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Genomic analysis of *Bacillus subtilis* lytic bacteriophage ϕ NIT1 capable of obstructing natto fermentation carrying genes for the capsule-lytic soluble enzymes poly- γ -glutamate hydrolase and levanase.

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Abstract

Bacillus subtilis strains including the fermented soybean (natto) starter produce capsular polymers consisting of poly- γ -glutamate and levan. Capsular polymers may protect the cells from phage infection. However, bacteriophage ϕ NIT1 carries a γ -PGA hydrolase gene (*pghP*) that help it to counteract the host cell's protection strategy. ϕ NIT1 had a linear double stranded DNA genome of 155,631-bp with a terminal redundancy of 5,103-bp, containing a gene encoding an active levan hydrolase. These capsule-lytic enzyme genes were located in the possible foreign gene cluster regions between central core and terminal redundant regions, and were expressed at the late phase of the phage lytic cycle. All tested natto origin *Spounavirinae* phages carried both genes for capsule degrading enzymes similar to ϕ NIT1. A comparative genomic analysis revealed the diversity among ϕ NIT1 and *Bacillus* phages carrying *pghP*-like and levan-hydrolase genes, and provides novel understanding on the acquisition mechanism of these enzymatic genes.

Introduction

Phage infection starts by adsorption of phage particles into the bacterial cells through the recognition of phage receptors on the host cell surface. Production of extracellular polymers such as polysaccharides has been known as one of the strategy of bacteria to avoid phage adsorption by covering cell surface phage receptors. For instance, capsular polysaccharides act as a barrier to phage infection in several bacteria.¹⁻⁴⁾

However, phages have evolved strategies to infect bacteria covered with capsules and biofilms.⁵⁾ Numerous phages possess capsular polysaccharide degrading enzymes associated with phage virion.⁶⁻⁹⁾ The *Escherichia coli* K1 strain produces the polysialic acid capsule known as the K1 antigen, on the surface of the host cell. The K1 antigen covers the LPS which is directly recognized by bacteriophage

T7, hence the capsule of strain K1 acts as a barrier to T7 infection.¹⁰⁾ In contrast, *E. coli* T7-like phages K1E and K1F recognize K1 antigen via their tail structural protein, and the endosialidase associated with the tail structural protein of the phages helps the phage approach the cell surface by degrading the K1 antigen.⁷⁾ The genomic analysis of K1E suggested that acquisition of an endosialidase domain in the tail structural protein allowed K1E and other K1 phages.⁹⁾

Some *Bacillus* species, including *Bacillus anthracis* and the *Bacillus subtilis* strains for natto (fermented soybeans) starter, produce a capsular polymer, poly- γ -glutamate (γ -PGA; poly glutamate with a γ -linkage), and levan.¹¹⁾ The composition of γ -PGA is different among the species, e.g., *Bacillus anthracis* has only D-glutamate polymer,¹²⁾ while γ -PGA of *Bacillus subtilis* consists of a mixture of a polymer of glutamate enantiomers.⁸⁾ In *B.*

anthracis, membrane associated CapABCE proteins are involved in γ -PGA production, and exported γ -PGA is anchored to the peptidoglycan via CapD.¹³⁾ *Bacillus subtilis* and *B. licheniformis* produce γ -PGA by PgsABCE, but their γ -PGA is not covalently-associated with peptidoglycan, because of absence of a CapD ortholog.¹⁴⁾ Despite whether γ -PGA is anchored to the host cell surface, the γ -PGA capsule plays an important role in prevention of phage infection of host cells.¹⁵⁻¹⁷⁾

Some *Bacillus* phages possess unique strategies to overcome the barrier. Lytic bacteriophage ϕ NIT1 has been isolated from natto product with suspected phage contamination.¹⁶⁾ ϕ NIT1 carries a gene for the soluble γ -PGA hydrolase, *pghP*, on a 3.2-kb EcoRV fragment of its genome. PghP participates in the phage infection, in spite of non-constituent of with phage virion. PghP internally and randomly cleaves both D- and L- γ -PGA substrates, eliminating the γ -PGA capsid from the cells, thereby allowing the progeny phages to approach the receptors on the host cell surface.¹⁶⁾ Because PghP is not homologous with poly- γ -glutamate hydrolases of *B. subtilis* and other bacteria, the origin and acquisition mechanism of the *pghP* gene is of interest. A possible transposase gene is found downstream of *pghP*, but further information is required before its function can be clarified.

Herein, we describe the determination of whole genome sequence of ϕ NIT1. It comprised a linear double stranded DNA with a central core region (CR), terminal redundancy regions (TR), and possible foreign gene clusters (FGCs) located between CR and both TRs. The *pghP* gene was located in one of the FGCs, and the gene for the novel levan-degrading enzyme LevP was found in another FGC. The existence of the *pghP* and *levP* genes among the nine phages isolated from independent natto products with suspected phage contamination was also investigated.

Materials and methods

Bacteria and phage strains, plasmids and media. Lytic bacteriophages, ϕ NIT1, FSG, KKP, MOP, ONPC, ONPB, P-1, SUP, SS2P and THP, were isolated from independent natto products with suspected phage contamination.^{16, 18)} *Bacillus* typing phage SP50 was obtained from Ackermann et al. *Bacillus subtilis* (*natto*) NAFM5 was used as propagation and experimental phage indicator strain,^{16, 19)} and strains Miyagino, Takahashi, Naruse, and Asahikawa were used for the host analysis of phages. *B. subtilis* strains 1046, 1149, 1174, 1243, 1346, 1395, and 1417 were also used for host analysis of phages. For the expression of recombinant enzymes, *E. coli* BL21 (DE3) cells harboring appropriate plasmids were used. Unless otherwise noted, *B. subtilis* and *E. coli* strains were cultured in LB medium. For the capsule production, *B. subtilis* (*natto*) strain Takahashi was cultured in the GSP medium (1.5% L-glutamic acid mono potassium salt (Nacalai, Japan), 1.5% sucrose, and 1.5% polypepton-S (a polypeptone derived from soybean; Nihon Pharmaceutical, Japan)).

Phage techniques. Phages isolated from a single plaque on the lawn of the indicator strain were amplified and used for the experiments. The phage particles were purified from liquid culture by the method described previously.²⁰⁾ Briefly, 10^8 pfu of phages (100 μ l) were added to 100 ml of host cell culture at early log phase ($OD_{660} = 0.2 - 0.4$), and were incubated at 37°C with vigorous shaking. When OD_{660} decreased to 0.1, culture was centrifuged at 5,000 x g for 10 min at 4°C to remove cell debris. Sodium chloride and polyethylenglycol 6000 were added to the supernatant at a final concentration of 0.7 M and 10% (w/v), respectively. After incubation at 4°C for 12h, the reaction mixture was centrifuged at 5,000 x g for 20 min. at 4°C, and the pellet containing phage particles was suspended into 2 ml of SMC buffer (50 mM Tris-HCL buffer (pH 7.0), containing 0.1 M NaCl, 8 mM MgSO₄, 1 mM CaCl₂, and 0.01% gelatin), then filtered through 0.20 μ m cellulose acetate (Advantec, Japan). For further purification, the phage suspension was treated with DNase I and RNase A at room temperature for 1 h, and then, subjected to CsCl₂ density gradient centrifugation. A white and opaque phage band was collected, and pelleted by centrifugation at 60,000 x g for 3h. The obtained pellet was resuspended in SMC buffer, and the genomic DNA from the phage particles was prepared according to the method described previously.²¹⁾

A spot test method was used for the host range analysis. Indicator strains cultured in LB medium until the OD at 660 nm reached 0.4 were mixed with LB medium containing 0.8% soft agar, and then overlaid on the LB plate. A total of 10 μ l of diluted phage particles were spotted on the soft agar, and incubated at 37°C.

For the one-step growth analysis, a log-phase culture of NAFM5 at 37°C in LB medium was used as a host, and phages were added at multiplicity of infection (MOI) of approximately 4. Cells were separated from free phages by centrifugation, and then cultured in new LB media at 37°C with vigorous shaking. Aliquots were taken sequentially from the culture and filtered through 0.45 μ m filter, and then the plaque forming units were counted by the soft agar overlay method.

Electron microscopic examination. Phage particles were negatively stained with 2% sodium phosphotungstic acid (pH = 7.4) and observed by using a transmission electron microscope (H-8100, Hitachi).

Phage genomic DNA sequencing. The DNA from ϕ NIT1 particles was prepared as described previously.^{16, 21)} The shotgun approach was used to determine the nucleotide sequence of the whole genomic DNA of ϕ NIT1.²²⁾ Phage genomic DNA was digested by sonication, and the resulting 1.5 to 3.0 kb fragments were cloned into pUC119 to make the shotgun genomic library. Gaps of contiguous fragments and flanking regions were amplified by PCR and inverse PCR, respectively. The cycle sequencing reaction and sequencing were done by the method described previously using an ABI 310 (Applied Biosystems, CA) automated sequencer.

Computer analysis. The obtained sequences were assembled using Genetyx Mac ATSQ software (Genetyx, Tokyo, Japan). *Orfs* of more than 150 bp, with ATG, GTG, and TTG as the initiation codons, were predicted for all six frames by the Genetyx Mac program package (Genetyx). A homology search was performed using FASTA and BLAST programs against sequences present in the DDBJ/EMBL/GenBank nucleotide sequence database and the SWISSPROT/NBRF-PIR protein database. Transfer RNAs were identified by tRNAscan SE 1.21 (<http://lowelab.ucsc.edu/tRNscan-SE/>). Promoters corresponding to bacterial sigma 70 were searched using BPROM programs available at the SoftBerry web site (<http://linux1.softberry.com/berry.phtml>). A possible promoter consensus sequence (TTGACa/t – 17 to 19-bp – (t/c/aATAAT) for host sigma A was also investigated by using the Genetyx program. Possible ρ -independent terminators were searched by using ARNold programs available at the University Paris-Nord 11 site (<http://rna.igmors.u-psud.fr/toolbox/arnold/index.php>). Comparison analysis of genomes among ϕ NIT1 and related phages was performed by using the GenomeMatcher program.²³⁾

Northern blotting and determination of the transcription initiation site. Strain NAFM5 cells were grown in LB medium. At early exponential phase (OD₆₆₀ = 0.4), ϕ NIT1 was infected with a moi of approximately 4. Aliquots of culture were collected sequentially, and phages in the supernatant and host cells were separated by centrifugation. Plaque forming units from the supernatant were counted by the overlay method. The total RNA fraction was prepared from the cells by phenol extraction (equilibrated in 20 mM sodium acetate (pH5.5), 0.5% SDS, and 1 mM EDTA) followed by treatment with DNase I. A total of 3 μ g of total RNA preparation was separated on a denaturing 1.5% agarose gel and blotted onto a nylon filter. Northern blotting and hybridization were done by standard methods using the PCR products of each gene as probes described in the legend of Fig. 6. Probe labeling and detection were performed using the DIG system (Roche, Basel, Switzerland).

Determination of the transcription initiation sites of *pghP* and *levP* was carried out by using the 5'-Full RACE core kit (Takara Bio, Shiga, Japan). As a template, the total RNA purified from infected cells by using the RNeasy Protect Bacteria Mini Kit (Qiagen, Venlo, Netherlands) was treated twice with DNase to remove the remaining DNA. Reverse transcription and 5'-RACE were performed according to the manufacturer's instructions.

Cloning of the levanase gene and measurement of the enzyme activity of the recombinant enzyme.

The putative levanase gene was amplified by PCR by using the ϕ NIT1 genome as a template. The amplified DNA fragment was digested with *Nde*I and *Bam*HI, and cloned into the corresponding sites of the pET15b expression vector. The resulting plasmid was then introduced into *E. coli* BL21(DE3), and the transformant was cultured in 100 ml of LB medium containing 100 mg/ml ampicillin at 37°C. When

the optimal density at 660 nm reached 0.8, isopropyl-b-D-thiogalactopyranoside was added to a final concentration of 1 mM. After incubation for an additional 3h at 30°C, the cells were harvested by centrifugation at 4°C, and resuspended in 50 ml of 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl and 20 mM imidazole (binding buffer). Cells were disrupted by sonication, and unbroken cells and large debris were removed by centrifugation at 16,000 x g for 15 min at 4°C. The supernatant was dialyzed twice against binding buffer, and filtered by 0.20 mm cellulose acetate filter. The resulting recombinant His6-tagged levanase was loaded onto a HisTrap HP (GE Healthcare, UK), and eluted with a linear gradient of 20 to 500 mM of imidazole. The eluate was dialyzed twice against more than a 100-fold volume of 20 mM potassium phosphate buffer (pH 5.5).

The levanase activity of purified recombinant LevP (rLevP) was evaluated by the analysis of product oligo-saccharides using thin-layer chromatography. The purified rLevP preparation (final concentration of 200 ng/ μ l) was mixed with an equal volume of 1% levan (from *Serratia levanicum*, Wako) in 20 mM potassium phosphate buffer (pH 5.5) and incubated at 37°C for 10, 30, 60 and 120 min. After denaturing the enzyme by heating at 98°C for 5 min, 5 μ l of the reaction supernatant was applied onto a silica gel 60 F₂₅₄ TLC plate (Merck). The TLC plate was developed with a solvent system of chloroform : methanol : acetic acid : water (5 : 12 : 1 : 3, v/v/v/v) and air-dried. After spraying 50% (v/v) sulfuric acid, the plate was dried and placed in a 110°C oven for 10 min to visualize the reaction spots.

Accession number. The genomic sequence of ϕ NIT1 was assigned accession number AP013029 in the DDBJ/GenBank/EMBL nucleotide sequence libraries.

Results and Discussion

General features of ϕ NIT1, and its genomic structure

ϕ NIT1 has typical morphology of *Myoviridae* phage with an isometric head of about 100 nm in diameter and a contractile tail 250 nm long (Fig. 1A and B). The complete nucleotide sequence of the ϕ NIT1 genome encased in the phage virion was determined to consist of a linear double

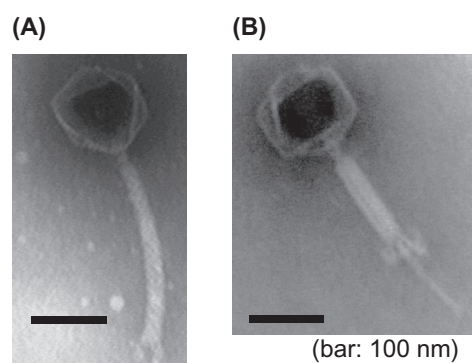


Fig. 1. Electron micrographs of phage ϕ NIT1 in a native state (A) and contracted state (B), negatively stained with phosphotungstic acid.

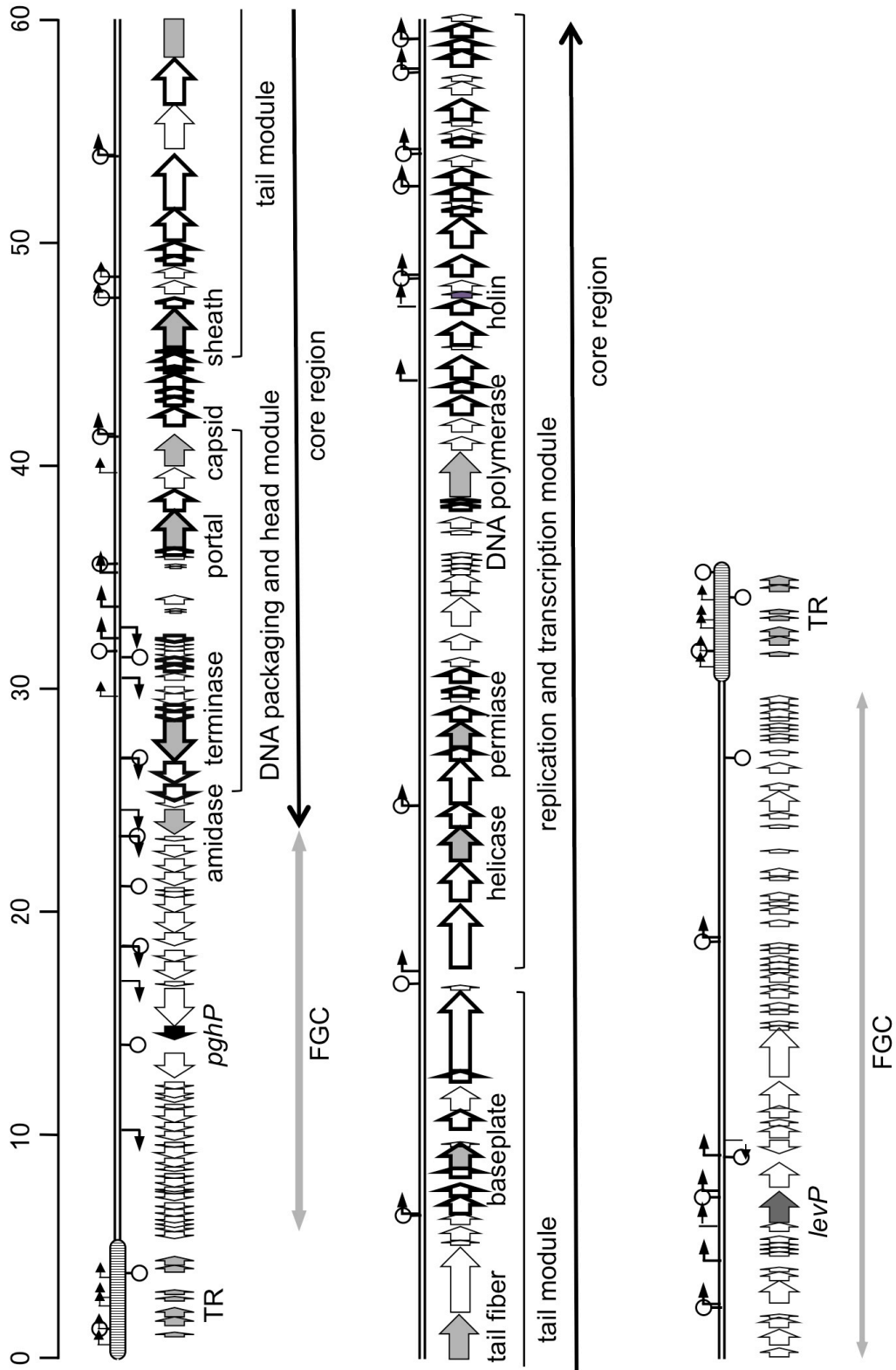


Fig. 2. Gene organization of ϕ NIT1. Predicted *orfs* are indicated by an arrow showing the direction of transcription. Open reading frames (*orfs*) are shown in arrows. Genes predicted as major functional proteins are shaded, and genes homologous to those of *Listeria* phage A511 are surrounded by heavy line. tRNAs are shown as small arrows. Upper and lower lines show the transcription units of sense and antisense strands, respectively. Possible promoters and putative rho-independent terminators are indicated by bent arrow and a lollipop, respectively. Terminal redundancy (TR) sequences in both ends are indicated as rounded squares.

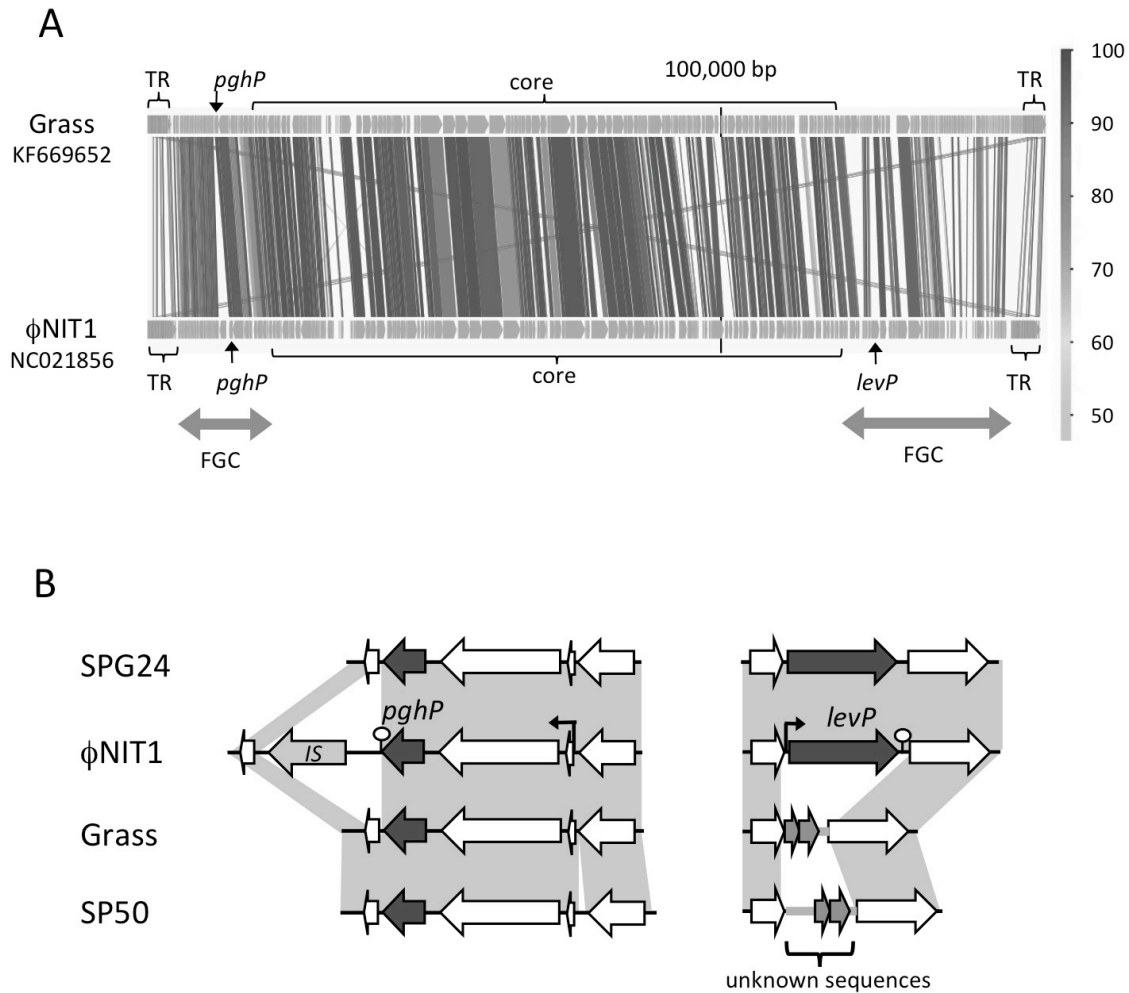


Fig. 3. Genomic structure of *Bacillus* phages carrying the *pghP* gene. (A) Comparison of the ϕ NIT1 genome to that of phage Grass. Predicted ORFs of ϕ NIT1 are indicated in the lower row, and those of phage Grass are in upper row. The gradation parameter represents the identity values of the predicted gene products (%). The regions of terminal repeats, core regions and FGCs are indicated beside each row. Positions of *pghP* and *levP* are shown by arrows. (B) Details of the gene structure around *pghP* and *levP*. Genes are indicated by an arrow and conserved regions are connected by gray shading.

stranded DNA of 155,631-bp, with a terminal redundancy (TR) of 5,103-bp, similar to that found in a SPO1-related group of Myoviridae of Ackermann's classification of tailed bacteriophages²⁴, SPO1 and A511 (Fig. 2). A part of the genome containing 6,230-bp a gene for poly-gamma-glutamate hydrolase of a phage (*pghP*) determined previously (AB469692) was confirmed.¹⁶ The total G+C content of the ϕ NIT1 genome was 42.1%, which was similar to that of the host *B. subtilis* (*natto*) chromosomal DNA, as well as other *B. subtilis* bacteriophages.

To confirm whether the structure of the genome in the capsid is circular or linear, the electrophoretic and *in silico* restriction maps of the ϕ NIT1 genome were compared. Among the 10 restriction enzymes investigated, *AccII* and *EcoRI* provided digested fragments having lengths different from that of the *in silico* digestion of the circular genome. Additionally, ligation of the genome before digestion and heat treatment of the digested DNA fragments before electrophoresis did not alter the fragment patterns, which suggested that ϕ NIT1 has a linear genome without cohesive

ends. Therefore, the 1.6 and 3.5-kb *EcoRI* fragments that might contain the ends of genome were extracted from the agarose gel, and both ends of these fragments were determined by run-off sequencing using the corresponding internal primers. As expected, the TR sequences of 5,103-bp in both ends of the linear genome were determined, and an *EcoRI* site was located at 1,625-bp and 3,478-bp from the left and right ends of the TR, respectively. *Bacillus* myophage SPO1²⁵ and *Listeria* myophage A511²⁶ are known to possess the TRs of 13,185-bp and 3,125-bp in their genome respectively, while the TR of ϕ NIT1 was different from them in terms of its length and nucleotide sequence. Although a number of *orf*s of *Staphylococcus* myophage Twort²⁷ and *Enterococcus* myophage ϕ EF24C²⁸ showed homology and synteny with those of phages A511 and ϕ NIT1, they have been reported as circular genomes.

Seventy-one ORFs in the ϕ NIT1 genome, which belong to head, tail morphogenesis and DNA replication modules, shared synteny and gene similarity with *Listeria* phage A511,²⁶ indicating ϕ NIT1 belongs to subfamily

Spounavirinae of *Myoviridae*.²⁹⁾ Similar to other *Spounavirinae* phages, four putative tRNA genes were found upstream of the head morphological gene cluster. The central approximately 100-kbp region consists of 116 *orfs* for morphogenesis, DNA replication and transcription genes, suggesting that this region is involving the phage life-cycle. In addition, all genes homologous to phage A511 genes were located within this region. Therefore we designated it as a core region (Fig. 2).

After the ϕ NIT1 genome was submitted to the DDBJ/GenBank/EMBL databases, the genome of two ϕ NIT1 related *Bacillus* myophages, phage Grass (KF669652) and ϕ SPG24 (AB930182) carrying *pghP* became available. The genome organization of ϕ SPG24 was almost the same as that of ϕ NIT1, and almost all open reading frames for the core region were conserved among these *pghP*-carrying phages. However, phage Grass did not possess the *levP* gene (Fig. 3A and B). Phage Grass has a TR sequence of 4208 bp,³⁰⁾ whereas the existence of a TR in ϕ SPG24 has not been investigated.

Structural proteins of the purified ϕ NIT1 phage virion were separated by SDS-PAGE (Fig. 4A). The N-terminal amino acid sequence of the 48 kDa protein was determined as SFTTG, which corresponded to the 31st to 35th residues of

the deduced amino acid sequence of a putative capsid gene (*orf481*), indicating that the peptide bond between K30 and S31 was digested during maturation. The molecular mass of resulting mature capsid protein was calculated as 48,805 Da, which was in agreement with the electrophoresis results. Parker and Eiserling reported that an approximately 2-kDa N-terminal fragment of a major capsid protein of phage SPO1 was proteolytically cleaved during phage head maturation.³¹⁾ Among the *Spounavirinae* phages, the pro-capsid proteins of *Listeria* phage A511, *Staphylococcus* phages K and Twort, and *Lactobacillus* phage LP65 are cleaved between K and S, the same cleavage site as ϕ NIT1, whereas the cleavage site of *Bacillus* phage SPO1 pro-capsid is K-A.²⁵⁾ The N-terminal amino acid sequence of the 60-kDa protein was AQSYG corresponding to the 2nd to 6th amino acids of ORF569, which has 49% and 44% identity with the tail sheath proteins of phage A511 (*gp93*) and phage K (ORF49), respectively. Phylogenetic analyses of the capsid and tail sheath proteins among sequenced *Spounavirinae* phages showed that ϕ NIT1 (and related phages Grass and ϕ SPG24) were placed independently from the phages of the genus *Twortlikevirus* (Fig. 4B).²⁹⁾

Two genes for capsule-lytic enzymes located at possible foreign gene clusters in the phage genome

Between the core region and both TRs of the ϕ NIT1 genome, the regions containing nonfunctional gene fragments and some atypical phage genes, were found (Fig. 2). Between the core region and both TRs of the ϕ NIT1 genome, the two regions containing some atypical phage genes, were found (Fig. 2). The average length of ORFs in these regions (143.17 amino acids) was significantly shorter than that in core region (248.4 amino acids), and a number of genes were presumed to be defective fragments. BLAST analysis of the deduced amino acid sequences of ORFs in these regions revealed that 50% (18/36) and 54% (29/54) ORFs were homologous to *Bacillus* species and other Gram-positive bacterial genes, whereas 73% (85/116) ORFs in core region were typical phage genes. These facts suggest that genes or gene fragments in these regions have been acquired from host organisms during phage infection cycles. Therefore we classified these regions as possible foreign gene clusters (FGCs). Possible FGCs of several dozen-kbp in size were also observed in the genomes of other *Spounavirinae* phages, SPO1,²⁵⁾ A511,²⁶⁾ ϕ EA24C,²⁸⁾ phage Twort²⁷⁾, phage K,³²⁾ and phage LP65.³³⁾ Only a few possible functional genes are found in the FGCs, suggesting genes encoding products unnecessary to phage growth might be disrupted.

Interestingly, *pghP* and a putative levanase gene (*orf477*) encoding a glycosyl hydrolase (GH) family 32 protein, which was designated as LevP, were found independently in different FGCs (Figs 2 and 3). Two ϕ NIT1 related phages Grass (KF669652) and ϕ SPG24 (AB930182) possess *pghP* in the same position of their FGCs as ϕ NIT1 (Fig. 3A and B). A possible IS1341-like³⁴⁾ insertion sequence (IS) sandwiched between two 7-bp direct repeats of AAATAGG, was found at downstream of *pghP* of ϕ NIT1 (Figs. 2 and 3). However, this IS was absent in other

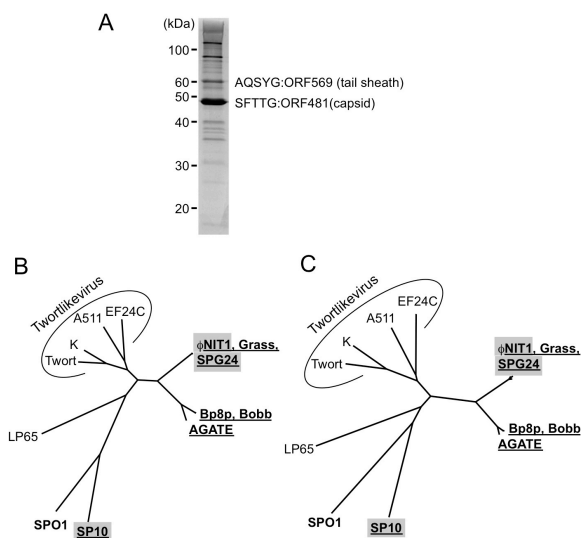


Fig. 4. Analyses of ϕ NIT1 virion proteins. SDS-PAGE of phage virion proteins (A). The protein components of ϕ NIT1 virions purified by CsCl density step gradient centrifugation were separated by 12.5% SDS-PAGE and stained with silver staining. A molecular weight scale is shown on the left, and the N-terminal amino acid sequences determined chemically are noted on the right. Phylogenetic trees of the capsid and tail sheath proteins. Amino acid sequences of capsid (B) and tail sheath (C) proteins of ϕ NIT1 and related *Spounavirinae* phages. All protein sequences were obtained from DDBJ/GenBank/EMBL databases, and alignment and tree construction were performed using ClustalW. *Bacillus* phages are written in bold. Phages that are underlined have *pghP* or *pghP*-like genes. Phages that are shaded have *levP* or *levP*-like genes.

ϕ NIT1-like phages, where an AAATAGG sequence remained at the corresponding site instead of the IS (Fig. 3B). In addition, this IS was found in other position of the ϕ SPG24 genome. These findings suggest that this IS is irrelevant to *pghP* acquisition.

During preparation of this manuscript, existence of a possible levanase gene corresponding to *levP* in ϕ SPG24 was reported.³⁵⁾ Masruufi and Levesque suggested that the ϕ SPG24 gene corresponding to *orf477* encodes a possible levansucrase by phylogenetic analysis, molecular docking and secretability predictions. However, its enzymatic activity has not been investigated. The *levP* gene was located at the same position in ϕ NIT1 and ϕ SPG24 phages, whereas two unknown genes were found instead of *levP* at the same position of the phage Grass genome (Fig. 3A and B). The levan degrading activity was detected in the supernatant of a ϕ NIT1-infected NAFM5 lysate, while a cell lysate without phage infection had no levanase activity. BLAST and domain analyses of the deduced amino acid sequence of *orf477* showed that ORF447 belongs to GH family 32 β -fructosidase (from the conserved domain database of the National Center for Biotechnology Information) with homology to putative levanases of Gram positive bacteria. All three active site amino acids for beta-fructofuranosidase activity, two aspartic acid and a glutamic acid residues, were conserved as D19, D150 and E198 of ORF477.

We assessed the levan hydrolyzing activity of ORF477 using recombinant protein. ORF447 was expressed in *E. coli* BL21(DE3) as a His6-tagged fusion protein, and a recombinant His6-ORF447 protein was purified homogenously (Fig. 5A). The levan hydrolyzing activity of His6-ORF447 was then confirmed by thin-layer chromatographic analysis of the reaction products from levan (Fig. 5B). After 60 min incubation, the spots of fructose and various lengths of fructo-oligosaccharides were observed, indicating that ORF477 acts as an endo-levanase. Thus, we designated the product of *orf447* as LevP, a levanase of phage.

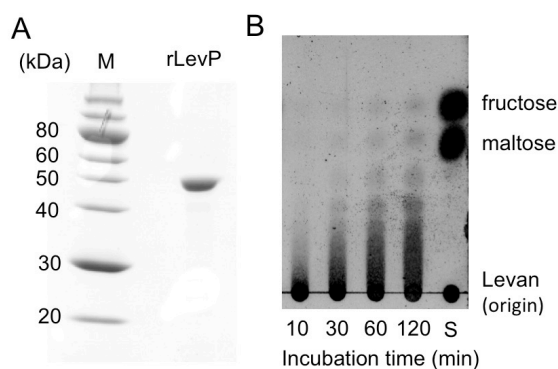


Fig. 5. Recombinant LevP preparation and its levan degradation activity. Recombinant LevP was expressed as a His6-tagged protein and purified by HisTrap HP column chromatograph. (A) SDS-PAGE of purified rLevP. (B) TLC profile of rLevP reaction products. The reaction was carried out with 0.5% levan at 37°C for the time indicated in the figure. Fructose, maltose and levan were used as standards.

Expression of *pghP* and *levP* genes during the phage lytic cycle.

To analyze the timing of expression of the enzyme genes in the phage lytic cycle, a one-step growth experiment of ϕ NIT1 was performed. Complete cell lysis occurred within 45 min after infection (Fig. 6A). The burst size of ϕ NIT1 was calculated as approximately 50, and this value was almost same as that of *Listeria* phage A511.³⁶⁾

Therefore, the expression of *pghP*, *levP*, capsid, and holin genes was then analyzed sequentially from 5 to 35 min after phage infection by northern blotting. In phage A511, the mRNA for the capsid gene, one of the phage structural proteins expressed in late phase of the lytic cycle, was detected at 20 min after infection to *L. monocytogenes*.³⁶⁾ The 1.5-Kb transcript for the ϕ NIT1 capsid protein was initially detected 20 min after infection, then it peaked after 25min (Fig. 6B). In ϕ NIT1, putative genes for a lytic enzyme and holin, a small protein that accumulates at programmed times in the phage lytic cycle to permeabilize the folded endolysin,³⁷⁾ were located distantly in its genome similar to those of *Myoviridae* phage T4³⁸⁾ and *Enterococcus* phage ϕ EF24C,²⁷⁾ and expressed at late phase (from 25 min after infection). If the *pghP* and *levP* genes are bacterial in origin, and were controlled under bacterial housekeeping sigma A, their expression might be observed immediately after infection. However, *pghP* (approximately 2.9 kb) and *levP* (approximately 1.5 kb) were expressed synchronously, and their expression patterns were similar to that of holin (Fig. 6B).

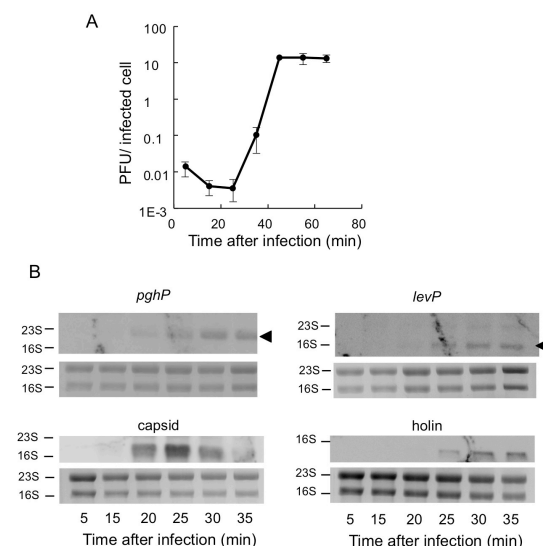


Fig. 6. Expression of *pghP* and *levP* during the phage lytic cycle. (A) One-step growth curve of NAFM5 infected with ϕ NIT1. Error bars show standard deviations. (B) Expression of *pghP*, *levP*, capsid (*orf481*) and holin genes. Total RNA was prepared from the NAFM5 cells infected with ϕ NIT1 at the time indicated. The sizes of the transcripts were estimated from the size of the 23S (2.9 kb) and 16S (1.45 kb) rRNA. PCR products of each gene were used as probes. The bands for the 23S and 16S rRNAs were used as the quantitative controls for the total RNA of each sample.

Hence, the transcription initiation sites of *pghP* and *levP* were determined from analysis of the promoter regions. The 2.9-kb mRNA for *pghP* was polycistronic, and *levP* was transcribed as a monocistronic mRNA of 1.5 kb. These results were supported by northern analysis data. No significant sigma A promoter consensus sequences³⁹ were observed upstream of the *pghP* and *levP* transcription initiation sites, suggesting that a phage dependent expression control mechanism for *pghP* and *levP* is present (Fig. 7). A common possible consensus sequence AAaAGa/t - TCCa/tAT was found in the promoter regions of *pghP* and *levP*. The transcription control mechanisms of *pghP* and *levP*

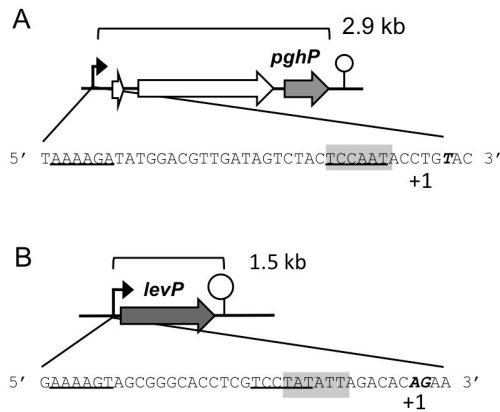


Fig. 7. Analysis of the transcription initiation sites of *pghP* and *levP*. Transcription initiation sites are indicated as bold type. The size of the transcripts was estimated from the transcription initiation site to the last base of the possible rho-independent termination sequence. The possible -10 sequence for sigmaA in *Bacillus subtilis* (TATAAT) is shaded. The underlined sequence indicates the possible consensus sequence, AAaAGa/t - TCCa/tAT, between the *pghP* and *levP* promoter regions.

including the consensus sequences are now being investigated.

Distribution and variation of *pghP* and *levP* genes among *B. subtilis* phages.

A loss of PGA production occasionally occurs during fermentation of natto products, and phage infection plays an important role for abnormal natto.¹⁶ Nagai and Yamasaki suggested that phages isolated from abnormal natto belong to *Myoviridae*.¹⁸ Transmission electron microscopy revealed that nine *B. subtilis* (*natto*) *Myoviridae* phages isolated from independent abnormal natto products in Japan, FSG, KKP, MOP, ONPC, ONPB, P-1, SUP, SS2P, and THP have ϕ NIT1-like hexagonal head and contractile tail. These phages all infected *B. subtilis* (*natto*) starter strains, whereas they did not form plaque on the lawn of *B. subtilis* laboratory strains (Table 1). Preliminarily Southern blot analysis showed that all the phages carry both *pghP* and *levP* genes (data not shown). Sequence analysis and the polycistronic structure of *pghP* and the flanking regions including upstream of *orf587* were highly conserved (more than 97% identity at the nucleotide level) among all of these 10 *B. subtilis* (*natto*) origin myophages including ϕ NIT1. The nucleotide sequences of the 2.3-kb PCR fragments including the *levP* gene of *B. subtilis* (*natto*) phages were also highly conserved (more than 99% identity at the nucleotide level). Additionally, restriction mapping and major capsid sequencing of these phages indicated that ϕ NIT1 and these *pghP/levP*-carrying Japanese phages were closely related to each other. We found that *Bacillus* typing phage SP50²⁴ infected both *B. subtilis* indicator and natto starter strains, and carried only the *pghP* gene at the same position of ϕ NIT1 (Table 1 and Fig. 3B). Sequence analyses of the *orfs* for the capsid and tail sheath proteins suggest that these *B. subtilis* (*natto*) phages and SP50 are closely related to ϕ NIT1.

Table 1. Host ranges of *Bacillus subtilis* (*natto*) infective phages

	Strains	Phages										
		ϕ NIT1	P-1	SUP	MOP	THP	FSG	SS2P	KKP	ONPB	ONPC	SP50
A	NAFM5	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
	Asahikawa	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	Nd.
	Miyagino	+++	+++	+++	++	+++	+++	++	+++	+++	+++	Nd.
	Naruse	+++	+++	+++	+++	+++	+++	++	+++	++	++	Nd.
	Takahashi	++	++	++	++	++	++	++	++	++	++	Nd.
B	1046											+++
	1149											++
	1174											++
	1243											+++
	1346	++	++	++					++			+++
	1395	+										++
	1417											++

Plaque formation of each phage was tested by spotting phage suspensions of different concentrations on the soft agar with indicator strains. +, ++, and +++ indicates that plaque formation was observed when 10^6 , 10^4 , and 10^2 pfu of phage suspension was spotted. Nd; not done.

A

φNIT1	NIT1	1	MAQTDTPYPIEALENAETVGVAYNIEVK-RQNPMSIYFSPHAGGIEVGTTELIYRV---V	56
	Grass	1	MAQTDTPYPIEALENAETVGVAYNIEVK-RQNPMSIYFSPHAGGIEVGTTELIYRV---V	56
	FSG	1	MAQTDTPYPIEALENAETVGVAYNIEVK-RQNPMSIYFSPHAGGIEVGTTELIYRV---V	56
	Sp50	1	MAQTDTPYPIEALENAETVGVAYNIEVK-RQNPMSIYFSPHAGGIEVGTTELIYRV---V	56
Bp8p	Bobb	1	--MADKYNNYAELRAAEQIGKDYNIECS-LHSDEMIMVVPHGGGIEPPTTELAYETELWI	57
	AGATE	1	--MADKYNNYAELRAAEQIGKDYNIECE-LFSDEMILVVPHGGGIEVGTTELSQEVDIRI	57
	Bp8p	1	--MADKYNNYAELRAAEQIGKDYHIECD-LFSDEMILVVPHGGGIEPPTTELSQEVDFRI	57
PM1	PM1	1	--MADKYINYAALKAAETIGVDYRIQAA-VQSDNFIIVAPHAGGIEVATTELTATL---	53
	SPP1	1	--MADVDFNFFDMESKAELCRDYWFEMR-RRSRDLVLLAIHGGGIEPRTTELLRGM---A	54
	SP10	1	--MADTFKNFADLKAVVDP-NDYSITYEKTENSEFLVLSPHGGGIEGGVSELVRAF----	53
Bsu	YoqZ	63	VLAADKYSNFEELKENESP-LSYNILTDD-VDRRVLLIAPHGGGIEGGTSELARELS---	117
	YndL	49	IYAEDVYQNFEEELKNNEDP-SDYGVVTKE-TGSPVLVLAIHGGGIEGGTSEVARELS---	103
	YmaC	30	SNQPDYTNFSSLAENESP-ADYDISYNEKKGSKVLIMSPHGGGIEGGVSELVRYFN---	85
	YjqB	1	--MEDMYSFAALSASESE---YRIIYEEKNGSELIVLGPHGGGIEPGVSELVRAFS---	52
	Bpu	2	RQMDITYQSYEELSASHERKIDYQILHER-KGDQLLVLSPHAGGIESSGVELIHEIS---	58
	Bli	35	SS-ADKYANFAELAEHETEGEDYQIEYTD-MGTELLILSPHGGGIEGGVSELVRAFS---	90
Sha	39	NRSLDYYSNFEELKENTEGKDWRIKTKNRKDNHILVTAIHGGGIEPPTTELLARRV---A	95	
φNIT1	NIT1	57	ELTGGSLYFQGLLPSGNSRLHVTSTHFDEPMAVCMLSKHTDAVSFHGYKDDYKNKTLVG	116
	Grass	57	ELTGGSLYFQGLLPSGNSRLHDTSTHFDEPMAVYMLSKHTDAVSFHGYKDDYKNKTLVG	116
	FSG	57	ELTGGSLYFQGLLPSGNSRLHDTSTHFDEPMAVYMLSKHTDAVSFHGYKDDYKNKTLVG	116
	Sp50	57	ELTGGSLYFQGLLSSGNSRLHDTSTHFDEPMAVYMLSKHTDAVSFHGYKDDYKNKTLVG	116
Bp8p	Bobb	58	KSGRISTYTFNSTKSSNNVDLHITSTANFDEPVGAVTNKHKAISFHGYADSKNENTIVG	117
	AGATE	58	KNRLISSYLFNAMKPSNNVDLHITSTANFDEPVGAVTNKHKAISFHGYAASRENTIVG	117
	Bp8p	58	KGLLISSYFNTTKSSNNLDLHITSTANFDEPIGVAVTNKHKAISFHGYADSKNENTIVG	117
PM1	PM1	54	AGNDTSYLYFEGLNSSGNDLHITSTHFDEPIALHMMONHEFGLSYHGYADSENEMTIIIG	113
	SPP1	54	AEWNDSYLYFEGR---NFDYHITSHKFDEPHALELVKHDYALSVDHGYKDDQESTIVG	110
	SP10	54	LVDNYSIYLFEGLRATGNQELHITSNFDEPTALRAVSEHDKTISFHGYKDTVKNKTLVG	113
Bsu	YoqZ	118	-KS-YSAYLFEGLRIPGASELHITSTNFDEPOALDLSKHDLTISIHGYAS-SKKHTLVG	174
	YndL	104	-KE-YSMYLFEGLKSAAGSVLHITSTHFDEPRALKMTGNHEVVISLHGYAE-EDOOIEVG	160
	YmaC	86	-NE-YSTYLFEGLKSHDNQTLHITSTNFDEPLAKKKIKEHQYVVSFHGYKGEN-KNTLVG	152
	YjqB	53	-DR-CSIYLFEGLKQRNRSRLHITSTIRFDEPLALEKVNDDHYALAFHGYYPKPIPTLVG	110
	Bpu	59	-SD-YSMYLFEGLVKGNHVLHITSTRFDEPLCLSEVSHHHHTFALHGYGETEVLQTLVG	114
	Bli	91	-AD-YSTYLFEGIKPSQNWDLHITSNHFDEPOALKAVKEHSYVLAHGYHDIIV-EHTLVG	147
Sha	96	NIGEYDFYTFEGLMPKHNERLHITSTVFDEPTLLKMLDHSDETIHGYSGS-GEDPIVYVG	154	
φNIT1	NIT1	117	GLNTELRNLIIVSKLNSKGIAAEVAT--DRFTATDPDNIIVNRCASGKGVOLHISSAORRAF	174
	Grass	117	GLNTELRNLIIVSKLNSKGIAAEVAT--DRFTATNPDNIIVNRCASGKGVOLHISSAORRAF	174
	FSG	117	GLNTELRNLIIVSKLNSKGIAAEVAT--DRFTATDPDNIIVNRCASGKGVOLHISSTORRAF	174
	Sp50	117	GLNTELRNLIIVSKLNSKGIAAEVAT--DRFTATDPDNIIVNRCASGKGVOLHISSKORRAF	174
Bp8p	Bobb	118	GLNEEFKQIVIKHLQAQGFNAEAAAT--TRFTGTDPKNIIVNRCHEGAGVQLELSTLQKRF	175
	AGATE	118	GLDEEFKQIVIKHLQAQGFNAEAAAT--TRFTGTDPKNIIVNRCHEGAGVQLELSTLQKRF	175
	Bp8p	118	GLDEEFKEIVIRHLQAQGFNAEAAAT--TRFTGTDPNNIIVNRCHEGAGVQLELSTLQKRF	175
PM1	PM1	114	GLSDTLKEAVYKSLSSYGFNVAYAT--DRFTATDPNNIIVNRAIR-RGVQLELSTAQRKAF	170
	SPP1	111	GLHEALEKNIISALNDAGFKAVAAT--DRFTATNPDNIICNRCATGLGVQLELSTLQKRF	168
	SP10	114	GTNRPALKIADDLVRQGFSAELIPEDHKFAGTNPENINNKRTGLSVQLELSTAQREAF	170
Bsu	YoqZ	175	GTDREKAAKITSLLTDAGFSAELLSSEDSRLAGTNEQNIANKNSTGMSIOLELSTEQRRM	234
	YndL	161	GTDRAADLVEKLQHAGFPVLLNMDHPHAGVSPNNIANKSKTGLSIOLELSTGFRKSL	220
	YmaC	153	GTDKRKAKMIVRALERRGFSAEALSSKSLAGLNAENINNOGETGLSIOLELSTREOREAF	202
	YjqB	111	GADRKKAKLICERLNEAGFSAELTNEKDRLAGVHPNNIIVNRTKREMGLOLELSTAQORNAL	170
	Bpu	115	GTDREAAETVKKRLTQNGFHALLAESDRFSGTHPDNIINNKCLTGKSVQLELSTQAORRAF	175
Bli	148	GTDTGAKAIVRALLNKGFSAEVSPSHRLAGGNPENINNOCKTQGSIOLELSTPQREAF	207	
Sha	155	GKDKKLAKSITKSLKNGFTVQKSP--KGIATSSSNIINRSDNDSGVQLELTTQORALF	212	
φNIT1	NIT1	175	FQNDWSKAN-RGNVTQEF-LDYAEAIKEAAEYYG----- 208	
	Grass	175	FQNDWSKAN-RGNVTQEF-LDYAEAIKEAAEYYG----- 208	
	FSG	175	FQNDWSKAN-RGNVTQEF-LDYADAIKEAAEYYG----- 208	
	Sp50	175	FENDDWSKGN-RGNVTQEF-LDYADAIKEAAEYYG----- 208	
Bp8p	Bobb	176	FTNDWSAGNRRRLNRTEVLTERRYAKAIALAIDEYTEGRVAFDDLDQL----- 222	
	AGATE	176	FYNDWSAGN-RHNRTEVL-ERYASAIALAIEEYTEGRIVTDEDNLVITSL----- 224	
	Bp8p	176	FFHDDWSAGN-RHYRTKVL-ERYASAVALAIEEYTDGRIVTDEDNLYISMQSHSNDL 230	
PM1	PM1	171	FEGGDWSKDN-RMNRDAF-YNYVTAVRYALT----- 200	
	SPP1	169	FEGQDWHSGK---MSGEF-YDYITAIGEGLTGL----- 197	
	SP10	171	FDVFTREERQ--YTKNDEF-HKYVASIYLSMLEGV----- 205	
Bsu	YoqZ	235	FNTFT----LAGRNGTQNOVFYDYIAVLTKFINENVYCMAGVAP----- 274	
	YndL	221	FGIFS----LKSRAVQNERFYEFTEVMFRFL-KNSY----- 252	
	YmaC	203	FDDFY----YKRNKYTKNSFEYAYVSAIKGVLEKEYS----- 235	
	YjqB	171	FRNFG----CRDKSIQNDLDFRYVEAVKLGFT----- 200	
	Bpu	176	FQDFR----RRYRDTQTEQFYQYTNVVKQVLALYE----- 207	
	Bli	208	FSEFG----LWTRASSKNETFQAYVSAVKEVLETRYK----- 240	
Sha	213	FKDKQ----LDQNIIRKNPDNYTHTF-YKFAKAVNKGIEDAQ----- 248		

Fig. 8. Analyses of the γ -PGA hydrolases encoded in the genome of *Bacillus* phages.

All protein sequences were obtained from the DDBJ/GenBank/EMBL databases. Alignment and tree construction were performed using ClustalW. (A) Comparison of the deduced amino acid sequences of γ -PGA hydrolases encoded in the genome of *Bacillus* phages and bacteria. The zinc binding motif and phosphate binding motif are indicated as black and open boxes, respectively. Amino acids identical with PghP are shaded. Bsu ; YoqZ, YndL, YmaC and YjqB proteins of *Bacillus subtilis* strain 168,⁴⁵ Bpu ; *Bacillus pumilus* hypothetical protein (accession No. WP_012009647), Bli ; *Bacillus Licheniformis* hypothetical protein (accession No. AAU24062), Sha ; *Staphylococcus haemolyticus* hypothetical protein (accession No. WP_011276332).

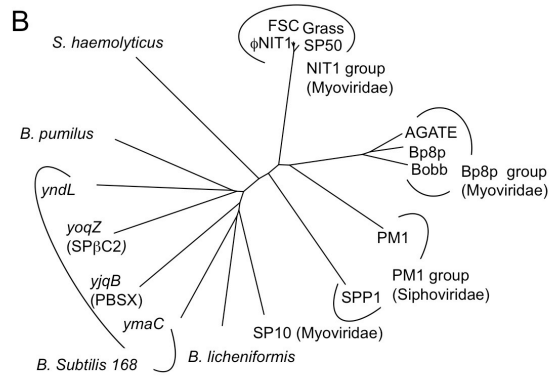


Fig. 8. (Continued)

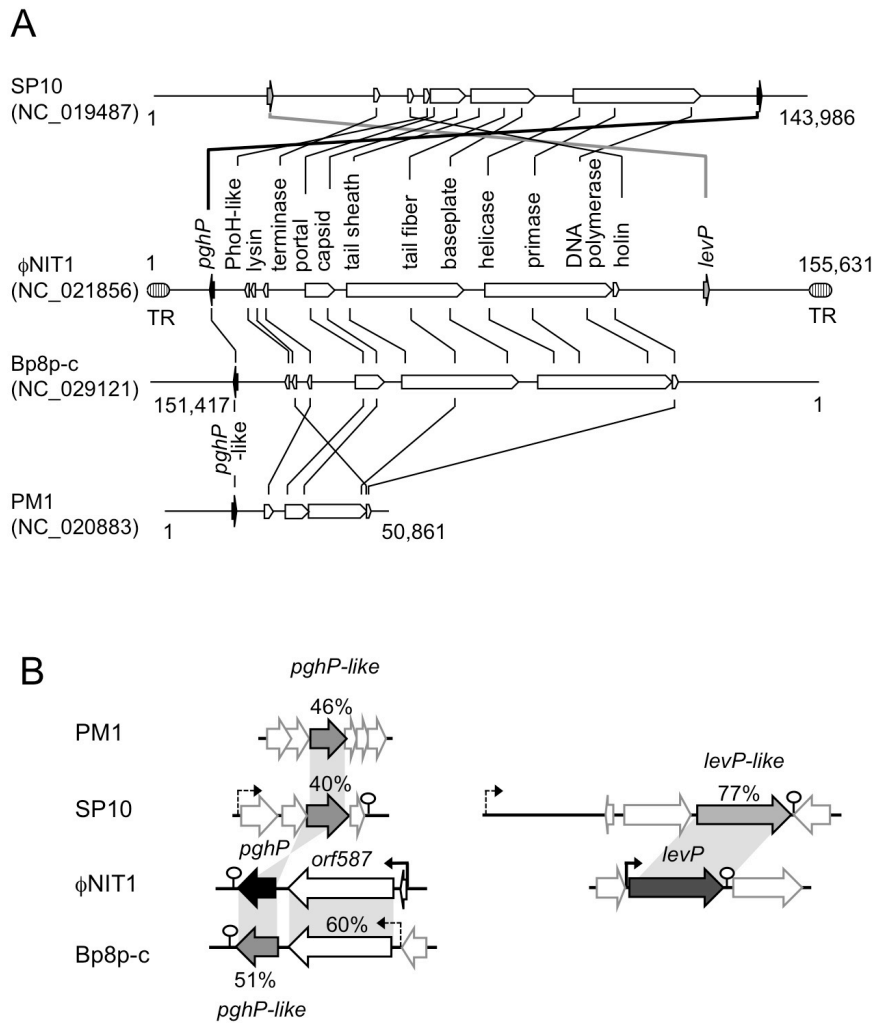


Fig. 9. Outline of the genome of *Bacillus* phages carrying genes for capsule-hydrolyzing enzymes.

(A) Comparative organization of genome structures of *Bacillus* phages carrying genes for capsule-hydrolyzing enzymes. Predicted modules are indicated by boxes. Positions of *pghP*-like and *levP*-like genes are indicated as arrows.

(B) Details of gene structure around the *pghP*-like and *levP*-like genes. Genes are indicated as arrows, and conserved regions are connected by gray shading. Identity values (%) of the predicted gene products are indicated.

In addition to ϕ NIT1 and its related phages, other phages carrying a possible poly- γ -glutamate hydrolase gene on their genomes have been reported. *Siphoviridae* bacteriophage PM1, which has been isolated from an abnormal natto product, is carrying a gene coding for a possible poly- γ -glutamate hydrolase.⁴⁰ BLAST analysis revealed that a related *Bacillus* siphophage SPP1⁴¹ (NC004166) also carries a different gene encoding a PghP-like protein. The deduced amino acid sequence of the PghP-like proteins of PM1 and SPP1 showed 51% and 42% identity with PghP, respectively (Fig. 8A). Most recently, *pghP*-like genes in the genomes of some *Bacillus Myoviridae* phages, such as AGATE⁴² (NC_020081) and Bp8p-c⁴³ (NC_029121) were reported, and a *pghP*-like gene was also found in the related phage Bobb (NC_024792) by BLAST analysis. The crystal structure of PghP indicated that a phosphate binding motif (His78-Thr89-Ser81), zinc binding motif (His40-Glu45-His103) and Glu249 are important for its enzymatic catalytic properties.⁴⁴ These residues were completely conserved among all of these PghP-like proteins (Fig. 8A). It is worthwhile to note that the deduced amino acid sequences of PghP-like proteins in these myophages showed around 50% identity with PghP, and those of the *Siphoviridae* phages (Fig. 8A). As the result, phage encoded PghP and PghP-like proteins were classified into four groups (Fig. 8B). Thus, the groups of *pghP*-carrying myophage were divided into two groups, coincident with the grouping of morphological protein groups indicated in Fig. 3B.

Recently, existence of four genes similar to *pghP* in the genome of *B. subtilis* strain 168 was reported.⁴⁵ Two of them are located near the phage-related region 5, and others are flanked by PBSX and SP β prophage elements, suggesting ancestral temperate phages of these elements may carry *pghP*-like gene. PghP related proteins were also found in the genome of several bacteria, but these PghP-like proteins were classified into a different group from the PghP and PghP-like proteins of myophages except for that of temperate phage SP10 (Fig. 8A and B).

The genomic structure of the *Bacillus Myoviridae* phages phiAGATE⁴³ and Bp8p-c⁴⁴ carrying a *PghP*-like gene was similar to that of ϕ NIT1, but the existence of TRs in their genomes was not investigated (Fig. 9A). The upstream *orf587*-like gene and *pghP*-like gene were thought to be transcribed into poly cistronic mRNA similar to ϕ NIT1 (Fig. 8B). In contrast, the genome structure of another myophage SP10⁴⁶ was different from those of ϕ NIT1 and Bp8p-c (Fig. 9A). Genes of *pghP*-like and *levP*-like proteins were found in the possible FGCs, but the structure of their transcriptional units differed from those of ϕ NIT1 (Fig. 9B). The *orf587*-like *orf* was absent at the flanking region of the *pghP*-like gene in SP1, and this structure was rather close to that of *Siphoviridae* PM1 (Fig. 9B).

The variations of primary and genetic structures among phage-carrying PghP, LevP and related proteins suggest that the genes encoding these proteins originated from different ancestor and/or evolved independently after acquisition by phages.

B. subtilis produces different types of biofilms, such as

colony biofilms, floating biofilms (robust pellicles) and submerged surface-adhered biofilms, depending on the culture condition.⁴⁷ γ -PGA is one of the components in the floating biofilms, but its production is not required for floating biofilms formation.⁴⁹⁸ Conversely, γ -PGA production is involved in mucoid colony morphology, and enhances submerged biofilm formation.⁴⁹ Recently, it was shown that levan can be a structural and possibly stabilizing component of *B. subtilis* floating biofilms.⁵⁰ Therefore, under certain conditions, having γ -PGA in the biofilm may be enough on its own to defend against phage infection, and levan may play an auxiliary role as a barrier. *B. subtilis* (*natto*) produces γ -polyglutamate and levan as the extracellular capsule polymers,⁵²¹ and they are thought to act as a barrier to phage adsorption to the cell. In fact, removal of capsular γ -PGA from the surface of the host cell by the γ -poly glutamate hydrolase activity of PghP in a supernatant of ϕ NIT1-infected NAFM5 lysate is important for the effective infection of encapsulated cell with ϕ NIT1.¹⁶ Namely, elimination of γ -PGA by PghP allows progeny phages approach to their receptors on the host cell. Existence of phages carrying only *pghP* (phages Grass and SP50) and *pghP*-like (phages AGATE and Bp8p-c) genes suggests their γ -PGA hydrolyzing enzymes are beneficial for the phage infection.

Unlike phages carrying a polysaccharide depolymerase on their virions, ϕ NIT1 requires the initial infection of the host cells to produce the capsule-hydrolyzing enzymes. As no signal sequence was contained in the deduced amino acid sequences of PghP and LevP, these enzymes are intracellularly produced by infected cells, and should be released by cell lysis concomitantly with the release of phage progenies to depolymerize the capsule polymers. In contrast to polyglutamate capsule of *B. anthracis*, the capsule of *B. subtilis* (*natto*) is not anchored to the peptidoglycan such some *B. subtilis* (*natto*) cells in the population may remain as naked cells allowing phage infection. Once ϕ NIT1 successfully infects the host cell, PghP and LevP should be expressed in parallel with the production of phage proteins before host cell lysis.

We tried to assess the importance of LevP in the phage infection using *Bacillus* phage SP50 that only has the *pghP* gene. The *Bacillus subtilis* (*natto*) starter strain Takahashi is known as a γ -PGA and levan producer in the presence of sucrose in the media.⁵¹² One-step growth analysis was used to evaluate the effect of PghP and LevP on the phage infection. When ϕ NIT1 and SP50 were added to the encapsulated stationary-phase cells of the Takahashi strain, phage progenies were detected at 120 min after phage addition, and treatment of recombinant PghP (rPghP) shortened the eclipse period to 60 min. These results support the previous observation that removal of γ -PGA from the surface of the cells by rPghP enabled the phage particles to easily access the host receptors. This apparently long eclipse of phage to the encapsulated cells might be related to the time required to produce the capsule depolymerase by the fraction of cellular population that were infected. Unexpectedly, recombinant LevP (rLevP) treatment of encapsulated host

cells did not show significant shortening of the eclipse time, and treatment of the cells with both rPghP and rLevP did not show any synergistic or additive effects for the phage growth. The role of the phage-encoded levanase in the phage life cycle remains to be investigated.

Author contributions

J. K. and N. A. designed this study, and T. O. and A. S. performed the experiment. T. O., N. A., K. K., A. S. and J. K. analyzed the data and discussed the results. J.K. wrote the manuscript. All authors have read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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REFERENCES

- Bernheimer HP, Tiraby JG. Inhibition of phage infection by pneumococcus capsule. *Virology*. 1976;73:308–309.
- Wilkinson BJ, Holmes KM. *Staphylococcus aureus* cell surface: capsule as a barrier to bacteriophage adsorption. *Infect. Immun.* 1979;23:549–552.
- Cescutti P, Paoletti S. On the specificity of a bacteriophage-borne endoglycanase for the native capsular polysaccharide produced by *Klebsiella pneumoniae* SK1 and its derived polymers. *Biochem. Biophys. Res. Commun.* 1994;198: 1128–1134.
- Ohshima Y, Schumacher-Perdreau F, Peters G, et al. The role of capsule as a barrier to bacteriophage adsorption in an encapsulated *Staphylococcus simulans* strain. *Med. Microbiol. Immunol.* 1988;177:229–233.
- Kostakioti M, Hadjifrangiskou M, Hultgren SJ. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold. Spring. Harb. Perspect. Med.* 2013;3:a010306.
- Bayer ME, Thurow H, Bayer MH. Penetration of the polysaccharide capsule of *Escherichia coli* (Bi161/42) by bacteriophage K29. *Virology*. 1979;94:95–118.
- Long GS, Bryant JM, Taylor PW, et al. Complete nucleotide sequence of the gene encoding bacteriophage E endosialidase: implications for K1E endosialidase structure and function. *Biochem. J.* 1995;309:543–550.
- Tanaka T, Fujita KI, Takenishi S, et al. Existence of an optically heterogeneous peptide unit in poly(γ -glutamic acid) produced by *Bacillus subtilis*. *J. Ferment. Bioeng.* 1997;84:361–364.
- Leiman PG, Battisti AJ, Bowman VD, et al. The structures of bacteriophages K1E and K1-5 explain processive degradation of polysaccharide capsules and evolution of new host specificities. *J. Mol. Biol.* 2007;371:836-849.
- Scholl D, Adhya S, Merrill C. *Escherichia coli* K1's capsule is a barrier to bacteriophage T7. *Appl. Environ. Microbiol.* 2005;71:4872-4874.
- Candela T, Fouet A. Poly-gamma-glutamate in bacteria. *Mol. Microbiol.* 2006;60:1091-1098.
- Hanby WE, Rydon HN. The capsular substance of *Bacillus anthracis*: With an appendix by P. Bruce White. *Biochem. J.* 1946;40:297–309.
- Candela T, Fouet A. *Bacillus anthracis* CapD, belonging to the gamma-glutamyltranspeptidase family, is required for the covalent anchoring of capsule to peptidoglycan. *Mol. Microbiol.*, **57**, 717–726 (2005).
- Candela T, Mock M, Fouet A. CapE, a 47-amino-acid peptide, is necessary for *Bacillus anthracis* poly-glutamate capsule synthesis. *J. Bacteriol.* 2005;187: 7765–7772.
- Thorne CB. *Bacillus anthracis*. In: Sonenshein AL, Hock JA, Losick R, editors. *Bacillus subtilis* and other Gram-positive bacteria. Washington, D.C.: American Society for Microbiology; 1993. pp. 113-124.
- Kimura K, Itoh Y. Characterization of poly-gamma-glutamate hydrolase encoded by a bacteriophage genome: possible role in phage infection of *Bacillus subtilis* encapsulated with poly-gamma-glutamate. *Appl. Environ. Microbiol.* 2003;69:2491–2497.
- Negus D, Burton J, Sweed A, et al. Poly- γ -(D)-glutamic acid capsule interferes with lytic infection of *Bacillus anthracis* by *B. anthracis*-specific bacteriophages. *Appl. Environ. Microbiol.* 2013;79:714-717.
- Nagai T, Yamasaki F. *Bacillus subtilis* (*natto*) bacteriophages isolated in Japan. *Food sci. Technol. Res.* 2009;15:293-298.
- Nagai T, Kimura K, Itoh Y. Chemical analysis of poly- γ -glutamic acid produced by plasmid-free *Bacillus subtilis* (*natto*): Evidence that plasmids are not involved in poly- γ -glutamic acid production. *J. Gen. Appl. Microbiol.* 1997;43:139–143.
- Kaneko J, Kimura T, Narita S, et al. Complete nucleotide sequence and molecular characterization of the temperate staphylococcal bacteriophage ϕ PVL carrying Panton-Valentine leukocidin genes. *Gene.* 1998;215:57-67.
- Kaneko J, Kimura T, Kawakami Y, et al. Pantone-valentine leukocidin genes in a phage-like particle isolated from mitomycin C-treated *Staphylococcus aureus* V8 (ATCC 49775). *Biosci. Biotechnol. Biochem.* 1997;61:1960-1962.
- Narita S, Kaneko J, Chiba J, et al. Phage conversion of Pantone-Valentine leukocidin in *Staphylococcus aureus*: molecular analysis of a PVL-converting phage, ϕ SLT. *Gene.* 2001;268:195-206.

- 23) Ohtsubo Y, Ikeda-Ohtsubo W, Nagata Y, et al. GenomeMatcher: a graphical user interface for DNA sequence comparison. *BMC Bioinformatics*. 2008;9, 376.
- 24) Ackermann H-W, Azizbekyan RR, Bernier RL, et al. Phage typing of *Bacillus subtilis* and *B. thuringiensis*. *Res. Microbiol.* 1995;146:653-657.
- 25) Stewart CR, Casjens SR, Cresawn SG, et al. The genome of *Bacillus subtilis* bacteriophage SPO1. *J. Mol. Biol.* 2009;388:48-70.
- 26) Klumpp J, Dorscht J, Lurz R, et al. The terminally redundant, nonpermuted genome of *Listeria* bacteriophage A511: a model for the SPO1-like myoviruses of gram-positive bacteria. *J. Bacteriol.* 2003;190:5753-5765.
- 27) Kwan T, Liu J, DuBow J, et al. The complete genomes and proteomes of 27 *Staphylococcus aureus* bacteriophages. *Proc. Natl. Acad. Sci. U S A*. 2005;102;5174-5179.
- 28) Uchiyama J, Rashel M, Takemura I, et al. In silico and in vivo evaluation of bacteriophage phiEF24C, a candidate for treatment of *Enterococcus faecalis* infections. *Appl. Environ. Microbiol.* 2008;74:4149-4163.
- 29) Lavigne R, Darius P, Summer EJ, et al. Classification of *Myoviridae* bacteriophages using protein sequence similarity. *BMC Microbiol.* 2009;9: 224.
- 30) Miller SY, Colquhoun JM, Perl AL, et al. Complete genome of *Bacillus subtilis* myophage Grass. *Genome Announc.* 2013;1:e00857-13.
- 31) Parker ML, Eiserling FA. Bacteriophage SPO1 structure and morphogenesis. III. SPO1 proteins and synthesis. *J. Virol.* 1983;46:260-269 (1983).
- 32) O'Flaherty S, Coffey A, Edwards R, et al. Genome of staphylococcal phage K: a new lineage of *Myoviridae* infecting gram-positive bacteria with a low G+C content. *J. Bacteriol.* 2004;186:2862-2871.
- 33) Chibani-Chennoufi S, Dillmann M-L, Marvin-Guy L, et al. *Lactobacillus plantarum* bacteriophage LP65: a new member of the SPO1-like genus of the family *Myoviridae*. *J. Bacteriol.* 2004;186:7069-7083.
- 34) Murai N, Kamata H, Nagashima Y, et al. A novel insertion sequence (IS)-like element of the thermophilic bacterium PS3 promotes expression of the alanine carrier protein-encoding gene. *Gene*, 1995;22:103-107.
- 35) Maaroufi H, Levesque RC. Glycoside hydrolase family 32 is present in *Bacillus subtilis* phages. *Virol. J.* 2015;12:157.
- 36) Loessner MJ, Scherer S. Organization and transcriptional analysis of the *Listeria* phage A511 late gene region comprising the major capsid and tail sheath protein genes *cps* and *tsh*. *J. Bacteriol.* 1995;177:6601-6609.
- 37) Young I, Wang I, Roof WD. Phages will out: strategies of host cell lysis. *Trends Microbiol.* 2000;8:120-128.
- 38) Miller ES, Kutter E, Mosig G, et al. Bacteriophage T4 genome. *Microbiol. Mol. Biol. Rev.* 2003;67:86-156.
- 39) Jarmer H, Larsen TS, Krogh A, et al. Sigma A recognition sites in the *Bacillus subtilis* genome. *Microbiology*. 2001;147:2417-2424.
- 40) Umene K, Shiraishi A. Complete nucleotide sequence of *Bacillus subtilis* (*natto*) bacteriophage PM1, a phage associated with disruption of food production. *Virus Genes*. 2013;46:524-534.
- 41) Alonso JC, Lüder G, Stiege AC, et al. The complete nucleotide sequence and functional organization of *Bacillus subtilis* bacteriophage SPP1. *Gene*. 1997;204:201-212.
- 42) Barylski J, Nowicki G, Goździcka-Józefiak A. The discovery of phiAGATE, a novel phage infecting *Bacillus pumilus*, leads to new insights into the phylogeny of the subfamily *Spounavirinae*. *PLoS One*. 2014;9:e86632.
- 43) Yuan Y, Peng Q, Wu D, et al. Effects of actin-like proteins encoded by two *Bacillus pumilus* phages on unstable lysogeny, revealed by genomic analysis. *Appl. Environ. Microbiol.* 2015;81:339-350.
- 44) Fujimoto Z, Kimura K. Proteins. Crystal structure of bacteriophage phiNIT1 zinc peptidase PghP that hydrolyzes gamma-glutamyl linkage of bacterial poly-gamma-glutamate. 2012;80:722-732.
- 45) Mmberti S, Prati P, Cremaschi P, et al. gamma-PGA Hydrolases of phage origin in *Bacillus subtilis* and other microbial genomes. *PLoS One*. 2015;10:e0130810.
- 46) Yee LM, Matsumoto T, Yano K, et al. The genome of *Bacillus subtilis* phage SP10: a comparative analysis with phage SPO1. *Biosci. Biotechnol. Biochem.* 2011;75:944-952.
- 47) Vlamakis H, Chai Y, Beaugard P, et al. Sticking together: building a biofilm the *Bacillus subtilis* way. *Nat. Rev. Microbiol.* 2013;11:157-168.
- 48) Morikawa M, Kagihiro S, Haruki M, et al. Biofilm formation by a *Bacillus subtilis* strain that produces gamma-polyglutamate. *Microbiology*. 2006;152:2801-2807.
- 49) Stanley NR, Lazazzera BA. Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly-gamma-dl-glutamic acid production and biofilm formation. *Mol. Microbiol.* 2005;57:1143-1158.
- 50) Dogsa I, Brložnik M, Stopar D, et al. Exopolymer diversity and the role of levan in *Bacillus subtilis* biofilms. *PLoS One*. 2013;8:e62044.
- 51) Shih IL, Yu YT. Simultaneous and selective production of levan and poly(gamma-glutamic acid) by *Bacillus subtilis*. *Biotechnol. Lett.* 2005;27:103-106.