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Viability of *Bacillus subtilis* (*natto*) Bacteriophages after Freezing and Thawing

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Abstract

Bacillus subtilis (*natto*) phage ONPA suspended in an SM buffer without a cryoprotectant is sensitive to freezing at −20°C to −40°C. Freezing with a cryoprotectant and/or at low temperatures (−80°C and −165°C) can dramatically improve the viability of ONPA. *B. subtilis* (*natto*) phage JNDMP, the other principal type of *B. subtilis* (*natto*) phage used in this study, was stable even without a cryoprotectant at −20°C. The observation of ONPA using electron microscopy, after freezing at −20°C and subsequent thawing, suggested that the phage tails contracted and the phage genome DNA remained in the heads. The contraction of the phage tails may have caused the inactivation of ONPA phage particles.

Discipline: Genetic resources
Additional key words: cryopreservation, cryoprotectant, lyophilization, skim milk

Introduction

Natto is a Japanese food made from soybeans fermented with *Bacillus subtilis* (*natto*) (Nagai & Tamang 2010). *B. subtilis* strains have been collected by many institutes because they have significant potential for producing enzymes and are used for fermenting foods.

A devastating risk in natto factories is the contamination by bacteriophages, which are often detected on the factories' floors (Nakajima 1995). Many natto factories use one of three commercial starter cultures with very similar bacteriological and biochemical properties (Kiuchi et al. 1987). This situation can lead to a rapid spread of phages throughout the factories, once the phages invade the natto production process. *B. subtilis* (*natto*) phages isolated in Japan were classified into two groups, whose representatives are ONPA (Group II) and JNDMP (Group I). ONPA and JNDMP differ substantially in both the length of the genome DNA and the shape (Nagai & Yamasaki 2009). ONPA is composed of a head and a contractile tail, whereas JNDMP has a head and a tail with a knob-like structure (Nagai & Yamasaki 2009). The former is a Myoviridae phage, and the latter is a Siphoviridae phage in morphology (Fauquet et al. 2005). The *B. subtilis* (*natto*) phage ϕNIT1 is also a Myoviridae phage, and the analysis of the genomic DNA revealed that it has a poly-γ-glutamate hydrolase gene (*pghP*) and levanase gene (Ozaki et al. 2017). The *B. subtilis* (*natto*) phage PM1 is a Siphoviridae phage, which harbors a *pghP*-like gene (Ozaki et al. 2017, Umene et al. 2009). Poly-γ-glutamate hydrolase decomposes capsular poly-γ-glutamate on *B. subtilis* (*natto*) cells to assist the phages in invading the cells (Kimura & Itoh 2003). ONPA and JNDMP might be ϕNIT1- and PM1-related phages, respectively, based on the grouping by their authors (Kimura & Itoh 2003, Umene et al. 2009).

Despite being harmful agents in natto factories, phages are very important genetic resources to be preserved. For example, a generalized transducing phage of *B. subtilis* (*natto*) was found in a laboratory phage collection (Nagai & Itoh 1997).

The stable preservation of microorganisms as active genetic resources is a primary mission of culture collections aiming to pass preserved microorganisms on to future generations. In the NARO Genebank (National Agriculture and Food Research Organization, Tsukuba,

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Japan), phages have been preserved by lyophilization (freeze-drying), of which the first step is freezing. During periodic checks to monitor surviving phages, however, the author observed that some types of *B. subtilis* (*natto*) phages became inactivated during preservation.

In this paper, the author investigated the viabilities of 20 *B. subtilis* (*natto*) phages after freezing at −20°C and thawing, the effect of freezing temperatures on the viabilities of representative phages (ONPA and JNDMP), improvement of the viabilities with common cryoprotectants, and the effect of dilution of phage suspensions on the viabilities, in order to establish effective preservation methods for the phages, and speculated one reason of inactivation of ONPA by electron microscopy observation.

Materials and methods

1. Microorganisms and cultural conditions

B. subtilis (*natto*) phages ONPA (MAFF 270115) and JNDMP (MAFF 270105) and other *B. subtilis* (*natto*) phages (MAFF 270101–270120), as well as *B. subtilis* (*natto*) HM (MAFF 212145) used as a host for the phages, were obtained from the NARO Genebank (the accession numbers of the organisms are indicated in parentheses). *B. subtilis* (*natto*) HM was grown overnight in nutrient broth (Nissui Pharmaceutical, Tokyo, Japan) at 37°C. The titer of the phages was determined by mixing *B. subtilis* (*natto*) HM cells (30 µL, ca. 1×10^9 CFU/mL), using a conventional double-layer method with 0.6% agarose and LBMg [1% tryptone (BD, NJ), 0.5% yeast extract (BD, NJ), 1% NaCl, and 10 mM $MgSO_4$] agar (Nagai & Yamasaki 2009). SM buffer (0.58% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl [pH 7.5], and 0.01% gelatin) was used for preparing the phage suspension (Sambrook et al. 1989).

2. Preparation of phage suspension

Four mL of the SM buffer and a few drops of chloroform were added to double-layer agar on which plaques developed. After 6.5 h at room temperature, the phage suspension was recovered and filtered through a 0.2 µm filter (crude phage suspension, Advantec, Tokyo, Japan). The suspension was used in experiments other than the dilution experiment on purified phage suspension and electron microscopy observation.

Purification of the crude phage suspension was carried out by ultrafiltration. Briefly, the crude phage suspension was filtered through a 0.2 µm filter again, and 1 mL of the filtrate was transferred into a USY-20 ultrafiltration unit (molecular weight cut-off: 200 kD; Advantec). An air pressure was applied to the USY-20 ultrafiltration unit at room temperature using a syringe. When the volume of the suspension was decreased, 1 or 2 mL of the SM buffer without gelatin (lest background macromolecules were included in specimens for electron microscopy observation) was poured into the unit and the pressure was applied again. The washing procedure was repeated several times. Finally, the volume of the recovered phage suspension from the unit was raised to 1 mL with the SM buffer without gelatin.

For dilution experiments, 1.5 mL of ONPA suspension was purified as above using the SM buffer for the washing procedures, and the recovered suspension was directly used in the experiments.

3. Cryoprotectants

Glycerol (15 g/90 mL) was dissolved in deionized water and sterilized at 121°C for 15 min. A skim milk sodium glutamate solution (skim milk-Glu), containing 10 g of skim milk (BD, NJ) and 1.5 g of sodium glutamate monohydrate in 90 mL of deionized water, was autoclaved at 115°C for 15 min, kept in a sealed autoclave overnight, and then autoclaved again at 110°C for 10 min. Dimethyl sulfoxide (DMSO, 3.5 mL/45 mL) was dissolved in deionized water and filtered through a 0.2 µm filter (Advantec).

The phage suspension (one-tenth volume of the cryoprotectant solution) was added to the above cryoprotectants, yielding final concentrations of 15% glycerol, 10% skim milk, 1.5% sodium glutamate, and 7% DMSO.

4. Freezing and thawing

Test phage suspension in a tube was dispensed into 1.5 mL microtubes (60 µL each), and they were set on plastic racks (240010; Tarsons, Kolkata, India; 96.7358.9.01, Treff AG, Degersheim, Switzerland) in groups of three. The microtubes were frozen at each indicated temperature for 1 or 2 h. The remainder of the test phage suspension (unfrozen phage suspension as a control) was kept in a refrigerator (5°C) until the determination of the phage titer. Freezers were used for freezing at −20°C (KGNv-3646HC; Nihon Freezer, Tokyo, Japan), −40°C (NF-140SF3; Nihon Freezer), and −80°C (MDF 592, SANYO, Osaka, Japan), and a vapor phase liquid nitrogen tank (DR-430LM; Taiyo Nippon Sanso, Tokyo, Japan) was used for freezing at −165°C.

Frozen phage suspensions were thawed in a refrigerator (5°C), and then the titers of the frozen suspensions (after thawing) and the control were determined using the double-layer method (Nagai & Yamasaki 2009). Phage viability was defined as the ratio of the mean titer of the frozen (and then thawed)

phage suspensions in three microtubes to the titer of the unfrozen phage suspension (control).

5. Temperature shift experiments

ONPA suspensions (60 µL) were frozen for 1 h at −20°C (in a freezer) or at −165°C (in a liquid nitrogen tank) as a first temperature and then transferred to a −20°C freezer or liquid nitrogen tank (−165°C) to be frozen at a second temperature and kept there for 2 h. When samples frozen at −20°C were transferred to the liquid nitrogen tank, they were carried in precooled ethanol at −80°C to prevent any rise in their temperature. For the transfer of samples from the tank (−165°C) to the −20°C freezer, the samples were carried with no refrigerant. Samples frozen at −20°C or −165°C for 3 h were included in the experiment as controls.

6. Electron microscopy observation

Phage particles purified by ultrafiltration were observed under a JEM 2000EX electron microscope (JEOL, Tokyo, Japan) at the Hanaichi Ultrastructure Research Institute (Aichi, Japan) by negative staining with 2% uranyl acetate. A frozen sample of purified phage (−20°C for 9 h) was transported to the institute in a dry ice box and thawed at an ambient temperature just before observation, and an unfrozen purified phage sample was transported in a coolant.

Results and discussion

1. Phage viability after freezing

Bacteriophages are typically preserved by lyophilization (freeze-drying), L-drying, and cryopreservation (Ackermann et al. 2004, Fujita 2010). The freeze-drying method adopted in the NARO Genebank has the advantages of low running costs and superior ease of handling and distribution compared to cryopreservation. As freezing is the first step in lyophilization, the viability of phage particles after freezing and thawing was investigated in detail.

Twenty *B. subtilis* (*natto*) phage suspensions in SM buffer were frozen at −20°C and then thawed after 1 h. The viabilities are plotted in Figure 1 and show that the phages in Group I were remarkably tolerant of freezing. Group II phages were very labile when subjected to freezing: the reduction of viabilities ranged from 10^{-4} to 0 (below the detection limit ≈ 10^{-8}), and lot-to-lot reproducibility was very poor.

Although the suspension of phages in the SM buffer is convenient for laboratory stocks, it should not be frozen directly without testing for the freezing tolerance of the phage.

Clark & Geary (1973) concluded that morphological type B phages were more resistant to freezing than type A phages. JNDMP and ONPA phages belong to type B and type A, respectively, on the basis of the morphological criteria of Clark & Geary (1973), and JNDMP clearly survived freezing better than ONPA. However, there were a significant number of exceptions in their data on the relationship between successful cryopreservation and phage morphology; thus, further and careful investigation remains to be conducted.

It may be noteworthy that thawing ONPA for 1 min at 50°C increased the viabilities tenfold in comparison with slower thawing at 5°C, though it caused a loss of 20% of ONPA active particles (data not shown).

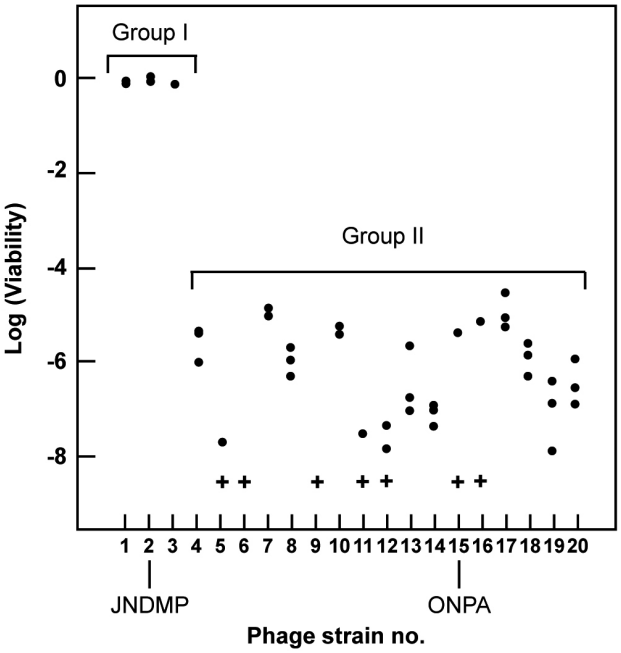

Fig. 1. Viabilities of *B. subtilis* (*natto*) phages suspended in SM buffer after freezing at −20°C.
The values of triplicate measurements are plotted as black circles on a semilogarithmic chart, and the plus signs indicate a viability of 0. Details of the group names, "Group I" and "Group II," are included in the text. Tested phage strains (numbers in parentheses are MAFF accession numbers) were 1, JNCHUP (270104); 2, JNDMP (270105); 3, JNHMP (270106); 4, P-1 (270101); 5, DMP (270102); 6, MIP (270103); 7, MOP (270107); 8, THP (270108); 9, THAP (270109); 10, SUP (270110); 11, KKP (270111); 12, KKP-GE (270112); 13, SS1P (270113); 14, SS2P (270114); 15, ONPA (270115); 16, ONPB (270116); 17, ONPC (270117); 18, FUKUSHOGUNP (270118); 19, ONPD (270119); and 20, SUP(SS1P) (270120). JNDMP and ONPA are representative phages in this study, as indicated in the figure. Freezing time: 2 h; phage concentration: 1×10^9 PFU/mL.

2. Effect of freezing temperature on viability

The freezing-thawing sensitivities of JNDMP (Group I) and ONPA (Group II) phages in the SM buffer were determined at temperatures ranging from −20°C to −165°C (Fig. 2). Almost all ONPA particles were inactivated by freezing at −20°C, and the viabilities at −20°C stretched over a wide range: from 10^{-8} to 0 (below the detection limit). However, the viabilities of ONPA were greatly improved by lowering the freezing temperature from −20°C to −80°C or −165°C.

The wide range of measured viabilities of ONPA may be due to measurement error owing to small differences in the experimental conditions (differences in the thickness of the sample tubes, positions of the tubes in the freezers, etc.), as was observed in a study on the cryopreservation of oomycetous fungi, in which the contact of sample tubes with the stainless-steel walls of their containers led to freezing failures (Nakagiri 2014). In the present study, no stainless-steel tube racks were employed, as recommended by Nakagiri (2014).

JNDMP in an SM buffer remained active after freezing, with viabilities of roughly 1 (100%) regardless of the freezing temperature (Fig. 2). Therefore, the cryopreservation of ONPA was focused on in the following experiments.

3. Temperature shift experiments

ONPA phages (1×10^{10} PFU/mL) frozen at −165°C were transferred to a −20°C freezer, and the viability was determined in triplicate (Table 1). The viability decreased to a thousandth of the former level, from 1.1×10^{-2} to 9.6×10^{-6}, but the temperature shift from −20°C to −165°C did not fully recover the viability (as compared to 1.1×10^{-2} after freezing at −165°C). These results indicated that the temperature during preservation was also an important factor for successful preservation: inactivation could occur not only during the freezing process, but also during preservation at −20°C. Once the phages were injured after freezing at −20°C, they could not become normal even though the freezing temperature was lowered to −165°C, where the phages were frozen successfully.

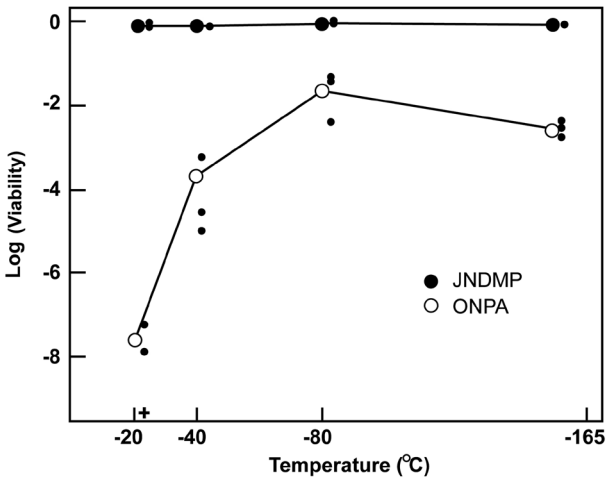

Fig. 2. Viabilities of *B. subtilis* (*natto*) phages, JNDMP and ONPA, in SM buffer after freezing at specified temperatures.
Averages (large circles) and measurements (small circles) are plotted in the figure ($n = 3$). The plus sign indicates a viability of 0. Freezing time: 2 h; phage concentration: 4-8 × 10^{10} PFU/mL.

4. Effect of cryoprotectants on viability

In order to protect microorganisms from freezing damage, cryoprotectants are often added to sample suspensions. For example, glycerol is added for freezing (often employed, as glycerol stock, in the freezing preservation of bacteria in deep freezers), and skim milk-Glu is added for freezing and freeze-drying (Nagai et al. 2005). DMSO is also used for freezing phages (Fujita 2010).

In the present study, cryoprotectants greatly improved the viability of ONPA at temperatures ranging from −20°C to −165°C (Fig. 3). The viability of ONPA in skim milk-Glu was roughly 100% over the tested temperature range. At −20°C and −40°C, DMSO and glycerol were not particularly effective. Although DMSO was more effective than glycerol at −20°C, both cryoprotectants demonstrated roughly identical effectiveness at −80°C and −165°C.

5. Effect of dilution on viability

The phage suspension with SM buffer was diluted with the SM buffer or skim milk-Glu, and the dilutions were frozen at the specified temperatures in order to investigate the effect of dilution (= phage concentration)

Table 1. Temperature shift experiments for cryopreservation of ONPA

1st Temp. (°C)	2nd Temp. (°C)	Viability
−20		0.0 [a]
−165		1.1×10^{-2}
−20	−165	1.9×10^{-8} [b]
−165	−20	9.6×10^{-6}

The samples were kept for 1 h at the first temperature, and 2 h at the second temperature. Temperatures for controls, which were frozen for 3 h, are given at the first temperature. $n = 3$. Phage concentration, 1×10^{10} PFU/ml.

[a] All samples were inactivated.

[b] Two of three samples were inactivated.

on the viability of ONPA. After thawing the dilutions, their viabilities were determined (Fig. 4).

Dilution with skim milk-Glu had no influence on viabilities after freezing at −20°C or −165°C as the viabilities were constant over the dilution range. However, when the phage suspension was diluted with the SM buffer, the viabilities of crude ONPA by the dilution decreased dramatically but reached a constant value below a phage concentration of 10^9 PFU/mL. This may be due to the existence of cryoprotectants in the crude phage suspension itself: the suspension contained cell lysates, culture media, and chloroform. Indeed, the viabilities of the purified suspension had a constant value over the dilution range. The presumed cryoprotectants in the crude phage suspension seemed to be removed by ultrafiltration. Phage–phage interaction, if any, did not appear to contribute to the viability, at least not below a concentration of 10^{10} PFU/mL.

Dilution with the SM buffer had no effect on the viability of JNDMP after freezing at −20°C, the highest cryopreservation temperature tested in this study (data not shown).

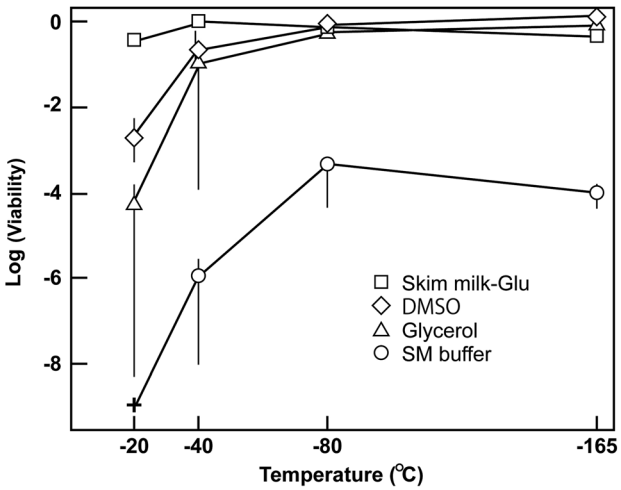

Fig. 3. Viabilities of *B. subtilis* (*natto*) ONPA in cryoprotectants.
Averages (open symbols) are plotted in the figure (n = 3). The plus sign indicates a viability of 0. Ranges of triplicate measurements (minimum value to maximum value) are indicated by vertical lines. Freezing time: 2 h; phage concentration: 4-7 × 10^9 PFU/mL.

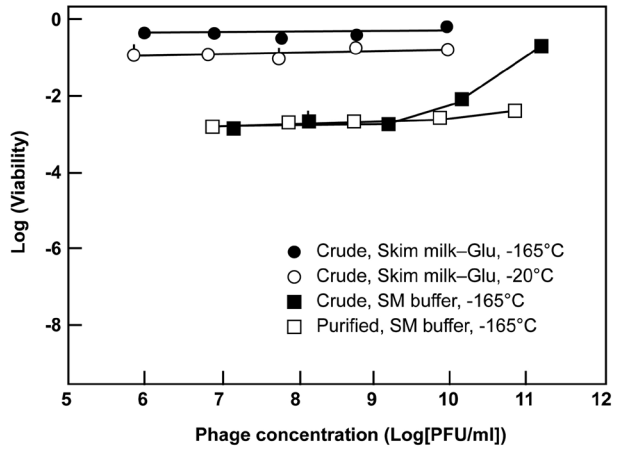

Fig. 4. Effect of dilution on the viability of ONPA.
Phage suspensions (crude and purified) were diluted with skim milk-Glu (circle) or SM buffer (square) and frozen at −165°C or −20°C. Averages (symbols) and ranges (vertical lines) are plotted in the figure (n = 3). Freezing time: 1 h. The initial concentrations of phages in the series of crude phage suspension diluted with skim milk-Glu (−165°C), crude phage suspension diluted with skim milk-Glu (−20°C), crude phage suspension diluted with SM buffer (−165°C), and purified phage suspension diluted with SM buffer (−165°C) were 9.8 × 10^9, 8.9 × 10^9, 1.6 × 10^{11}, and 6.2 × 10^{10} PFU/mL, respectively.

6. Electron microscopy observation of phage ONPA after freezing at −20°C

Almost all ONPA particles were found to be inactivated after freezing at −20°C and then thawing as indicated in the above experiments. Electron microscopy images of the inactivated ONPA particles clearly showed a structural transformation in the phage particles. The tails (sheaths) of the frozen phages contracted, but the DNA was not ejected from the heads (Fig. 5). Steel (1976) observed the contraction of the phage sheath by freezing and thawing under certain conditions and DNA ejection from the contracted tail, making a “cloud” at the tip of the tail, but such ejection of DNA was not seen with ONPA in the present study. It is noted that the freezing conditions in this part of the present study differed from those in the other parts with respect to timing. Here, the frozen sample was transported in a dry ice box to an

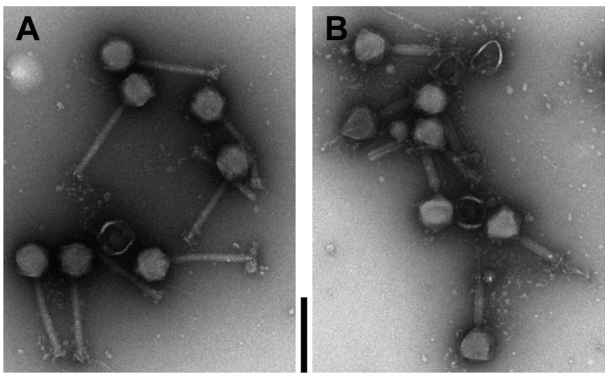

Fig. 5. Electron microscopy observation of ONPA (A) before and (B) after freezing and thawing.
Bar: 200 nm.

institution prior to observation.

Freezing seemed to trigger sheath contraction for injection of phage DNA into host cells as reported by Watson et al. (1987) on the *E. coli* T4 phage. Here, the tail of the frozen ONPA contracted before entering a lytic cycle, and the phages could not function normally.

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