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ORIGINAL PAPER

Characterization of *Bacillus* phage-K2 isolated from chungkookjang, a fermented soybean foodstuff

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Abstract An investigation of a virulent *Bacillus* phage-K2 (named Bp-K2) isolated from chungkookjang (a fermented soybean foodstuff) was made. Bp-K2 differed in infectivity against a number of *Bacillus subtilis* strains including starter strains of chungkookjang and natto, being more infectious to *Bacillus* strains isolated from the chungkookjang, but much less active against a natto strain. Bp-K2 is a small DNA phage whose genome size is about 21 kb. Bp-K2 is a tailed bacteriophage with an isometric icosahedral head (50 nm long on the lateral side, 80 nm wide), a long contractile sheath (85–90 nm × 28 nm), a thin tail fiber (80–85 nm long, 10 nm wide), and a basal plate (29 nm long, 47 nm wide) with a number of spikes, but no collar. The details of the structures of Bp-K2 differ from natto phage ϕBN100 as well as other known *Bacillus* phages such as SPO1-like or ϕ 29-like viruses. These data suggest that Bp-K2 would be a new member of the *Myoviridae* family of *Bacillus* bacteriophages.

Keywords *Bacillus* phage-K2 (Bp-K2) · *Bacillus subtilis* · Chungkookjang · DNA phage · Isometric icosahedral head · *Myoviridae*

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Introduction

Recently, fermented soybean foodstuffs have drawn great attention from many people and is considered a well-being, healthy food. Korean chungkookjang, a traditional fermented soybean foodstuff, is comparable to natto (Japan), kinema (Nepal), and thuanao (Thailand) [10]. Fermented soybean food products such as chungkookjang are known to affect a number of physiological activities such as fibrinolytic activity, lowering of blood pressure, prevention of osteoporosis, etc. [6, 8, 16]. Chungkookjang is usually manufactured by fermenting steam-cooked soybeans with *Bacillus subtilis* for a couple of days [7]. A high-grade chungkookjang is covered with a copious amount of a sticky substance whose chemical nature is poly-γ–glutamate (γ-PGA). Poly-γ–glutamate is known to exert various bioactivities of chungkookjang. Productivity of γ-PGA, a metabolic product of *Bacillus subtilis*, is highly affected by *Bacillus* physiology as well as *Bacillus* phages infecting the host strains [9]. However, there is almost no information on the bacteriophage responsible for chungkookjang spoilage, although there is a report on the presence of the bacteriophage of *Bacillus subtilis* var. 816, which is an industrial bacterial strain employed in manufacturing soybean paste associated with poor quality of products [11]. Contrary to the very meager knowledge about the bacteriophage of *B. subtilis* (chungkookjang), investigations of *Bacillus subtilis* (natto) bacteriophages have been conducted intensively over the past few decades in Japan [5, 9, 14, 15]. To gain insights into the Bacillus phages infecting the starter bacterial strain of chungkookjang fermentation, a virulent bacteriophage (named Bp-K2) isolated from chungkookjang products was investigated with respect to infectivity toward a number of *B. subtilis* strains, morphology, and its genomic nature, including genome size and DNA restriction pattern.

Materials and methods

Bacterial strains and culture medium

Strains of *Bacillus subtilis*, K1 (laboratory strain), K2, K3, and K4 isolated from chungkookjang products sold in local markets (Korea) and J1, an isolate from Japanese natto, were employed as the host for the *Bacillus* phage. LB broth or LB agar containing 5 mM $MgSO_4$ was used in *Bacillus* propagation, and LB soft agar containing 0.7% agar and 10 mM $MgSO_4$ were used in overlaying the phage on the LB agar.

Phage isolation and preparation of high titer phage stock

A small potion of spoiled chungkookjang (Hankooknatto, Youngin, Korea) suspended in SM buffer (50 mM Tris-HCl, 100 mM NaCl, 8 mM $MgSO_4$) was centrifuged (12,000 rpm, 15 min), and then the supernatant was filtered through a 0.2-μm Millipore filter. The filtrate was mixed with *Bacillus* strain K2 in LB soft agar and overlaid on a LB plate to check phage infectivity. The bacteriophage was harvested from confluent plaques by pouring an aliquot of SM buffer, and then the phage suspension was filtered to remove bacterial cell debris. The phage stock was stored in chloroform (0.2%) at 4°C.

Infectivity of Bp-K2 to various *Bacillus* strains

B. subtilis strains, such as K1 (CBNU laboratory strain), K2, K3, and K4, isolated from Korean chungkookjang, and J1 (isolate from Japanese natto) were employed to check cross infectivity. Each 100 μl of the overnight-grown cells in LB broth was mixed with 100 μl of the diluted phage solution (10^6–10^7 PFU/ml) in LB soft agar and overlaid on a LB plate. The plates were incubated at 37°C for 24 h.

Energy filtering transmission electron microscopy

Phage stock fixed on a Formvar-coated EM grid and strained with 2% phosphotungstic acid (aqueous, pH 7.0) was viewed with the energy filtering™ (Leo 912AB Omega, Leo, Oberkochen, Germany) [19]. This work was carried out at the Korean Basic Science Institute, Chunchun Branch, Korea.

Bio-TEM (Bio-Transmission electron microscopy)

Phage stock fixed on a Formvar-coated EM grid and strained with 2% phosphotungstic acid (aqueous, pH 7.0) was viewed with Bio-TEM (Tecnai™ TEM, FEI Company, Hillsboro, OR) [19]. This work was carried out at the Korean Basic Science Institute, Daejon, Korea.

Preparation of nucleic acid from *Bacillus* phage K2 (=Bp-K2)

Nucleic acid of Bp-K2 was extracted with phenol-chloroform isoamyl alcohol (25:24:1). Nucleic acid precipitated with cold absolute ethanol was suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) [12].

Treatment of Bp-K2 nucleic acid with nucleases

Ten μl of Bp-K2 nucleic acid (980 ng/μl) was incubated with either 4 units of DNase I (1 unit/μl) or 40 μg of RNase A (10 μg/μl) for 1 h at 37°C [12]. Then the products were resolved on agarose gel (0.8%). The molecular size of Bp-K2 DNA was estimated with a parallel run of a DNA size ladder. The appropriate treatment of Bp-K2 DNA with a number of restriction enzymes, such as *Hin*dIII, *Bam*HI, *Eco*RV, *Sac*I, *Spe*I, *Sma*I, *Kpn*I, *Xba*I, *Bcl*I, and *Sac*II (10 units each), was carried out according to the suppliers' suggestions. Restriction enzyme treated-Bp-K2 DNA was resolved on 1.0% agarose gel [3].

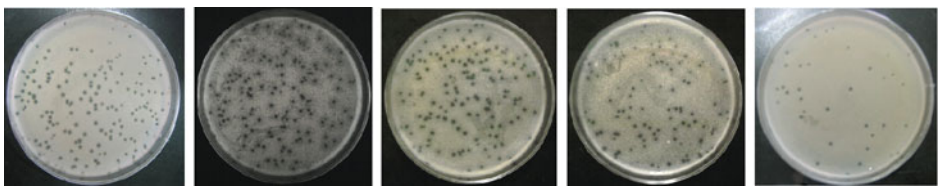

Fig. 1 Plaque formation of Bp-K2 on various *Bacillus subtilis* strains. One hundred μl of an overnight-grown *B. subtilis* strain in LB medium and 100 μl of the diluted phage solution (10^6–10^7 PFU/ml) were mixed in LB soft agar and overlaid on a *LB plate*. The *plates* were incubated at 37°C for 24 h. K1: CBNU laboratory strain, K2, K3, and K4: isolates from Korean chungkookjang, J1: isolate from Japanese natto

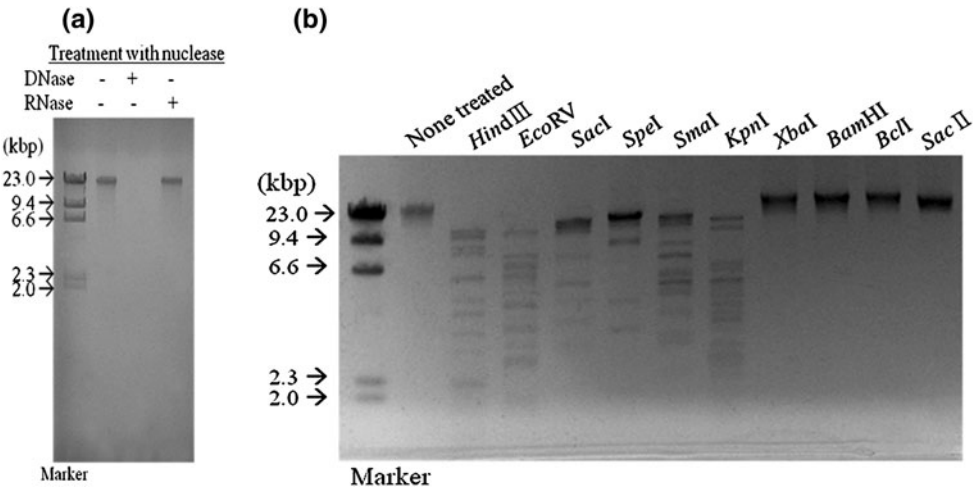

Fig. 2 Agarose gel electrophoresis of Bp-K2 genomic nucleic acid. **a** The products of Bacillus phage K2 nucleic acid treated with either DNAse or RNAse were resolved on 0.8% agarose gel. **b** Restriction digest patterns of Bacillus phage K2 DNA after treatment with restriction enzymes and followed by resolution on 1.0% agarose gel

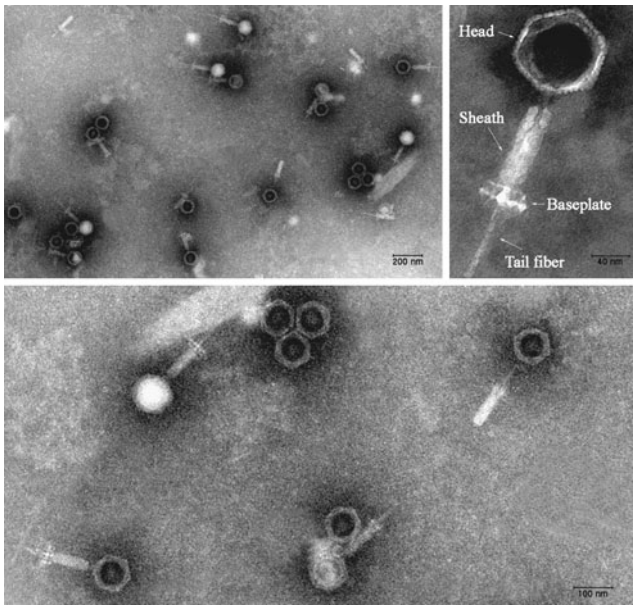

Fig. 3 Electron micrographs of *Bacillus* phage Bp-K2. A lateral side and diameter of the head were: ca. 50 × 80 nm. The length and width of the sheath were: ca. 85–90 × 28 nm. The length and width of the base plate were: ca. 29 × 47 nm. The length and width of the tail fiber were: ca. 80–85 × 10 nm

Results and discussion

Infectivity of *Bacillus* phage K2 isolated from spoiled chungkookjang for *Bacillus subtilis* varied from strain to strain. Bp-K2 was found to be highly active against *B. subtilis* starter strains isolated from the Korean chungkookjang, but relatively less active against a Japanese natto strain as shown in Fig. 1. The different infectivity was rather conceivable since a great variance exists among *Bacillus subtilis* strains including *Bacillus* natto strain and *Bacillus* chungkookjang strain [2].

As shown in Fig. 2a, the nucleic acid of *Bacillus* phage-K2 treated with DNase I was completely digested, whereas that treated with RNase A remained as intact. The data clearly revealed that Bp-K2 is a small DNA phage whose genome size is about 21 kb. The appropriate treatment of Bp-K2 DNA with a number of restriction enzymes, such as *Hin*dIII, *Bam*H, *Xba*I, *Eco*RV, *Sac*I, etc., resulted in restriction digest patterns as depicted in Fig. 2b. Bp-K2 DNA seemed to be resistant to a certain kind of restriction enzymes, i.e., *Bam*H I, *Bcl* I, *Xba*I, and *Sac*II.

The morphological study of Bp-K2 revealed an isometric icosahedral head (50 nm long on the lateral side, 80 nm wide), sheath (85–90 nm × 28 nm), tail fiber (80–85 nm), and baseplate, but no collar. The natto research team in Japan determined the heterogeneity of *Bacillus subtilis* natto phages present in Japan and classified into two groups, JNDMP (group I) or ONPA (group II), based on host ranges and comparative whole-genome hybridization [15]. JNDMP natto phage, i.e., ϕBN100, consisted of a hexagonal head and a long non-flexible tail with a knob at the tip [14], whereas the ONPA natto phage consisted of a bigger hexagonal head, a tail with a sheath, and a base [15]. The morphology of Bp-K2 is quite distinguishable from that of JNDMP. The general features of Bp-K2 and ONPA would be somewhat similar, but with a head diameter of Bp-K2 (80 nm), which is smaller than that of ONPA (89 nm). Besides, the most significant difference is in their genome sizes: ca. 21 kb of Bp-K2 and 91 kb of ONPA.

On the basis of electron micrographs (Fig. 3), a schematic diagram of Bp-K2 was depicted as shown in Fig. 4a. However, an argument would be possible that the tail fibers seen in Bp-K2 particles might be the naked tail tube that was revealed by contracting the sheath. Nevertheless, Bp-K2 seems to be different from other *Bacillus* phages, i.e., SPO1-like viruses (Fig. 4b) or ϕ29-like viruses (Fig. 4c) in

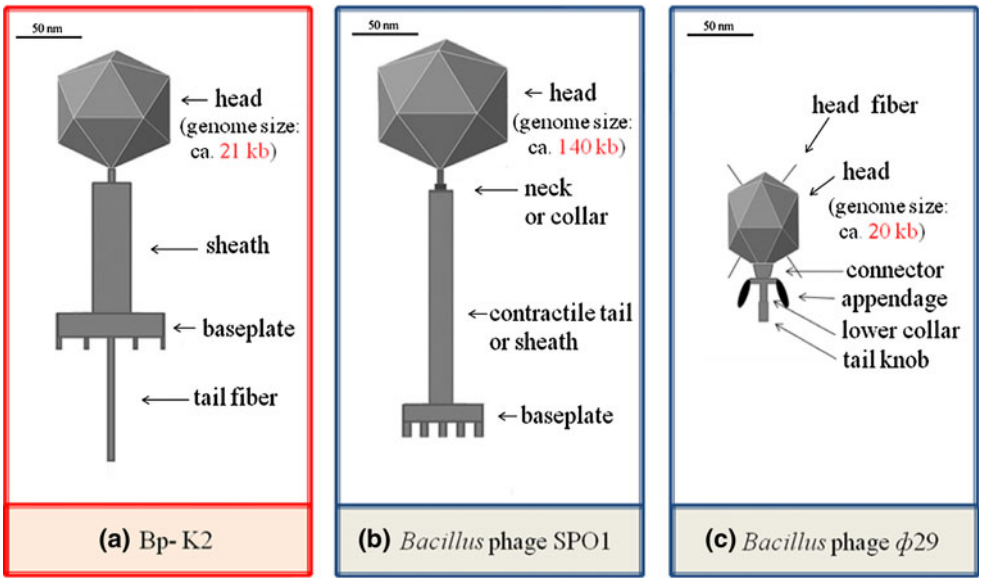

Fig. 4 Schematic representations of the *Bacillus* phages

structure and size [13, 17, 18]. These data suggest that Bp-K2 would be a smaller phage belonging to the *Myoviridae* family (phages with contractile sheath) [1, 4]. Currently, the Bp-K2 genome sequencing is being undertaken. The sequence data would provide better understanding of the molecular structure of Bp-K2.

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References

1. Ackerman H-W, Azizbekyan RR, Emadi Konjin HP, Lecadet M-M, Seldin L, Yu MX (1994) New *Bacillus* bacteriophage species. Arch Virol 135:333–344
2. Chun J, Bae KS (2000) Phylogenic analysis of *Bacillus subtilis* and related taxa based on partial *gyr*A gene sequences. Antonie van Leeuwenhoek 78:123–127
3. Davis LG, Dibner MD, Battey JF (1994) Basic methods in molecular biology, 2nd edn. Prentice-Hall Int. Inc., Norwalk, pp 86–95
4. Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) (2005) Virus taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, USA, pp 16–79
5. Fujii H, Shiraish A, Kaba H, Shibagaki M, Takahashi S, Honda A (1975) Abnormal fermentation in *natto* production and *Bacillus natto* phages. J Ferment Technol 53:424–428
6. Ikeda Y, Ike M, Morita A, Kajita E, Kagamimori S, Kagawa Y, Yoneshima H (2006) Intake of fermented soybeans, *natto*, is associated with reduced bone loss in postmenopausal women: Japanese population-based osteoporosis (JPOS) study. J Nutr 136:1323–1328
7. Kim KJ, Ryu MK, Kim SS (1982) Chungkookjang koji fermentation with rice straw. Kor J Food Sci Technol 14:301–308
8. Kim W, Choi K, Kim Y, Park H, Choi J, Lee Y, Oh H, Kwon I, Lee S (1996) Purification and characterization of a fibrinolytic enzyme produced from Bacillus sp strain CK 11–4 screened from chungkookjang. Appl Environ Microbiol 62:2482–2488
9. Kimura K, Itoh Y (2003) Characterization of poly-γ-glutamate hydrolase encoded by a bacteriophage genome: possible role in phage infection of *Bacillus subtilis* encapsulated with poly-γ-glutamate. Appl Environ Microbiol 69:2491–2497
10. Kimura K, Fuzimoto Z (2009) Bacteriophage contamination and fermentation of Natto by *Bacillus subtilis*(natto)-Study of phage related key enzyme that spoils sticky texture of natto. Book of abstracts, BioMicroWorld 2009, p 269
11. Lee Z-S (1978) Studies on the isolation and characterization of bacteriophage of *Bacillus subtilis* var.816. Kor J Microbiol 16:71–78
12. Ma YL, Lu CP (2008) Isolation and identification of a bacteriophage capable of infection *Streptococcus suis* type 2 strains. Vet Microbiol 132:340–347
13. Meijer WJJ, Hotcajadas JA, Salas M (2001) ϕ29 family of phages. Microbiol Mol Biol Rev 65:261–287
14. Nagai T, Itoh Y (1997) Characterization of a generalized transducing-phage of poly-γ–glutamic acid-producing *Bacillus subtilis* and its application for analysis of Tn917-LTV insertional mutants defective in poly-γ–glutamic acid production. Appl Environ Microbiol 63:4087–4089
15. Nagai T, Yamasaki F (2009) *Bacillus subtilis (natto)* bacteriophages isolated in Japan. Food Sci Techbnol Res 15:293–298
16. Park E, Shin JI, Park OP MH, Kang MH (2005) Soy isoflavone supplementation alleviates oxidative stress and improves systolic blood pressure in male spontaneously hypertensive rats. J Nutr Sci Vitaminol (Tokyo) 51:254–259
17. Parker ML, Eiserling FA (1983) Bacteriophage SPO1 structure and morphogenesis I. Tail structure and length regulation. J Virol 46:239–249
18. Parker ML, Ralston EJ, Eiserling FA (1983) Bacteriophage SPO1 structure and morphogenesis II. Head structure and DNA size. J Virol 46:250–259
19. Stenholm AR, Dalsgaard I, Middelboe M (2008) Isolation and characterization of bacteriophages infecting the fish pathogen *Flavobacterium psychrophilum*. Appl Environ Microbiol 74:4070–4078