Short Communication

Isolation of suppressors of the temperature-sensitive growth caused by a nonsense mutation in gene 1 of *Bacillus subtilis* phage ϕ 29 using hydroxylamine

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Bacillus subtilis phage ϕ 29 has a linear double-stranded DNA genome with a terminal protein covalently linked to each 5' end (Hirokawa, 1972; Ortín et al., 1971; Yanofsky et al., 1976; Yoshikawa and Ito, 1981; Yoshikawa et al., 1981), and has been studied extensively as a model system of the protein-priming DNA replication in which a viral-encoded protein provides the hydroxyl group needed by viral DNA polymerase to initiate the DNA synthesis (Matsumoto et al., 1983, 1984; Peñalva and Salas, 1982; Watabe et al., 1982, 1984). The detailed mechanism and proteins involved in the reaction has been clarified *in vitro* (Blanco et al., 1986, 1992, 1994; Gutiérrez et al., 1991). ϕ 29 gene 1 mutants which do not produce functional gene 1 product (gp1) can replicate their genome at 30°C but not at 42°C, indicating that gp1 is required for ϕ 29 DNA replication in a growth-temperature dependent fashion (Bravo and Salas, 1998; Carrascosa et al., 1976; Tone et al., 2012a). Although several characteristics of gp1 have been reported (Bravo and Salas, 1997, 1998; Bravo et al., 2000; Takeuchi et al., 1998), its role in viral DNA replication is still unclear. By isolating temperature-resistant revertants with spontaneous mutations from ϕ 29 gene 1 mutants, we have identified several second-site mutations that suppressed the defect of the gene 1 mutations at 42°C (Tone et al., 2012a); these suppressor mutations were located in gene 3 and gene 5. ϕ 29 gene 3 product (gp3) is a key protein for ϕ 29 DNA replication: it acts as a protein primer for the initiation of DNA replication and is also the component of the replication origin as a terminal protein (Matsumoto et al., 1983, 1984; Peñalva and Salas, 1982; Watabe et al., 1982, 1984). On the other hand, ϕ 29 gene 5 product (gp5) is a single-stranded DNA binding (SSB) protein required for viral DNA replication in a growth-temperature dependent fashion (Martín et al., 1989;

Tone et al., 2012b). Gp5 is an extremely abundant protein in infected cells (about 2.5×10^6 copies/cell), representing about 3% of total protein contents (Martín et al., 1989). Biochemical studies have shown that purified gp5 is a stable monomer in solution and occludes 3.4 nucleotides per monomer upon binding to single-stranded DNA (Soengas et al., 1994). Gp5 has also a helix-destabilizing activity and greatly stimulates dNTP incorporation *in vitro* ϕ 29 DNA replication (Gutiérrez et al., 1991; Martín et al., 1989; Soengas et al., 1995). The suppressor analyses suggest functional relationships between gp1, gp3, and gp5 and further investigations of these suppressors would be helpful to understand the role of gp1 in viral DNA replication (Tone et al., 2012a).

The structure of gp3 in complex with DNA polymerase has been resolved (Kamtekar et al., 2006). By mapping the suppressor mutations in gene 3 on the structure of gp3-DNA polymerase, we proposed the existence of a regulatory subdomain in the intermediate domain of gp3 (Tone et al., 2012a); in this model, gp1 regulates gp3's interactions by acting as a bridging molecule or as a competitive inhibitor, or by inducing a conformational change of gp3. On the other hand, the structure of gp5 is not yet determined. Therefore, the functional and structural relationships of gp5 still remain unclear. Furthermore, functional correlation of gp5 with gp1 is also unknown. In this study, we used hydroxylamine to mutagenize ϕ 29 expecting that a variety of suppressors of a gene 1 mutation would be obtained.

As a parental strain, a ϕ 29 gene 1 mutant ns1, which has an ochre codon at position 6 in gene 1 and does not produce gp1 in infected cells (Tone et al., 2012a), was used (Viral and bacterial strains used in this study are listed in Table 1). To avoid accumulation of numerous mutations

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Table 1. Bacterial and viral strains used in this study.

Strains	Relevant characteristic(s)	Source or reference
<i>B. subtilis</i>		
168	trpC2	Laboratory stock
SR22	trpC2 spo0A12	Laboratory stock
FR32	metB5 thr5 spo0A3 trnSLys3 (su ⁺)	Laboratory stock
TT535	trpC2 amyE:: (Pxyl-gene 5 wild type-gene 1 wild type, spc) Δ spo0A::neo	Tone et al. (2012a)
TT1143	trpC2 pHT01-gene 5T27I cat Δ spo0A::neo	This study
TT1145	trpC2 pHT01-gene 5G28S cat Δ spo0A::neo	This study
TT1149	trpC2 pHT01-gene 5G28D cat Δ spo0A::neo	This study
TT1155	trpC2 pHT01-gene 5T67I cat Δ spo0A::neo	This study
TT1156	trpC2 pHT01-gene 5G109E cat Δ spo0A::neo	This study
TT1169	trpC2 pHT01-gene 5wild type cat Δ spo0A::neo	This study
$\phi 29$		
wild type		Laboratory stock
ns1	gene 1 Ochre6	Tone et al. (2012a)
ns1/ns5	gene 1 Ochre6, gene 5 Ochre21	Tone et al. (2012a)
ns1-HAT1	gene 1 Ochre6, gene 5 T67I	This study
ns1-HAT2	gene 1 Ochre6, gene 5 T27I	This study
ns1-HAT6	gene 1 Ochre6, gene 5 G28D	This study
ns1-HAT9	gene 1 Ochre6, gene 5 G28S	This study
ns1-HAT11	gene 1 Ochre6, gene 5 G109E	This study

in the $\phi 29$ genomes, $\phi 29$ ns1 was incubated with 0.2 M hydroxylamine at 37°C for 20 hours to give a survival of 10^{-2} ; on average, one mutation per genome is introduced in this condition (Matsumoto et al., 1986). To fix mutations, the hydroxylamine-treated ns1 phages were propagated at 37°C in an LB medium using *B. subtilis* FR32 (a suppressive host of nonsense mutations), which permits the growth of $\phi 29$ ns1. The lysate was then plated at 42°C using *B. subtilis* SR22 as an indicator (a non-suppressive host) to select temperature-resistant revertants, which appeared at a frequency of 0.15%. Among them, 26 plaques were randomly picked up, and these $\phi 29$ strains, named as ns1-HAT (hydroxylamine-treated temperature resistant) 1~26, were investigated further.

To identify the suppressor mutations on $\phi 29$ genomes, each of their left-end early regions (5,300 bp from the left-end of the genome; Yoshikawa and Ito, 1982), where most viral replication genes including gene 1 locate, was PCR-amplified and the DNA sequences were determined. All the ns1-HAT mutants retained the original mutation in gene 1, indicating that they have second-site mutations but were not revertants to the wild type gene 1. As shown in Table 2, several second-site mutations were found in the viral DNA replication genes. Among them, the gene 3-E100K mutation was already confirmed as a suppressor of the gene 1 mutation (Tone et al., 2012a). Moreover, the ns1-HAT strains with the mutation in gene 1 (A27V) or in gene 6 (V58I) have other suppressor mutations in gene 3 or gene 5 (Tone et al., 2012a) (see the legend of Table 2). Thus, we further analyzed the five kinds of gene 5 mutations (T27I, G28S, G28D, T67I, and G109E) in this study.

To examine the effect of the secondary mutations on the viral growth at 42°C, one-step growth experiments were carried out using representative strains bearing the gene 5 mutations (ns1-HAT1, 2, 6, 9, and 11). Logarithmically growing *B. subtilis* SR22 in an LB medium with 5 mM MgSO₄ was infected with each of the ns1-HAT mutants at

Table 2. Identified second-site mutations in the left-end early region of ns1-HAT mutants.

Affected genes	Codon changes	Predicted amino acid changes	Number of clones obtained
gene 1 ^a	GCA to GTA	A27V	1
gene 3 ^b	GAG to AAG	E100K	4
gene 5	ACA to ATA	T27I	3
	GGC to AGC	G28S	4
	GGC to GAC	G28D	1
	ACT to ATT	T67I	13
	GGA to GAA	G109E	1
gene 6 ^c	GTT to ATT	V58I	1

^aThe ns1-HAT strain with the gene 1-A27V mutation has also a suppressor mutation (gene 5-T67I) identified in this study. The gene 1-A27V mutation is irrelevant to the suppression because the mutation locates downstream of the nonsense mutation at position 6 in gene 1.

^bThe gene 3-E100K mutation have been already obtained in the spontaneous isolation of temperature-resistant revertants from mis1 (L57S) (Tone et al., 2012a).

^cThe ns1-HAT strain with the gene 6-V58I mutation has also a suppressor mutation (gene 3-E100K) (Tone et al., 2012a).

42°C. At the indicated durations of infection, aliquots were taken and the production of infective particles was determined by plating on LTT plates using *B. subtilis* FR32 as an indicator. As shown in Fig. 1A, the parental strain ns1 did not grow at all. In contrast, the growth of the ns1-HAT mutants was completely or partially restored. Western blot analysis using an anti-gp1 serum showed that gp1 was not produced in the ns1-HAT mutants infected cells (Fig. 1B).

We have already reported four kinds of gene 5 mutations (L16I, S77Y, T82N, and A71T/A86V) which suppress the nonsense mutation in gene 1 at 42°C (Tone et al., 2012a). To confirm whether the newly identified gene 5 mutations (T27I, G28S, G28D, T67I, and G109E) are also suppressors of the gene 1 mutation, a

Fig. 1. The growth defect of $\phi 29$ due to the absence of gp1 can be compensated by the amino acid substituted gp5.

A, ns1-HAT mutants grow at 42°C. B, *subtilis* SR22 cells (about 10^8 cells/ml) were infected with each of $\phi 29$ strains at a multiplicity of infection of 0.1. After 7 minutes of incubation with shaking at the same temperature, the infected cells were diluted 10,000-fold into the same medium pre-warmed and incubated further. The production of infective particles was determined by plating on LTT plates using *B. subtilis* FR32 as an indicator. After overnight incubation of the plates at 30°C, plaques were counted. Symbols: hollow circle, wild type; cross, ns1; solid circle, ns1-HAT 1; hollow triangle, ns1 HAT-2; solid triangle, ns1 HAT-6; hollow box, ns1 HAT-9; solid box, ns1 HAT-11. B, Western blot analysis of gp1 in infected cells. Infected cells were prepared as (A) except that the multiplicity of infection was changed to 5 and that 10,000-fold dilutions were not carried out. After 30 minutes of incubation, 1 ml of the culture was centrifuged, and the cells were re-suspended and disrupted in 0.2 ml loading buffer (50 mM Tris-HCl pH 6.8, 4% SDS, 15% glycerol, 2.5% 2-mercaptoethanol, 0.005% BPB) by sonication. After separation by 11% SDS-Tricine-PAGE (Schägger and Von Jagow, 1987), proteins were transferred to a membrane at 70 mA for 30 minutes. gp1 was detected using a rat anti-gp1 serum and an Alexa Fluor 680 secondary antibody (Molecular Probes). As a loading control, an unknown host protein (about 45 kDa), which was nonspecifically detected by the anti-gp1 serum used, was also shown. A hyphen represents non-infected cells. C, Growth of $\phi 29$ ns1/ns5 at 42°C in various gp5-producing cells. *B. subtilis* strains carrying IPTG-inducible pHT01-gene 5 vectors were grown to a logarithmic phase at 42°C in an LB medium containing 5 mM $MgSO_4$ and 5 μ g/ml of chloramphenicol, and infected with $\phi 29$ ns1/ns5 mutant. At the same time, IPTG was added to the culture at a final concentration of 1 mM. The following experimental procedures were the same as that described in (A), except that *B. subtilis* TT535 was used as an indicator. Symbols: hollow circle, TT1169 (gp5-WT); solid circle, TT1155 (gp5-T67I); hollow triangle, TT1143 (gp5-T27I); solid triangle, TT1149 (gp5-G28D); hollow box, TT1145 (gp5-G28S); solid box, TT1156 (gp5-G109E). D, Western blot analysis of gp5 produced by IPTG induction. IPTG-induced cells were prepared as in (C). Thirty minutes after the addition of IPTG, cells were collected and processed as described in the legend of (B). Gp5 proteins were detected using a rat anti-gp5 serum. Note that the amounts of gp5 produced from the vector were equal to, or slightly higher than, that produced in infected cells (data not shown).

complementation assay was carried out. We used $\phi 29$ ns1/ns5; it has ochre codons at position 6 of gene 1 and at position 21 of gene 5, so that gp1 and gp5 are not produced in infected cells. To construct gene 5-expression vectors, each of the gene 5 was PCR-amplified from phage genome carrying wild type or mutant gene 5 using *Bam*HI-gene 5-Fw (5'-cgcggatccatggaaaacacaaacatcgta-3') and gene 5-AatII-Rv (5'-gacgtcttataggatagttgtaagctaaagaat-3') primers. These PCR products were cloned into an isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible expression vector pHT01 (Nguyen et al., 2007) using *E. coli* DH5 α cells. *B. subtilis* 168 was transformed with these variants of pHT01 containing gene 5. These *B. subtilis* strains were then infected with $\phi 29$ ns1/ns5 phage and the ability of gp5 variants to complement the growth of the phage at 42°C was determined. As shown in Fig. 1C, all gp5 variants with T27I, G28S, G28D, T67I, and G109E amino acid substitutions supplied from the vector did sup-

port the growth of the ns1/ns5 phage in the condition that wild type gp5 did not support the growth (Fig. 1C). Western blot analysis indicated that all the gp5 including wild type were produced in the cells in the presence of IPTG (Fig. 1D). In the absence of IPTG, the phage did not grow in these cells (data not shown). These results indicate that the five gene 5 mutations (T27I, G28S, G28D, T67I, and G109) can suppress the defect of gp1 at 42°C.

In the present and previous studies (Tone et al., 2012a), we have reported that gene 5 mutations leading to L16I, T27I, G28S, G28D, T67I, S77Y, T82N, A71T/A86V, and G109E can complement the defect of gp1 at 42°C, showing functional correlation between gene 1 and gene 5. To gain an insight into the mechanism of the suppression and the structure-function relationship of gp5, a model structure of gp5 was constructed (Fig. 2A) with CPHmodels 3.2 Server (Nielsen et al., 2010). Although the reliability is low, we think it reflects the gp5's structure because the

Fig. 2. A model structure of gp5 and sequence alignment.

A. A model structure of gp5. This model was made with CPHmodels 3.2 server (Nielsen et al., 2010) from amino acid sequence of gp5 using phosphoribosylaminoimidazolesuccinocarboxamide synthase Wit Atp (PDB: 2Z02) as a template and displayed as a ribbon diagram. The positions of the amino acid substitutions of the suppressors were colored in black. **B.** Sequence alignment of C-terminal region of $\phi 29$ gp1 and N-terminal region of GA-1 SSB. Amino acid sequences of $\phi 29$ gp1 (46–85 residues) and GA-1 SSB (1–27 residues) were aligned using Clustal W program. Numbers indicate the amino acid position. Bold letters indicate the most important region for the self-interaction of GA-1 SSB. The asterisks indicate the identical residues. The double dots and dots are strongly and weakly conserved residues, respectively, according to Gonnet Pam250 matrix.

DALI program (Holm and Rosenström, 2010) for the model structure returned *pfuThermoDBP-RP1*, an SSB protein of *Pyrococcus furiosus* DSM 3638 (Ghalei et al., 2014). The gp5 is composed of four-stranded, anti-parallel β -sheets connected to a long α -helix (Fig. 2A). Most SSB family proteins contain conserved OB (oligonucleotide/oligosaccharide binding)-fold domain (Murzin, 1993), such a domain organization was not seen in the gp5 structure. According to the model structure, the amino acid substitutions of the suppressors locate in various regions of gp5 (Fig. 2A). Although the reason for the broad distribution of amino acid substitutions in the suppressors remains unknown, we consider that these amino acid substitutions are affected in an oligomeric state or protein-protein interactions rather than the ssDNA binding ability of gp5 because the ssDNA binding and helix-destabilizing activities of one of the gp5 suppressors, gp5-S77Y, was not affected *in vitro* compared with wild type gp5 (unpublished data).

Although the biological significance is unclear, various oligomeric states of SSB proteins have been reported; T4 gp32 is monomeric (Chase and Williams, 1986), T7 gp2.5 is homodimeric (Kim et al., 1992), *E. coli* SSB is homotetrameric (Bujalowski and Lohman, 1991), and eukaryotic replication protein A is heterotrimeric (Wold, 1997). $\phi 29$ gp5 was reported to be a stable monomer in solution (Soengas et al., 1994). Interestingly, SSB of a $\phi 29$ -related phage GA-1 has different characteristics from that of $\phi 29$. It contains an insert at its N-terminal region which is not present in $\phi 29$ gp5, and self-interacts and forms filamentous structures similar to $\phi 29$ gp1 (Gascón et al., 2000a, b, 2002). Biochemical study using N-terminal deletion mutants of GA-1 SSB showed that the N-terminal insert was important for the self-interaction, the effective

binding to ssDNA, and the stimulatory effect on viral DNA replication *in vitro* (Gascón et al., 2002). In the N-terminal region of GA-1 SSB, a KDYIKVY sequence was especially important because the deletion of this region diminished the functionality of the GA-1 SSB (Gascón et al., 2002). We found that the KDYIKVY sequence is almost perfectly conserved in the C-terminal region of $\phi 29$ gp1 (Fig. 2B). The C-terminal region of gp1 is composed of the putative coiled-coil and the hydrophobic regions which were reported to be important for the self-association of gp1 (Bravo and Salas, 1998; Hashiyama et al., 2005). Furthermore, no ortholog of $\phi 29$ gene 1 has been found in the genome of GA-1 (Meijer et al., 2001). These findings suggest that the function of GA-1 SSB is separated into two genes, gene 1 and gene 5 in $\phi 29$. Thus, gp5 may work cooperatively with gp1, and gp1 may regulate the oligomeric state of gp5 by its self-association ability. It is intriguing that the gp5 suppressors gain the self-association property without gp1.

Another possibility of the functional correlation between gp1 and gp5 is that gp1 may regulate protein-protein interactions of gp5 with other DNA replication proteins. In most cases, prokaryotic SSB proteins have an acidic tail at the C-terminal (Lohman and Ferrari, 1994) and the acidic tails have been reported to be important for protein-protein interactions between SSB and other proteins involved in DNA replication, repair, and recombination (Burke et al., 1980; Hurley et al., 1993; Kelman et al., 1998). In $\phi 29$ SSB protein gp5, such an acidic tail is not present and the interaction partners of gp5 have not been reported. Thus, gp1 may regulate the interaction between gp5 and other proteins by acting as a bridging molecule. The gp5 suppressors may obtain the direct interactions with DNA replication proteins without gp1. Further biochemical investigations using the gp5 suppressors will clarify the role of gp1 and will reveal unknown aspects of $\phi 29$ DNA replication.

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