–287.
- Meile, J. C., Wu, L. J., Ehrlich, S. D., Errington, J., and Noirot, P. (2006) Systematic localisation of proteins fused to the green fluorescent protein in *Bacillus subtilis*: identification of new proteins at the DNA replication factory. *Proteomics* **6**, 2135–2146.
- Mellado, R. P., Peñalva, M. A., Inciarte, M. R., and Salas, M. (1980) The protein covalently linked to the 5' termini of the DNA of *Bacillus subtilis* phage ϕ 29 is involved in the initiation of DNA replication. *Virology* **104**, 84–96.
- Muñoz-Espín, D., Daniel, R., Kawai, Y., Carballido-López, R., Castilla-Llorente, V., Errington, J., Meijer, W. J., and Salas, M. (2009) The actin-like MreB cytoskeleton organizes viral DNA replication in bacteria. *Proc. Natl. Acad. Sci. USA* **106**, 13347–13352.
- Muñoz-Espín, D., Holguera, I., Ballesteros-Plaza, D., Carballido-López, R., and Salas, M. (2010) Viral terminal protein directs early organization of phage DNA replication at the bacterial nucleoid. *Proc. Natl. Acad. Sci. USA* **107**, 16548–16553.
- Pérez-Arnaiz, P., Longás, E., Villar, L., Lázaro, J. M., Salas, M., and de Vega, M. (2007) Involvement of phage ϕ 29 DNA polymerase and terminal protein subdomains in conferring specificity during initiation of protein-primed DNA replication. *Nucleic Acids Res.* **35**, 7061–7073.
- Prieto, I., Méndez, E., and Salas, M. (1989) Characterization, overproduction and purification of the product of gene 1 of *Bacillus subtilis* phage ϕ 29. *Gene* **77**, 195–204.
- Reilly, B. E., Zeece, V. M., and Anderson, D. L. (1973) Genetic study of suppressor-sensitive mutants of the *Bacillus subtilis* bacteriophage ϕ 29. *J. Virol.* **11**, 756–760.
- Serna-Rico, A., Illana, B., Salas, M., and Meijer, W. J. (2000) The putative coiled coil domain of the ϕ 29 terminal protein is a major determinant involved in recognition of the origin of replication. *J. Biol. Chem.* **275**, 40529–40538.
- Serna-Rico, A., Muñoz-Espín, D., Villar, L., Salas, M., and Meijer, W. J. (2003) The integral membrane protein p16.7 organizes *in vivo* ϕ 29 DNA replication through interaction with both the terminal protein and ssDNA. *EMBO J.* **22**, 2297–2306.
- Serrano-Heras, G., Salas, M., and Bravo, A. (2003) *In vivo* assembly of phage ϕ 29 replication protein p1 into membrane-associated multimeric structures. *J. Biol. Chem.* **278**, 40771–40777.
- Shereda, R., Kozlov, A., Lohman, T., Cox, M., and Keck, J. (2008) SSB as an organizer/mobilizer of genome maintenance complexes. *Crit. Rev. Biochem. Mol. Biol.* **43**, 289–318.
- Takeuchi, A., Makino, O., and Hirokawa, H. (1998) Gene 1, one of the *dna* genes of bacteriophage ϕ 29, represses other *dna* genes through binding to mRNA. *Biochem. Biophys. Res. Commun.* **243**, 566–571.
- Talavera, A., Salas, M., and Viñuela, E. (1972) Temperature-sensitive mutants affected in DNA synthesis in phage ϕ 29 of *Bacillus subtilis*. *Eur. J. Biochem.* **31**, 367–371.
- Tone, T., Takeuchi, A., and Makino, O. (2012) Single-stranded DNA binding protein gp5 of *Bacillus subtilis* phage ϕ 29 is required for viral DNA replication in growth-temperature dependent fashion. *Biosci. Biotechnol. Biochem.* **76**, 2351–2353.
- Young, F. E., and Spizizen, J. (1961) Physiological and genetic factors affecting transformation of *Bacillus subtilis*. *J. Bacteriol.* **81**, 823–829.
- Zaballos, A., and Salas, M. (1989) Functional domains in the bacteriophage ϕ 29 terminal protein for interaction with the ϕ 29 DNA polymerase and with DNA. *Nucleic Acids Res.* **17**, 10353–10366.