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REVIEW
Overview of Studies on *Bacillus subtilis* (*natto*) Bacteriophages and the Prospects

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Abstract
Natto, a Japanese soybean food fermented by *Bacillus subtilis* (*natto*), are often spoiled by bacteriophages. The contamination of natto by phages causes a rapid loss of viscosity of poly-γ-glutamic acid (PGA), which is a key factor affecting the quality of natto. *B. subtilis* (*natto*) phages were classified into 2 groups (I and II), based on the homology of their genomic DNA. A phage JNDMP (Group I) has a head (diameter, 60 nm) and a flexible tail (7 × 200 nm) and requires magnesium ions for propagation. JNDMP was found to be a generalized transducing phage. A virulent phage ONPA (Group II) has a head (diameter, 89 nm) and a contractile tail (9 × 200 nm) with a sheath (width, 23 nm) and requires no additional magnesium ions for propagation. The loss of PGA due to contamination of the phages is attributed to a PGA hydrolase, PghP, coded in the phage genome. The enzyme hydrolyzes PGA to oligomers via an endopeptidase-type action and rapidly reduces the PGA viscosity. These days, although contamination of natto with phages is relatively infrequent, phages still exist on the floors of factories. Controlling them via thorough cleaning of factories and hygiene education for workers seem to be the most important solutions.

Discipline: Food
Additional key words: contamination, fermentation, hygiene, poly-γ-glutamic acid, soybean

Introduction

Natto is a Japanese soybean food fermented by *Bacillus subtilis* (*natto*), which produces a very viscous polymer, poly-γ-glutamic acid (PGA) (Fig. 1 A)[17]. Japanese people usually eat natto with cooked rice after seasoning it, e.g. with soy sauce. Sawamura isolated a strain from natto wrapped in rice straws and named it *B. natto* after the fermented food[21]. *B. natto* was reclassified as *B. subtilis* based on bacteriological characterization in Bergey's Manual of Determinative Bacteriology, 8th edition and the scientific name "*B. natto*" was abolished[6]. However, its informal name, "*B. subtilis* (*natto*)", is often used for natto-producing *B. subtilis* strains, especially in the food industry, to distinguish them from typical *B. subtilis* strains which cannot produce natto. Seed cultures of *B. subtilis* (*natto*) have been supplied as spore suspensions by 3 companies and strains suitable for natto fermentation were isolated from the seed cultures and characterized in detail[14]. The limited number of natto seed cultures and the simplicity of the natto fermentation process explain the rapid spread of *B. subtilis* (*natto*) phages in natto factories.

Until the early 20th century, natto was produced by wrapping boiled soybeans in rice straws, inhabited by *B. subtilis* (*natto*) (Fig. 1B). After the discovery of *B. subtilis* (*natto*) and as clean packages were developed rather than bags of rice straws, natto was produced by inoculating steamed soybeans with a suspension of *B. subtilis* (*natto*) spores and depositing them in clean packages[15,17]. Presently, many such packages are made of polystyrene. The general natto fermentation process is shown as a schematic flowchart on Fig. 1C and natto products are delivered to retail stores via cold chain systems after being cooled and maturated by natto manufacturers. Although simplifying the natto fermentation process facilitates the process of cleaning the natto factories and keeping them clean, phages can still invade factories via airborne dust, raw soybean material, and workers and remain the most harmful agents for natto suppliers even now.

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Classification of *B. subtilis* (*natto*) phages

Abnormal natto, on which no sticky PGA at all was observed, were previously often detected in the market. In some cases, PGA soon lost its viscosity when mixed with seasoning. Many *B. subtilis* (*natto*) phages were isolated by several researchers independently from such abnormal natto and factories having produced the same. In 1967, Fujii *et al.* isolated a *B. subtilis* (*natto*) phage from an abnormal natto and experimentally showed that abnormal natto was associated with the phage, named PN-1, having contaminated the natto, and resulting in a loss of PGA[3]. They isolated many natto-spoiling *B. subtilis* (*natto*) phages and classified them into 3 serological groups (PN-3, PN-6 and PN-19 respectively)[4]. Yoshimoto *et al.* collected 42 *B. subtilis* (*natto*) phages from factories nationwide and classified them serologically and ultimately into 2 groups (NP-4 and NP-38) based on reaction with 4 anti-phage serums[28]. Tabei classified his own isolates into 4 groups (BNP_1, BNP_2, BNP_3 and BNP_4) based on their morphologies and the results of one-step growth tests[22]. Nagai and Yamasaki reclassified the phages of Tabei's collection into 2 groups (I and II) independently via DNA-DNA hybridization (Fig. 2)[19]. A phage JNDMP from Group I has a head (diameter, 60 nm) and a flexible tail (7 × 200 nm) (Fig. 3 A). Amplification of JNDMP requires magnesium ions. JNDMP (a synonym of ΦBN100) can transduce the genes at frequencies ranging from 10^{-8} to 10^{-6} transductants/phage particle[16] and is used to construct mutants lacking the ability to produce branched short-chain fatty acids, which generate the malodor of natto[24]. A phage ONPA from Group II has a head (diameter, 89 nm) and a contractile tail (9 × 200 nm) with a sheath (width, 23 nm) and requires no additional magnesium ions for amplification (Fig. 3 B). The plaques of ONPA were clearer than those of JNDMP, which developed turbid plaques. Genome sizes of JNDMP and ONPA were 42 and 91 kb, respectively. The other characteristics of JNDMP and ONPA are summarized in Table 1. Group I consists of BNP_4 of Tabei's classification, while Group II consists of BNP_1, BNP_2, and BNP_3 (Table 2). Tabei's collection is deposited at the NIAS Genebank (Tsukuba, Japan; http://www.gene.affrc.go.jp/index_en.php) with accession numbers from MAFF 270101 to 270120.

NP-4 and NP-38 groups of Yoshimoto *et al.* could be the same as Groups I and II, respectively, judged on their morphologies alone, because of the lack of preservation of NP-4 and NP-38. Meanwhile, PN-3, PN-6 and PN-19 groups of Fujii *et al.* corresponded to Groups I, I and II, respectively, as the results of DNA-DNA hybridization[19].

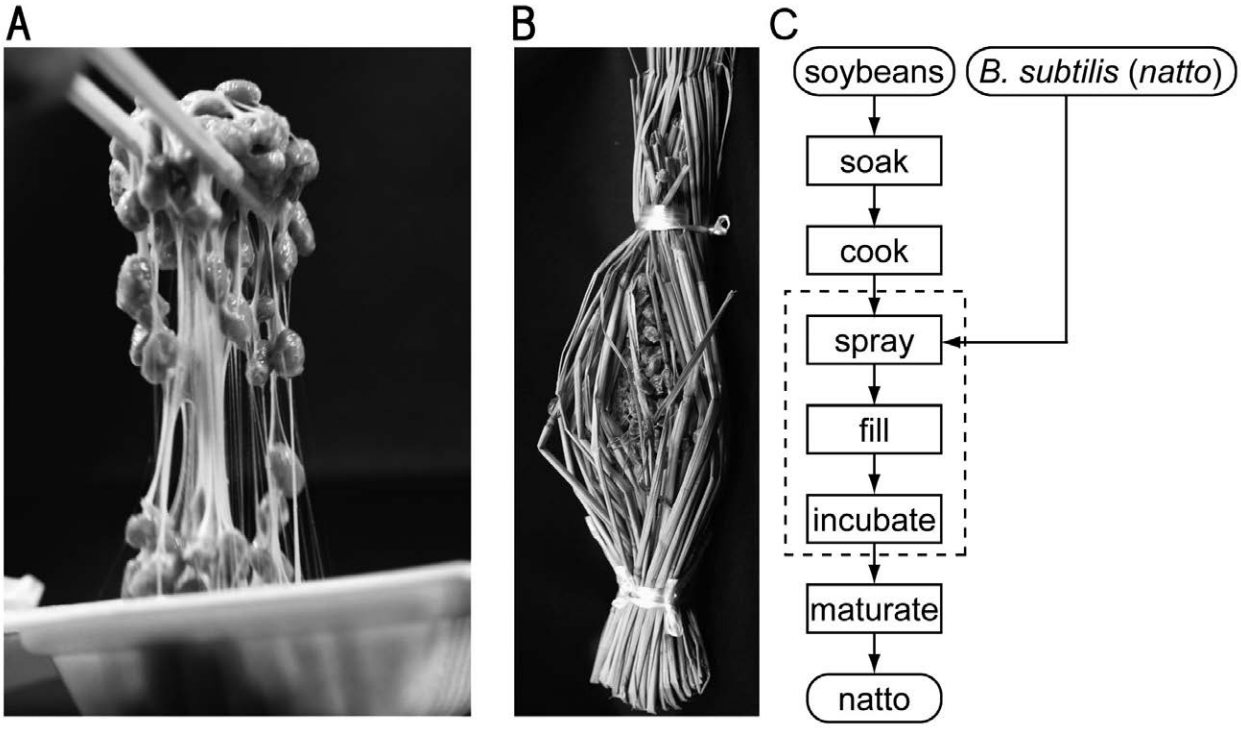

Fig. 1. Natto

A: Natto picked up with chopsticks. The strings between the soybeans mainly comprise PGA, highly viscous polymer. B: A classic natto for a souvenir. After sterilizing a bag of rice straws for hygienic reasons, soybeans are inoculated with *B. subtilis* (*natto*). C: A schematic presentation of the natto fermentation process. Contamination by phages is frequently detected on the floors of rooms used for processing, as shown in the dotted box.

Recently, 2 novel *B. subtilis* (*natto*) phages, ΦNIT1 and PM1, were isolated[13,26]. ΦNIT1 belongs to Group II, because the gene of PGA depolymerase PghP was hybridized to the genomes of phages belonging to Group II. PM1, conversely, belongs to Group I because of PCR detection from DNAs of PN-3 and PN-6 group phages using a primer set for PM1 and its morphological similarity to JNDMP. Fujii *et al.* also isolated a phage PM, but the phage seemed to be a mixture of Group I and II phages based on electron microscopic photographs in their report[5]. The *B. subtilis* (*natto*) phages reported to date and their classification are summarized in Table 2.

There are many fermented soybean foods worldwide, for example, kinema in Nepal and toa-nao in Thailand, from which *B. subtilis* (*natto*)-type strains have been isolated. It is plausible to consider the likelihood of *B. subtilis* (*natto*) phages being present in such fermented foods. Surveying and investigating *B. subtilis* (*natto*) phages from the fermented soybean foods might shed light on the origin of the fermented soybean foods and their spread. In 2011, a *Bacillus* phage was isolated from chungkookjang, a Korean fermented soybean food, the host of which remained indeterminate[11]. Comparative studies on the *Bacillus* phage and *B. subtilis* (*natto*) phages will offer interesting insights into the relationship between the phages.

Occurrence of Contamination by *B. subtilis* (*natto*) phages

Following the discovery of a *B. subtilis* (*natto*) phage from abnormal natto without PGA[3], the first survey of the contamination of natto factories by *B. subtilis* (*natto*) phages in Japan was carried out in 1970 and showed that 28 of 60 factories were contaminated by *B. subtilis* (*natto*) phages[28]. The phages were detected more often in old

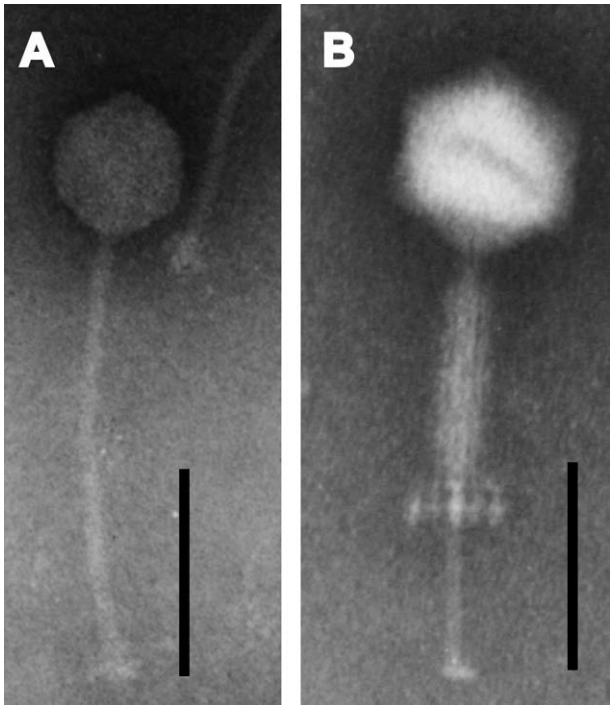

Fig. 3. *B. subtilis* (*natto*) phages
A: JNDMP (Group I), B: ONPA (Group II). Bar represents 100 nm. (from Nagai and Yamasaki, 2009, © Japanese Society for Food Science and Technology)

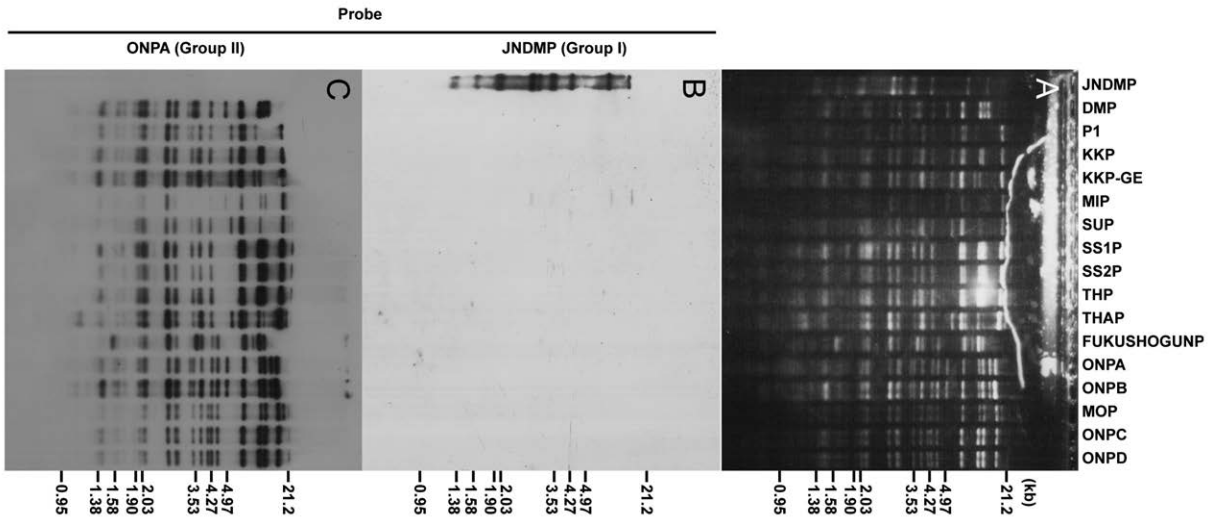

Fig. 2. DNA-DNA hybridization of phage genome DNAs
A: Agarose gel electrophoresis of phage DNA fragments digested with *Hin*dIII. B and C: DNA-DNA hybridization using genomic DNA of JNDMP (B) or ONPA (C) as probes. (from Nagai and Yamasaki, 2009, © Japanese Society for Food Science and Technology)

factories because of their clay walls, thorough cleaning of which was difficult. In such factories, soybean debris remains on the rough clay walls, where it encourages the propagation of *B. subtilis* (*natto*) and thus *B. subtilis* (*natto*) phages. In more modern factories, the clay walls have been replaced in favor of stainless steel, and phage contamination of natto products is rare. Fujii *et al.* also reported 25% of factories (2/8) in Kyushu Island had been contaminated by *B. subtilis* (*natto*) phages[4].

Tabei obtained natto products from 54 manufacturers located in 20 prefectures but failed to detect *B. subtilis* (*natto*) phages in any, soon after obtaining them[23]. However, following preservation of these products for 1 or 3 months at 4°C, phages were detected in 4 products (7.4%). He also isolated phages from 4 natto products from manufacturers to investigate the cause of PGA loss.

Although the occurrence of natto without PGA is recently rare[27], there are *B. subtilis* (*natto*) phages outside factories and on the floors of inoculation and incubator rooms, even in modernized factories (Fig. 1C)[20]. Although Fujii *et al.* showed that some disinfectants, namely benzalkonium chloride, chloramine-T and sodium hypochlorite etc., could effectively suppress *B. subtilis* (*natto*) phages, the key action remained the thorough cleaning of factories to remove soybean debris and hygiene education for works in natto factories[25]. A system of detecting the *B. subtilis* (*natto*) phage, which was recently developed based on a PCR technique, will also function as a powerful tool to control phages in factories[26].

PGA digesting enzymes of *B. subtilis* (*natto*) phages

Unlike proteins, PGA is a polymer in which D- and L- glutamic acids are bonded between a γ-carboxyl group and the α-amino group of an adjoining glutamic acid. PGA is synthesized by a membranous enzyme complex via hyperphosphorylated intermediates[1]. Genes related to PGA production, *pgsBCA*, were cloned and characterized

Table 1. Characteristics of JNDMP and ONPA

	JNDMP (Group I)	ONPA (Group II)
Head (diameter, nm)	60	89
Tail (width × length, nm)	7 × 200	9 × 200
Sheath (width, nm)	No	23
Genomic DNA (kb)	42	91
Latent time (min)	35	50
Burst size (progeny particles/particle)	46	72
Heat stability (°C)[a)]	53	63
Mg requirement	+	-
Host range[b)]		
B. subtilis (*natto*) Miura	-	+
B. subtilis (*natto*) Naruse	+	+
B. subtilis (*natto*) Takahashi	+	+
B. subtilis Marburg	-	-
B. megaterium, *B. cereus*, *B. brevis*	-	-

[a)] Temperature where survival rate reaches 1%
[b)] Mirua, Naruse and Takahashi are isolates from 3 commercial seed cultures for natto fermentation

Table 2. Re-classification of *B. subtilis* (*natto*) phages reported to date

Authors & reference numbers		Nagai & Yamasaki (19)	Tabei (22)	Fujii et al. (4)	Yoshimoto et al. (28)	Kimura & Itho (13)	Umene et al. (26)	Fujii et al. (5)
Groups or phages	Group I	JNDMP	BNP_4	PN-3, PN-6	NP-4		PM1	PM (a mixture of Group I & II phage?)
	Group II	ONPA	BNP_1, BNP_2, BNP_3	PN-19	NP-38	ΦNIT1		

as homologous with genes for the capsular PGA production of *B. anthracis*[1,2]. The PGA production is controlled by a regulatory gene *comP* coding a sensor protein kinase of the ComP-ComA two-component signal transduction system in *B. subtilis*[18].

Hongo *et al.* found that a *B. subtilis* (*natto*) strain lysogenized with a *B. subtilis* (*natto*) phage could not accumulate PGA in the culture[8]. From the culture of *B. subtilis* (*natto*) infected with a *B. subtilis* (*natto*) phage, they partially purified extracellular PGA depolymerase[9]. The PGA depolymerase digested PGA into di- and tri-γ-glutamates via an endo-type reaction, and thus reduced the PGA viscosity very rapidly[10].

Conversely, Kimura *et al.* purified a PGA depolymerase, PghP, from the culture of *B. subtilis* (*natto*) infected with a phage ΦNIT1 isolated from an abnormal natto[13]. PghP is a monomeric enzyme with a molecular weight of 22.9 kDa, which digested PGA into tri-, tetra- and penta-γ-glutamates, indicating that PghP has a different mode of reaction from the PGA depolymerase reported by Hongo *et al.* The gene of PghP was cloned and found to be a novel protein using BLAST searching in a DNA database. The *pghP* gene was detected on the genomes of other *B. subtilis* (*natto*) phages, including *B. subtilis* phages. PghP functions to break down the barrier of PGA on host cells and render them easily accessible to phages. Conversely, Hara *et al.* reported that susceptibility to *B. subtilis* (*natto*) phage required PGA productivity of the host cells[7]. This apparent discrepancy remains to be elucidated. PghP was crystallized and analyzed preliminarily[29].

PghP was analyzed in detail, but Hongo's depolymarase has not been purified enough to be clarified in detail. The 2 types of PGA depolymarase must be analyzed and compared to elucidate *B. subtilis* (*natto*) phage's strategies for breaking the PGA barrier. PghP is also of interest in the food industry. Generally, natto without PGA is of no value in food markets. However, such natto has an advantage when used as an ingredient for Chinese fried rice or snacks because of its ease of handling in food factories over natto with very sticky PGA[12]. PghP might be used as an enzyme for food processing in the near future.

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