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Bacteriophages of *Bacillus subtilis* (*natto*) and Their Contamination in Natto Factories

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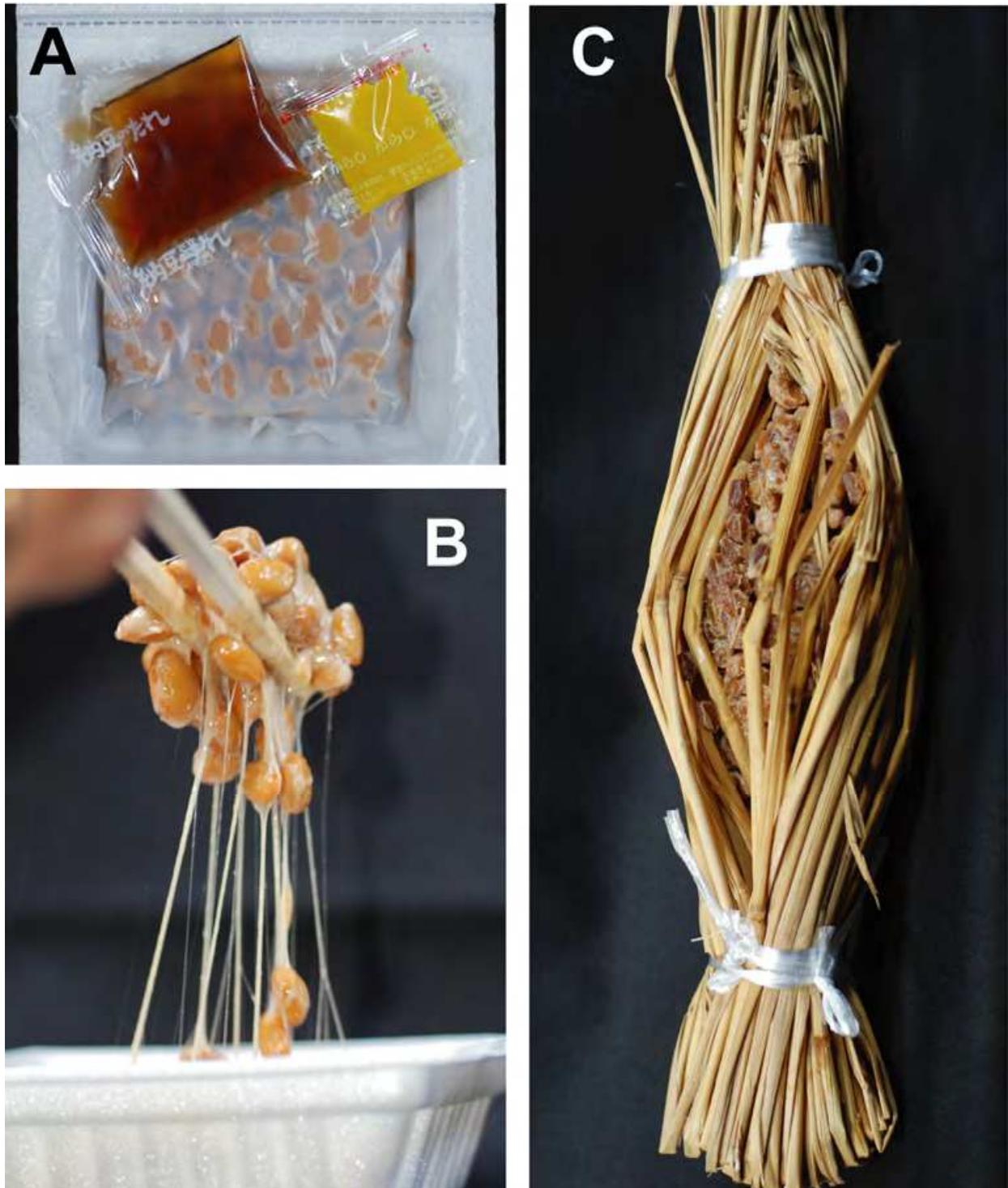
1. Introduction

Natto is a fermented soybean food, which is produced and consumed mainly in Japan (Nagai & Tamang, 2010). The Japanese usually eat natto with cooked rice, after mixing it with seasonings attached in a package or with soy sauce (Fig. 1). Natto has a characteristic odour of short-chain fatty acids and ammonia (Ikeda *et al.*, 1984; Kanno & Takamatsu, 1987), and a highly viscous polymer, poly- γ -glutamate (PGA, see Section 3).

Bacillus natto, named after “natto” when isolated from it for the first time, is the sole microorganism used for natto fermentation (Sawamura, 1906). However, the species was regarded as a “probable synonym” of *B. subtilis* in *Bergey's Manual of Determinative Bacteriology*, 8th Edition (Gibson and Gordon, 1974). This classification was supported by the fact that the chromosomal DNA of *B. natto* has a high level of homology with that of *B. subtilis* (Seki *et al.*, 1975). Phylogenetic analysis of *B. natto* (meaning *Bacillus* isolates from natto) and typical *B. subtilis* strains by sequencing of the 16S rRNA gene also showed that *B. subtilis* (*natto*) and *B. subtilis* are the same species (Tamang *et al.*, 2002). Although the scientific name “*B. natto*” was abandoned, the informal name “*B. subtilis* (*natto*)” is often used in the food industry, and even in the scientific field, to emphasize that *B. subtilis* (*natto*) isolates have the ability to produce natto unlike the type strain of *B. subtilis*.

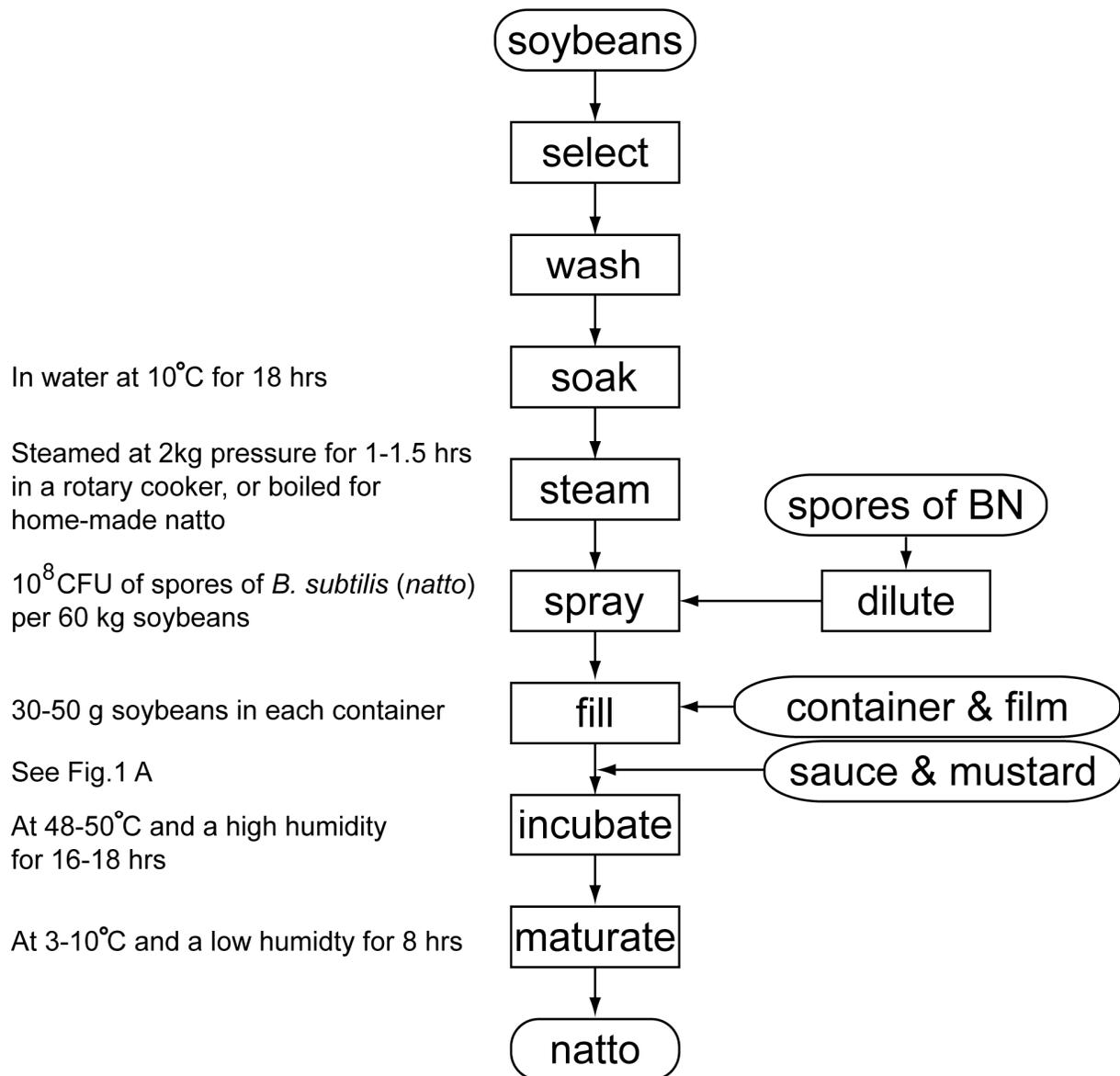
Commercial *B. subtilis* (*natto*) starters for natto fermentation are sold by three companies (Miura, Naruse and Takahashi) in Japan. The key strains for natto fermentation were isolated from the starters and characterized (Kiuchi *et al.*, 1987; Sulistyo *et al.*, 1988), and the characteristics, including PGA production and flavor, were found to be very similar among the strains.

Until the early 20th century, natto had been produced by packing boiled soybeans in a bag made of rice straw, which *B. subtilis* (*natto*) inhabit as soil bacteria (Fig. 1 C). Since the discovery of *B. subtilis* (*natto*) by Sawamura (1906) and the development of sanitary containers (Fig. 1 A & B) as substitutes for bags made of rice straw, the process of natto fermentation has been modernized and automated.



A) brown package, sauce; yellow package, mustard. B) Natto is very stringy because of PGA production (see Section 3). C) This natto is for a souvenir, so the straw is sterilized for hygiene unlike the real classic type of natto. After sterilization, a spore suspension of *B. subtilis* (*natto*) is inoculated as shown in Fig. 2.

Fig. 1. Modern natto (A and B) and a classic type of natto (C)



BN, *Bacillus subtilis* (*natto*). The production conditions shown on the left of the flow chart are cited from reviews on natto fermentation (Ueda, 1989; Kiuchi & Watanabe, 2004).

Fig. 2. Process of natto fermentation

The process of natto fermentation is so simple that very small factories, even homes, can produce natto in 2 or 3 days (Ueda, 1989; Kiuchi & Watanabe, 2004) (Fig. 2). After being selected and washed, soybeans are soaked in water at 10°C for 18 hr. Soybeans are cooked (steamed or boiled) and a suspension of spores of *B. subtilis* (*natto*) is sprayed on the boiled soybeans while they are hot to prevent the soybeans from being contaminated with other kinds of bacteria or phages. The soybeans are packed in containers made of polystyrene paper together with packed seasonings (typically, sauce and mustard; Fig. 1 A) and incubated at 50°C for 16–18 hr and then kept at 3–10°C for 8 hr to mature the natto further. Natto products are delivered to markets or stores through a cold chain system, keeping the products at a low temperature until consumers buy them. This simplicity of the process of natto fermentation, including a limited number of starters of natto, can result in devastating damage to a factory that becomes contaminated with bacteriophages.

2. Classification of *Bacillus subtilis* (natto) phages

The viscous polymer PGA on natto is an important characteristic. However, natto products without the polymer were often found in the market, despite normal synthesis of PGA just after fermentation in a factory. In some cases, the viscosity of natto decreased rapidly while mixing with seasonings. Fujii *et al.* (1967) found a bacteriophage from such an abnormal natto and named it PN-1. Fermentation of soybeans with *B. subtilis* (natto) and PN-1 resulted in the production of natto with no viscous polymer, indicating that PN-1 is attributed to a loss of polymer on natto. This was the first report on a *B. subtilis* (natto) phage.

Yoshimoto *et al.* (1970) surveyed contamination by *B. subtilis* (natto) phages in natto factories all over Japan. Among 60 factories, 28 were contaminated with phages at densities ranging from 5 PFU/cm³ sample to 2000. Forty-two phages were isolated from the samples and classified into 4 groups based on host ranges, and finally into two serological groups, NP-4 and NP-38 groups, using four anti-phage serums.

Group	MAFF no.	Strain	Source & month and year of isolation
I	270104	JNCHUP	natto, Oct. 1980
	270105	JNDMP	natto, Feb. 1981
	270106	JNHMP	natto, Feb. 1981
II	270101	P-1	abnormal natto, Jan. 1980
	270102	DMP	natto, May 1980
	270103	MIP	natto, May 1980
	270107	MOP	abnormal natto, Jul. 1981
	270108	THP	abnormal natto, Jul. 1981
	270109	THAP	abnormal natto, Dec. 1981
	270110	SUP	abnormal natto, Dec. 1981
	270111	KKP	abnormal natto, Mar. 1982
	270112	KKP-GE	industrial sewage, Mar. 1982
	270113	SS1P	abnormal natto, Dec. 1983
	270114	SS2P	abnormal natto, Dec. 1983
	270115	ONPA	abnormal natto, Aug. 1984
	270116	ONPB	abnormal natto, Aug. 1984
	270117	ONPC	abnormal natto, Sep. 1985
	270118	FUKUSHOGUNP	abnormal natto, Oct. 1985
	270119	ONPD	abnormal natto, Aug. 1986
270120	SUP-SS1P	not recorded	

Note: These phage isolates are distributed by the NIAS Genebank (acronym, MAFF; web site, http://www.gene.affrc.go.jp/index_en.php).

Table 1. *Bacillus subtilis* (natto) phages used in classification

Twenty *B. subtilis* (*natto*) phages were isolated from abnormally fermented natto, effluent from natto factories and soil of paddy fields in Kyushu Island, southern Japan (Fujii *et al.*, 1975). The phages, including PN-1, were classified into three groups based on host ranges, immunological reaction, and morphologies. All tested *B. subtilis* (*natto*) strains were infected by 21 *B. subtilis* (*natto*) phages, and some strains of *B. subtilis* also were infected by 17 phages. Three representative phages, PN-3, PN-6 and PN-19, from three serological groups were studied using an electron microscope. They had a head (diameter, 80–90 nm) and a contractile tail (length, 165–175 nm). Although PN-3 and PN-6 did not resemble each other in shape, judging from their morphologies on somewhat obscure photographs, they were found to belong to the same group (Nagai & Yamasaki, 2009).

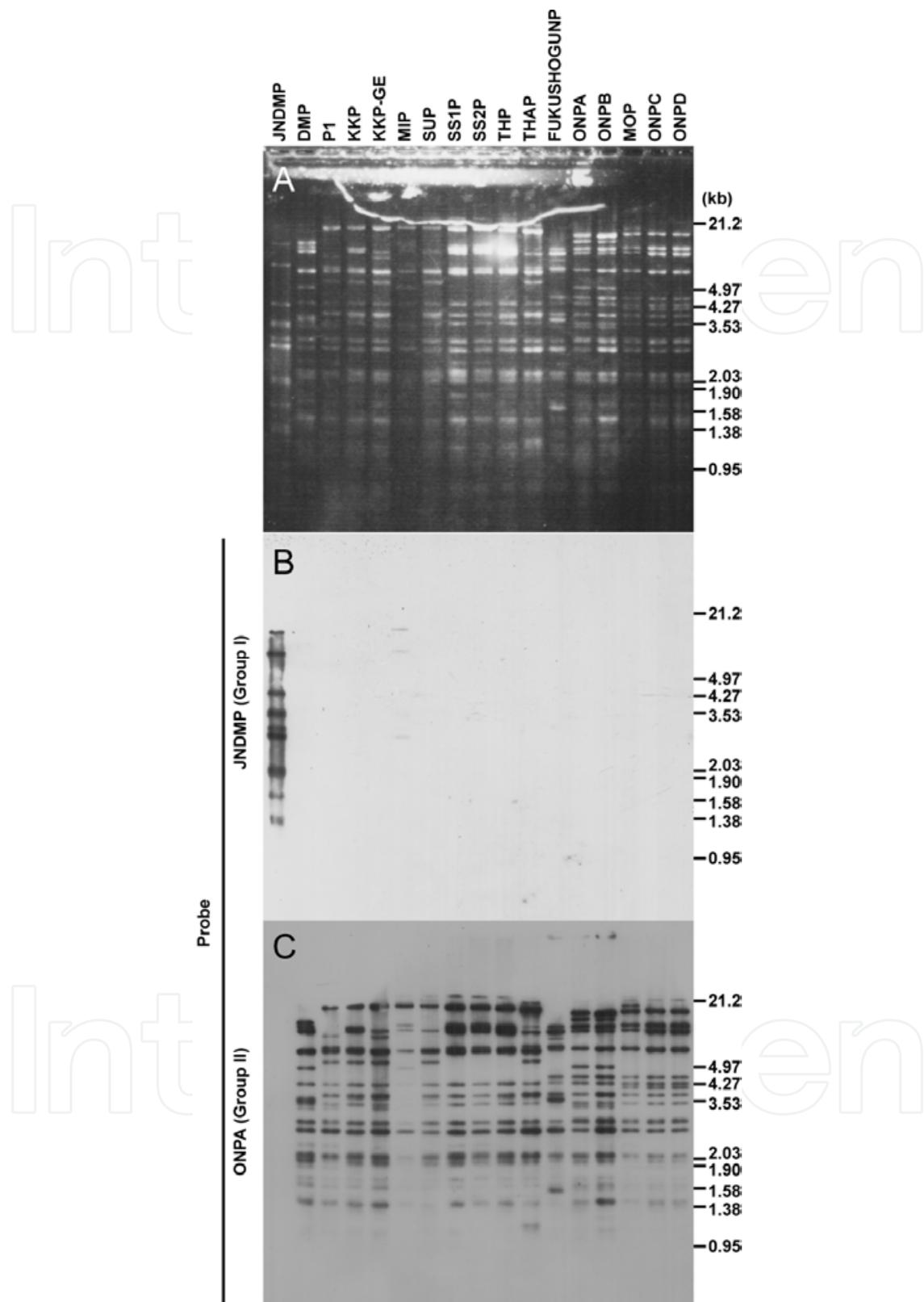
Nagai and Yamasaki (2009) classified 20 phages [deposited in the NIAS Genebank (Tsukuba, Japan) with accession numbers from MAFF 270101 to MAFF 270120 (Table 1)], mainly isolated from abnormally fermented natto, into two groups based on DNA-DNA hybridization (Fig. 3). No cross hybridized band is visible in the photograph, indicating that group I phages and group II phages are genetically independent of each other. Representative phages from the two groups were further characterized. Phage JNDMP (Group I) has a head (diameter, 60 nm) and a flexible tail (7 x 200 nm) (Fig. 4 A) and requires magnesium ions for amplification. Phage ONPA (Group II) has a head (diameter, 89 nm) and a contractile tail (9 x 200 nm) with a sheath (width, 23 nm) and does not require additional magnesium ions (Fig. 4 B). Plaques of ONPA were clearer than those of JNDMP. Other characteristics of JNDMP and ONPA are summarized in Table 2.

Group type	I	II
	JNDMP(MAFF 270105)	ONPA (MAFF 270115)
Head (nm)	60	89
Tail (nm)	7 x 200	9 x 200
Genome DNA (kb)	42	91
Latent time (min)	35	50
Burst size	46	72
Heat stability (°C) ¹⁾	53	63
Mg ion requirement	+	-
Host range ²⁾		
Miura (MAFF 118100)	-	+
Naruse (MAFF 118103)	+	+
Takahashi (MAFF 118105)	+	+
Marburg	-	-

1) Temperature at which about 1% of phage particles in suspension can survive after 10-min heating in a water bath.

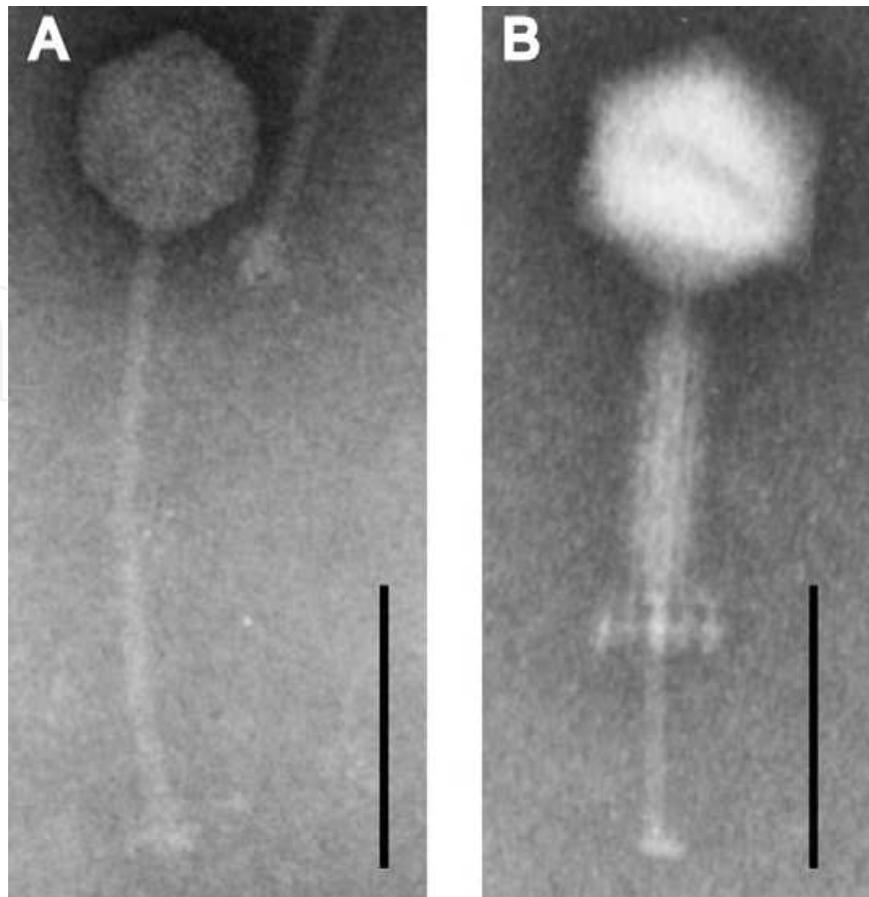
2) Miura, Naruse and Takahashi are commercial starters for natto fermentation.

Table 2. Other characteristics of JNDMP and ONPA



A) An agarose gel electrophoresis of fragments of phage DNA after digestion with a restriction enzyme, *Hind*III. B and C) Southern hybridization of the gel using genomic DNA of JNDMP (B) or ONPA (C) as a probe. (from Nagai & Yamasaki, 2009, with permission)

Fig. 3. Analysis of Southern hybridization of phage genome DNA



A) JNDMP, B) ONPA. Bar = 100 nm. (from Nagai and Yamasaki, 2009, with permission)

Fig. 4. Electron microscope photographs of *B. subtilis* (*natto*) phages

The phages of Yoshimoto's group (1970) had been discarded, and so genetic relationships among their two types of phages and ONPA or JNDMP could not be investigated. However, NP-4 and NP-38 had the same morphologies as JNDMP (Group I) and ONPA (Group II), respectively. Using DNA-DNA hybridization, type strains of Fujii *et al.* (1975), PN-3, PN-6 and PN-19, were found to belong to Group I, I and II, respectively (Nagai & Yamasaki, 2009).

3. Polyglutamate degrading enzyme

3.1 Polyglutamate

B. subtilis (*natto*) produces a very viscous polymer of DL-glutamic acid, which has two carboxyl groups on α - and γ -carbons (Fig. 5A), with extremely high degrees of polymerization. Unlike proteins, in which amino acid residues bind via α -carboxyl groups and amino groups (Fig. 5B), the glutamic acids in this polymer, poly- γ -glutamate (PGA, Fig. 5C), are synthesized by binding a γ -carboxyl group and an amino group of an adjacent glutamic acid via a hyperphosphorylated intermediate (Fig. 5D) (Ashiuchi *et al.*, 2001). Genes related to the production of PGA were cloned and expressed well in *Escherichia coli* cells (Ashiuchi *et al.*, 1999). The gene *pgsBCA* is homologous with genes for capsular PGA production of *B. anthracis* and codes for a membranous enzyme complex (Ashiuchi *et al.*,

2001). On the other hand, a regulatory gene for PGA production was cloned and found to be *comP*, which codes for a sensor protein kinase of the ComP-ComA two-component signal transduction system (Nagai *et al.*, 2000). Recently, PGA cross-linked by γ -radiation was found to hold a large quantity of water and to be useful for greening of desert areas by scattering the PGA resin in which seeds (e.g. soybeans) are embedded (Hara, 2006). Thus, PGA is becoming an important industrial material.

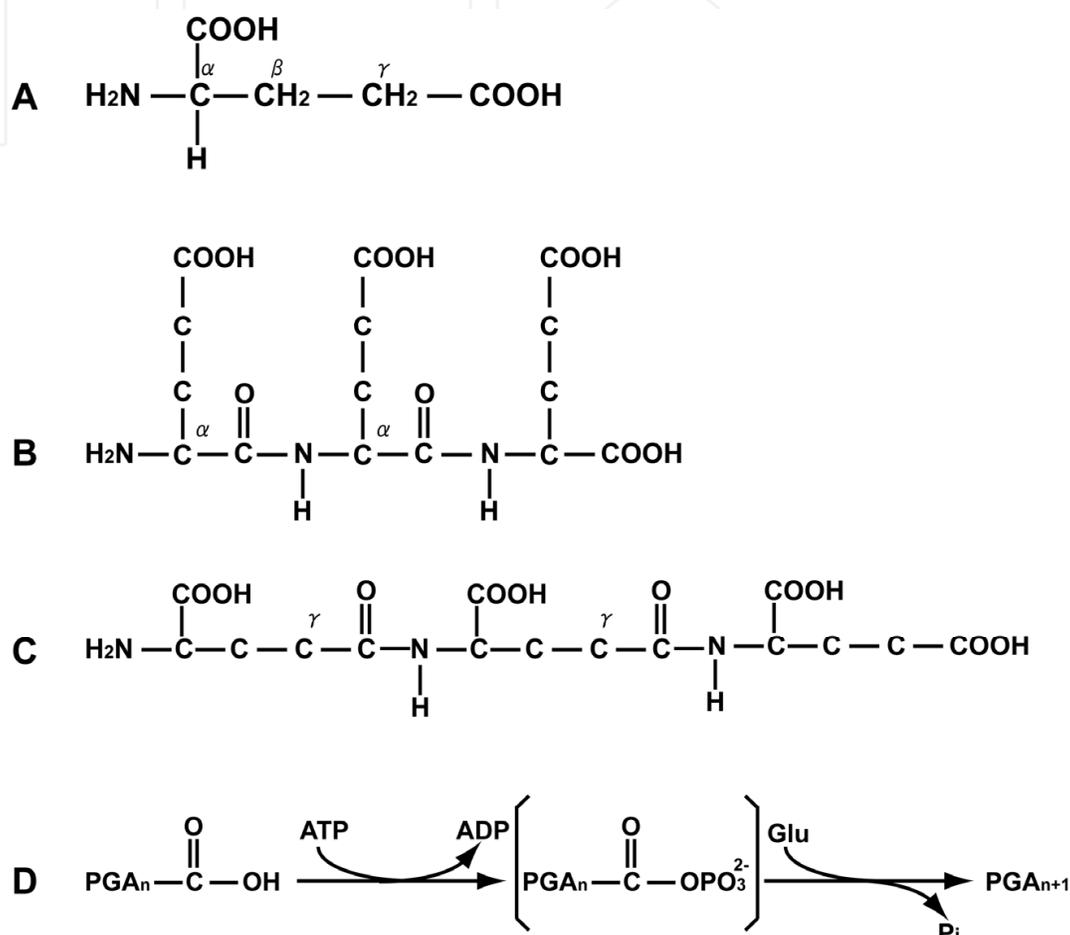


Fig. 5. Glutamic acid (A), tri- α -glutamic acid (B), tri- γ -glutamic acid (C), and the mechanism of synthesis of poly- γ -glutamic acid (D)

3.2 Polyglutamate depolymerase

It was found that a *B. subtilis* (*natto*) lysogenic strain could not accumulate PGA in the culture, whereas a nonlysogenic strain accumulated it under the same conditions (Hongo & Yoshimoto, 1968). In the culture of the lysogenic strain, bacteriophages were induced at a high density of 10^9 PFU/ml from the early stage of the experiment. Around the peak of production of phages, PGA depolymerase appeared to be released from the lysogenic strains to the culture. The enzyme was also produced extracellularly, when *B. subtilis* (*natto*) was infected by phages. After infection with phage NP-1 cl (Yoshimoto & Hongo, 1970), depolymerase was synthesized in parallel with the production of phage particles in host cells (Hongo & Yoshimoto, 1970a). The depolymerase digested PGA by endopeptidase-type action, resulting in the rapid loss of viscosity of PGA. Chromatographic studies showed that

the products of the reaction were di- γ -glutamate and tri- γ -glutamate (Hongo & Yoshimoto, 1970b). Optimal pH and temperature of the depolymerase were pH 6–8 and 40–50°C, respectively. The depolymerase was stable at pH values ranging from 4 to 9 and below 70°C. The depolymerase was not linked to phage particles.

3.3 Poly- γ -glutamate hydrolase, PghP

Culture supernatant of *B. subtilis* (*natto*) infected with a phage Φ NIT1, which had been isolated from natto containing a small amount of PGA, caused the viscosity of PGA to decrease rapidly, and degraded PGA with a molecular weight of 10^6 Daltons to oligo- γ -glutamyl peptides (Kimura & Itoh, 2003). From the culture supernatant, a 25-kDa monomeric enzyme was purified through five column chromatographic steps and was named PghP for “ γ -PGA hydrolase of phage”. Analysis of the products of enzymatic reaction on PGA showed that they were tri- γ -glutamate, tetra- γ -glutamate and penta- γ -glutamate. PghP was inhibited with monoiodoacetate and EDTA and the activity inhibited by EDTA was restored by adding Zn^{2+} or Mn^{2+} , indicating that a cysteine residue(s) of PghP and these ions participated in the hydrolase reaction.

The gene for PghP was cloned based on a nucleotide sequence predicted from the N-terminal amino acid sequence of purified PghP, and sequenced (Kimura & Itoh, 2003). The predicted PghP was a 22.9-kDa protein with 203 amino acid residues, in which the first methionine was eliminated posttranslationally. PghP was a unique protein: similar proteins were not detected in the database by a BLAST search program. PghP is distributed in a variety of *B. subtilis* (*natto*) phages including some phages isolated from *B. subtilis* strains which produce no PGA. PghP of Φ NIT1 has a different substrate specificity from the PGA depolymerase in Section 3.2 (Hongo & Yoshimoto, 1970b).

Φ NIT1 could amplify in both encapsulated and non-encapsulated *B. subtilis* (*natto*). On the other hand, *B. subtilis* phage BS5 (Ackermann *et al.*, 1995), which produced no PghP, could amplify only in non-encapsulated *B. subtilis* (*natto*). BS5, however, could amplify in encapsulated *B. subtilis* (*natto*) in the presence of additive PghP. These results indicate that *B. subtilis* (*natto*) produces PGA for physical protection from attacks by phages (Kimura & Itoh, 2003).

Apparently opposite results were also reported: that PGA production made the cells susceptible to *B. subtilis* (*natto*) phages (Hara *et al.*, 1984). In their study, *B. subtilis* (*natto*) phages could infect *B. subtilis* (*natto*) producing PGA. After curing of plasmid pUH1, which harboured genes controlling PGA production, of *B. subtilis* (*natto*) strains, the cured strains could no longer be infected by the phages. *B. subtilis* Marburg, which was not infected by the *B. subtilis* (*natto*) phages and did not produce PGA by nature, and the cured *B. subtilis* (*natto*) strains became susceptible to the phages after transformation with DNA from *B. subtilis* (*natto*). Hara *et al.* thought that “PGA might be associated with phage absorption” from the results. However, a cured *B. subtilis* (*natto*) strain was susceptible to phage Φ BN100, indicating basically that the plasmid did not control phage absorption (Nagai & Itoh, 1997). The experiments conducted by Hara *et al.* examined three factors: the existence of pUH1-type plasmids, PGA productivity and γ -glutamyl transpeptidase (γ -GTP) (at the time, γ -GTP and pUH1 were thought to be a PGA synthesizing enzyme and a plasmid coding γ -GTP

genes, respectively [Aumayr *et al.*, 1981; Hara *et al.*, 1981], but pUH1 was found to harbour no genes for PGA production [Nagai *et al.*, 1997]), so the situation might make the results difficult to interpret accurately. This discrepancy remains to be elucidated.

Natto without PGA has no commercial value as a food on the market, but might have a value as an ingredient of natto fried rice or natto snack foods because of its ease of manufacturing in food factories (Kimura, 2008). Thus, PghP could be useful in the food industry.

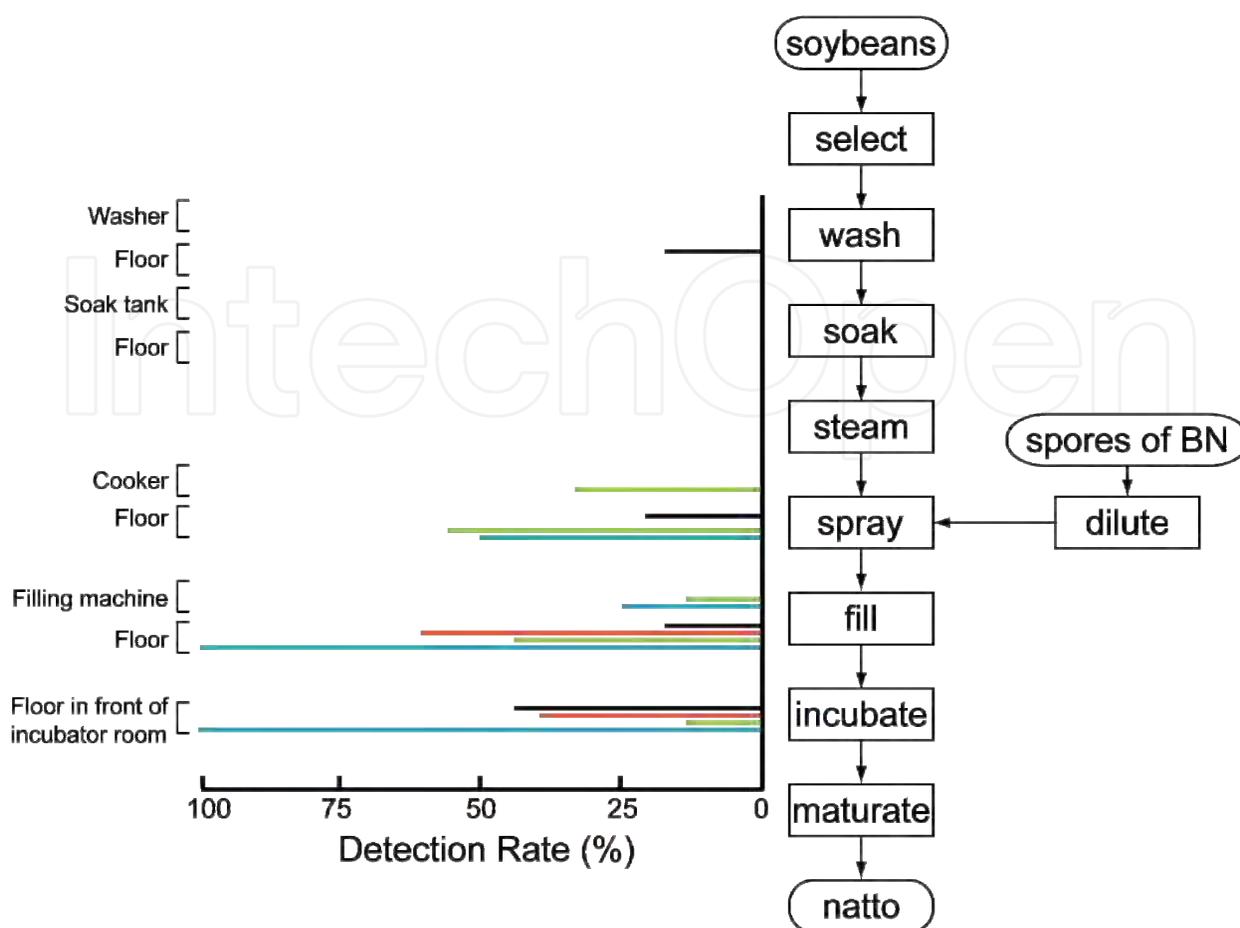
4. Phage contamination in natto factories

The first report on contamination of natto by phages was reported by Fujii *et al.* (1967). At that time, the authors did not search the factory for phages where the contaminated natto had been made. In 1975, the authors investigated contamination by phages in the same factory. Phages were not detected in the factory, but in effluent from it because of modernization of the factory (Fujii *et al.*, 1975).

Yoshimoto *et al.* (1970) searched natto factories throughout Japan for contamination by phages. Phages were detected in 28 factories (47%) among 60 factories at a density ranging from 5 to over 1000 PFU/cm³ of sample. Phages were very often detected in old factories, the walls of which were made of clay (common in old Japanese buildings). The surfaces of clay walls have too many asperities to clean off soybean debris perfectly, resulting in amplification of phages in their host cells on soybean debris. The walls of modern factories are made of clean stainless steel, so phages were rarely detected. In factories contaminated by phages, the phages were detected most frequently in the fermentation rooms. Fujii *et al.* also reported that 25% of factories (2/8 factories in Kyushu Island, west Japan) were polluted with phages (Fujii *et al.*, 1975).

Nakajima (1995) investigated contamination by *B. subtilis* (*natto*) phages in four natto factories in Ibaraki prefecture, central Japan (Fig. 6). Before inoculation of natto starter to soybeans, phages were detected only on the floor of the washing room in a factory and its detection rate was very low. The phages might have been brought in with raw soybeans or dust in the air. After inoculation, the detection rate rose to 100%. These results indicate that perfect cleaning to ensure that no soybean debris remains in machines or on floors is essential, especially after soybeans have been sprayed with a spore suspension of *B. subtilis* (*natto*). The author did not mention contamination by phages of natto products made by the four factories. Another report showed that phage contamination was not detected in any natto made in factories in Iwate prefecture, northern Japan (Yamamoto, 1986). In total, contamination of natto products by phages has decreased drastically, but *B. subtilis* (*natto*) phages still exist in natto factories, fields, and waste water.

Improvement of a factory highly polluted with phages and bacteria was reported (Takiguchi *et al.*, 1999). When phages and bacteria were detected in natto made by the factory, a manual was compiled to ensure strict separation of the entrance and exit, hand-washing, removal of abnormal natto from the factory as soon as possible, dilution of starter with sterilized water, installation of UV lighting, replacement of wooden parts with those made of stainless steel, including periodical cleaning and hygiene education. After these efforts, no contamination was detected in the natto.



Four independent factories (indicated by different color bars) were surveyed for *B. subtilis* (*natto*) phages in several facilities in the process of natto fermentation (the right flow chart, also see Fig. 2) and the floors near the facilities.

BN, *Bacillus subtilis* (*natto*)

Detection rate (%) = no. of detections / no. of samples \times 100

Fig. 6. Contamination by *B. subtilis* (*natto*) phages in natto factories (adapted from Nakajima, 1995)

The following disinfectants were effective against *B. subtilis* (*natto*) phages: benzalkonium chloride, chloramine-T, sodium hypochlorite, TEGO-51 and Vantocil IB (Fujii *et al.*, 1983). The most important measure is cleaning the machines and floors of natto factories to remove soybean debris on which *B. subtilis* (*natto*) and phages can propagate.

5. Other topics on *B. subtilis* (*natto*) phages

5.1 Generalized transducing phage for *B. subtilis* (*natto*)

For genetic transfer of DNA between *B. subtilis* (*natto*) strains by transduction, a phage Φ BN100 was screened in laboratory stock strains (Nagai & Itoh, 1997). The phage could transduce prototroph genes (for adenine, uracil or leucine requirement) to auxotrophs at rates ranging from 3.8×10^{-8} to 1.6×10^{-6} (number of transductants per phage particle). The phage was also used for analysis of transposon insertional mutagenesis on a gene responsible for the regulation of PGA production (Nagai & Itoh, 1997) and construction of

mutants on production of branched short-chain fatty acids for preparation of odorless natto (Takemura *et al.*, 2000). Φ BN100 is a synonym for JNDMP (see Section 2).

5.2 *B. subtilis* (natto) phage PM1 and a phage detection system by PCR

A phage was newly isolated from natto producing no PGA and characterized (Umene *et al.*, 2009). The morphology of the phage, PM1, was very similar to that of JNDMP, and the size of its genomic DNA was found to be 50 kb using field inversion gel electrophoresis, 10 kb smaller than that of JNDMP. The genome of PM1 was a linear double-stranded DNA, and might be circularly permuted and have no definite termini, like T4 phage.

Based on a sequence of a 1.1-kb *Eco*RI fragment of genomic DNA, which did not have significant homology with any sequences deposited at the DNA database so far, the following pair of primers for PCR to amplify a 0.53-kb region in the 1.1-kb *Eco*RI fragment was designed:

5'-CGCACTGGAAGCAATCAAGTCGG-3' (corresponding to nt 33–55)

5'-CAACCCTCTGACCGACTTTTCCC-3' (corresponding to nt 538–560)

Among ten *B. subtilis* (natto) phage isolates in the authors' laboratory, eight were target sequences of amplified with the primer set, suggesting that PM1 phages are distributed over a large area of Japan.

5.3 *Bacillus* phage isolated from chungkookjang

Chungkookjang is a Korean soybean food fermented by *Bacillus subtilis*. From the fermented soybeans, a virulent *Bacillus* phage was isolated and named Bp-K2 (Kim *et al.*, 2011). Bp-K2 resembled ONPA in morphology, but had a smaller head (width, 80 nm) and genomic DNA (21 kb). Bp-K2 had a contractile tail with a sheath (85–90 nm x 28 nm), a tail fiber (80–85 nm x 10 nm) and a basal plate (29 nm x 47 nm). Bp-K2 could develop plaques on not only *B. subtilis* strains isolated from chungkookjang but also *B. subtilis* (natto).

5.4 Defective phage of *B. subtilis* (natto)

As *B. subtilis* Marburg strain produces a defective phage PBSX (Seaman *et al.*, 1964; Anderson & Bott, 1985; Zahler, 1993 for a review), which cannot amplify in host cells, *B. subtilis* (natto) IAM 1207 produces defective phage PBND8 after induction with bleomycin (Tsutsumi *et al.*, 1990). Although PBND8 resembled PBSX in morphology, the size of DNA contained in heads of PBND8 was 8 kb, 5 kb smaller than that of PBSX (13 kb). SDS-polyacrylamide gel electrophoresis of component proteins of the phage particles showed that PBND8 was clearly distinct from PBSX and PBSY, a defective phage from *B. subtilis* W23.

Seaman *et al.* (1964) also showed the production of PBSX-like particles from *B. natto*. The particles neutralized antiserum against PBSX particles, indicating that the PBSX-like particles from *B. natto* were very closely related to PBSX. At least two kinds of defective phages might be produced by strains belonging to a *B. subtilis* (natto) group (i.e., PBND8 and PBSX-like defective phage).

6. Conclusion

B. subtilis (*natto*) phages that have been isolated in Japan are classified into two groups (Groups I and II), which are genetically independent of each other judging from DNA-DNA hybridization analysis. Phage JNDMP (Group I) has a head (diameter, 60 nm) and a flexible tail (7 x 200 nm) and requires magnesium ions for amplification. Phage ONPA (Group II) has a head (diameter, 89 nm) and a contractile tail (9 x 200 nm) with a sheath (width, 23 nm) and does not require additional magnesium ions. JNDMP was found to be a generalized transducing phage for *B. subtilis* (*natto*). Natto contaminated with phages is not covered with PGA, which is an important factor of the quality of natto. The loss of PGA is attributed to PGA hydrolase, PghP, or its relevant enzyme, which is expressed from a gene on phage genomic DNA in infected host cells. The enzymes digest PGA by endopeptidase-type action, resulting in a rapid loss of viscosity of PGA. Contamination of natto products by phages can be prevented by cleaning the facilities and floors of natto factories. Until 1980, contamination by phages had caused devastating damage to natto factories, but such trouble is now rare thanks to the modernization of natto factories and hygiene education for workers.

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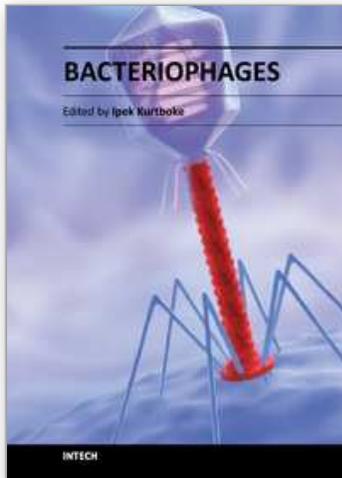
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Bacteriophages have received attention as biological control agents since their discovery and recently their value as tools has been further emphasized in many different fields of microbiology. Particularly, in drug design and development programs, phage and prophage genomics provide the field with new insights. Bacteriophages reveals information on the organisms ranging from their biology to their applications in agriculture and medicine. Contributors address a variety of topics capturing information on advancing technologies in the field. The book starts with the biology and classification of bacteriophages with subsequent chapters addressing phage infections in industrial processes and their use as therapeutic or biocontrol agents. Microbiologists, biotechnologists, agricultural, biomedical and sanitary engineers will find Bacteriophages invaluable as a solid resource and reference book.

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