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Characterization of *Bacillus subtilis* Strains Isolated from Fermented Soybean Foods in Southeast Asia: Comparison with *B. subtilis* (*natto*) Starter Strains

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Abstract

We compared the production of poly-γ-glutamate (γ-PGA), protease and amylase, the phage type, and inheritance of the insertion sequence (IS) IS4*Bsu*1among 90 *Bacillus subtilis* strains isolated from fermented soybean foods from Southeast to East Asia with those of a *B. subtilis* (*natto*) starter strain. All the isolates produced high levels of protease but various levels of amylase. None of them belonged to the standard phage type of *B. subtilis*, but 11 strains appeared to belong to the same phage type as *B. subtilis* (*natto*). Sixty-three isolates produced γ-PGA, while 28 isolates carried the IS element, the copy number of which ranged from 1 to 17. The IS appeared to be widely distributed among *B. subtilis* strains from non-salted types of fermented soybeans, such as "Tua Nao" of Thailand (90%) and "Kinema" of Nepal (56%), while a relatively small fraction (15%) of *B. subtilis* strains from salted types of fermented soybeans, e.g. Chinese "Douchi", harbored the IS. No relationship was apparent between the IS element and γ-PGA production. Nucleotide sequencing demonstrated that the IS elements of the randomly selected 9 isolates had a sequence identical with that of *B. subtilis* (*natto*) and those of another 4 strains showed only one to three mutations. These results together imply that IS4*Bsu*1 was recently distributed among *B. subtilis* strains that predominantly occur in cooked soybeans.

Discipline: Food
Additional key words: natto, phage-typing, protease, amylase, insertion sequence

Introduction

A variety of fermented soybean foods produced in Southeast and East Asia are used for seasoning; some are sun-dried for long-term preservation[16,21]. To produce these soybean foods, boiled or steamed soybeans are fermented immediately (non-salted type), or after soaking in salted water (salted type). Unlike Japanese fermented soybeans of the non-salted type (natto), which are fermented using a pure starter strain of *Bacillus subtilis*, naturally occurring microorganisms or seeds (a portion of the product) are used to produce fermented soybeans in other Asian countries[13,15,16]. Since salt prevents bacterial growth, fungi play a major role in the fermentation of salted soybeans[18,22]. On the other hand, *B. subtilis* strains are considered to contribute significantly to the fermentation of non-salted soybeans, and they are predominantly isolated from the products[13,15,16] .

B. subtilis starter strains of natto, traditionally called *B. subtilis* (*natto*), produce a polymer of glutamate with a γ-peptide linkage, poly-γ-glutamate (γ-PGA), as the major slimy material of natto[10]. Production of γ-PGA in these strains is regulated by the ComQXPA quorum sensing system[7,19,20], and is genetically unstable. This genetic instability is caused by the translocation of the insertion sequence (IS) IS4*Bsu*1 on the chromosome into the *comP* gene at a high frequency[11,12]. The *comP* gene encodes a bifunctional protein, having both sensor (for ComX pheromone) and transmitter functions that transfer the pheromone-signal to the ComA response regulator[19]. IS4*Bsu*1 was the first IS element to be identified in *B. subtilis*. We attempted to determine whether or not *B. subtilis* strains responsible for the fermentation of soybean foods in other countries shared similarity with, or harbored IS4*Bsu*1 like the *B. subtilis* (*natto*) starters . We accord-

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ingly isolated and characterized 90 *B. subtilis* strains from foods made of fermented soybeans in Southeast and East Asia.

We compared the biotin requirement, productivity of γ-PGA protease and amylase, phage type, and inheritance of IS4*Bsu*1 among the *B. subtilis* isolates with those of *B. subtilis* (*natto*). Our results indicated that some *B. subtilis* isolates displayed common properties with *B. subtilis* (*natto*), including phage type and inheritance of IS4*Bsu*1.

Materials and methods

Fermented soybeans

Most of the fermented soybeans (Table 1) were purchased at local markets in different countries or imported from Taipei, Kuwararunpool and Jakarta and obtained in Tokyo.

Isolation and sources of *B. subtilis* strains, typed phages and media

B. subtilis strains were isolated from soybean food samples as follows. Food samples (10 g) were homogenized in 90 mL of 0.9% NaCl, and a portion was incubated at 80ºC for 20 min. After dilution with 0.9% NaCl, aliquots were spread on GSP agar plate[10], and colonies that developed were randomly selected and purified. We confirmed that the isolated strains were *B. subtilis* using Bergey's manual[2]. *B. subtilis* strains from Kinema were isolated by Nikkuni[14]. *B. subtilis* (*natto*) Miyagino is a commercial starter strain. *B. subtilis* (*natto*) Sawamura (IAM1212) was isolated from commercial natto[17]. *B. subtilis* IFO13719 (type strain) and 168 (*trpC2*) were obtained from the Institution for Fermentation (Osaka) and from Dr. F. Kawamura, respectively. Ten typed phages (SP50, SP10, ϕ105, SPP1, CS1, ϕ3T, S-a, F, BS5 and ϕ29) were obtained from H.-W. Ackermann and used to phage-type *B. subtilis* strains as described[1].

Cells were cultured in antibiotic medium 2 (Difco Laboratories) and Trypto-soya broth (TSB; Difco Laboratory) for the preparation of chromosomal DNA and phage-typing. We assayed γ-PGA production on GSP agar and TSB agar. The biotin requirement was tested on Spizizen's minimal medium[19] containing 0.1% vitamin-free casamino acids (SMMC). The production of amylase and protease was assayed on SMMC agar containing biotin (10 μg/mL), nutrient broth (NB; Difco Laboratory) agar or on NB agar containing 1% glucose (NBG). Soluble starch or skim milk (each 0.5%) was embedded in agar medium as the substrate for amylase and protease, respectively. Amylase activity was detected using iodine-potassium iodide.

DNA manipulations

Chromosomal DNA[7] was purified from *B. subtilis* cells as described by Lovett et al.[8]. Restriction endonuclease digestion, agarose gel electrophoresis, and polymerase chain reaction (PCR) were performed as described[12]. DNA fragments were Southern-blotted using the 1.2-kb *Bam*HI-*Pst*I fragment of IS4*BSu*1 as a probe[12]. To determine the IS4*Bsu*1sequences, the *tnpA* region from nucleotide positions (nt) 124 to 1301 of the IS[12] was amplified from the chromosomal DNAs of *B. subtilis* strains using the oligonucleotide primers, 5'-AAGGACAATAAGCATGGATAAG-3' (nt 124 to 145) and 5'-ACTATAATCTTTGACGAGTGCA-3' (complementary to nt 1280 to 1301), and KOD DNA polymerase (Toyobo Biochemicals). Amplified 1.2-kb DNA fragments were then purified by agarose gel electrophoresis and used as sequencing templates along with the above primer pair, the following primers, 5'-GTTCATAATC-CAAGTAACCCG-3' (nt 746 to 725), 5'-AGGTGCT-GCTTCAGCCATTTAA-3' (complementary nt 1032 to 1053) and a Dye-Terminator Cycle Sequencing Kit (Perkin-Elmer). Nucleotides were sequenced using an ABI310 DNA sequencer (Perkin-Elmer).

Results and discussion

Isolation of *B. subtilis* strains from fermented soybean foods

Thirty-six fermented soybean foods were obtained from 21 cities in 7 Asian countries (Table 1). Of those, 12 consisted of non-salted types of food that included Thai "Tua Nao", Chinese "Douchi" and Nepalese "Kinema". *B. subtilis* was predominantly isolated (> 10^8 cells/g) from these soybean foods as described[4–6]. "Mac Tua Nao" is a raw and slimy non-salted type of Laotian fermented soybeans similar to Japanese natto, which contained 1.6×10^8 *B. subtilis* spores per 1 g. Similar numbers of *B. subtilis* spores were detected in "Chine Pepoke" and "Douchi", which correspond to the semi-dried non-salted type of fermented soybeans from Myanmar and China, respectively. Sun-dried non-salted types, such as "Tua Nao" of Thailand and "Shan Pepoke" and "Karpun Doucha" of Myanmar, contained from 2.6 to 6.9 $\times 10^7$ cells/g of *B. subtilis* spores. We also isolated *B. subtilis* strains from 14 salted fermented soybean foods (Table 1). Significantly fewer *B. subtilis* spores (<10^3/g of sample) were isolated that might have been contaminants during the fermentation process.

A total of 90 spore-forming strains isolated from the samples listed in Table 1 were confirmed to be *B. subtilis* according to the following taxonomical criteria: facultative aerobic, catalase-positive reaction, Gram-positive

rod, endospore formation, growth in 7% NaCl, nitrate reductase-positive reaction, production of acetylmethylcarbinol from glucose and acid formation from glucose, mannitol and maltose[2].

Characterization of the isolated *B. subtilis* strains

B. subtilis (*natto*) strains require biotin for growth[3]. About 20% of the isolated strains required biotin, but this phenotype appeared to be irrelevant to other phenotypes. All the strains produced protease, and amylase on SMMC agar plates, while 38% of the strains did not produce this enzyme on NB agar, and of these, 29% did not produce the enzyme on NBG agar due to catabolite repression. These differences in amylase production were independent of the origin of the strains. The production of γ-PGA by *B. subtilis* (*natto*) is an important characteristic. *B. subtilis* (*natto*) produces γ-PGA in the absence of L-glutamate, although L-glutamate apparently enhances the γ-PGA production by some strains[9]. We therefore tested the γ-PGA productivity of the isolates and the effect of L-glutamate on this process. Two-thirds of the isolates produced γ-PGA on GSP agar plates containing L-glutamate, but 70% of them required L-glutamate for γ-PGA production. These strains did not produce γ-PGA on TSB agar without L-glutamate. The production of γ-PGA and its regulation by L-glutamate were apparently not associated with other properties of the strains.

We used the system developed by Ackermann to phage-type the *B. subtilis* isolates. Fifty strains were resistant to all the 10 typed phages and the remainder were classified into 19 types according to the sensitivity to the typed phages, that was distinct from the phage type defined by Ackermann[1]. *B. subtilis* (*natto*) Miyagino, a commercial starter strain of natto, was sensitive only to the SP10 phage. We also identified 11 isolates with simi-

Table 1. Fermented soybean foods used as sources of *B. subtilis* strains

Fermented soybeans	Fermentation-type/appearance	City/country	Sample name
Chine Pepoke	Non-salted/semi-dried block	Yangon/ Myanmar	MyaA2
		Tungny/Myanmar	MyaB2
		Boban/Myanmar	MyaC2
		Lasho/Myanmar	MyaD
Chungkuk Jang	Non-salted/raw paste	Seoul/Korea	KorC1, C2
Douchi	Salted/semi-dried block	Hong Kong/China	ChiB
		Chung Shan/China	ChiC
		Chang Meng/China	ChiD
		Yang Chang/China	ChiE1, E2
		Chyon Tsuu/China	ChiF
		Dar Li/China	ChiH
		Man Shii/China	ChiG
		Lui Rii/China	ChiJ1, J2
Karpun Doucha	Non-salted/sun-dried beans	Taungy/Myanmar	MyaB3
		Boban/Myanmar	MyaC3
Kecap Manis	Salted/raw source	Jakarta/Indonesia	IndA
Kinema	Non-salted/sun-dried block	Hille/Nepal	NepD1-D9
Mac Tua Nao	Non-salted/raw beans	Ruanpanpan/Laos	LaoA1-A3
Mejyu	Non-salted/sun-dried block	Seoul/Korea	KorA1
Rar Jar Jang	Salted/liquid	Koi Rin/China	ChiG
Shan Pepoke	Non-salted/sun-dried chips	Yangon/Myanmar	MyaA1
		Taungy/Myanmar	MyaB1
		Boban/Myanmar	MyaC1
Shui Douchi	Salted/liquid	Taipei/Taiwan	TawA
Tao Jiao	Salted/liquid	Bangkok/Thailand	ThaA
Tua Cheo	Salted/liquid	Kuala Lumpur/Malaysia	MalA
Teng Jang	Salted/raw paste	Seoul/Korea	KorB1, B2
Tua Nao	Non-salted/sun-dried chips	Chiang Mai/Thailand	ThaB
		Chiang Rai/Thailand	ThaC1-C4

lar phage sensitivity to that of the *B. subtilis* (*natto*) strain. Other strains displayed similar phage sensitivities: 7 strains to SP50, 5 to both SP50 and SP10 and 2 to both SP10 and CS1. We concluded that the biotin requirement is random, that protease production is associated with all the isolates, although 35% of them did not produce amylase, and that particular phage types occur among *B. subtilis* strains in fermented soybeans.

Occurrence of IS4*Bsu*1 in the *B. subtilis* isolates

B. subtilis (*natto*) Miyagino and other *B. subtilis* (*natto*) strains harbor IS4*Bsu*1, an insertion sequence of the IS4 family[12]. We investigated the presence of this IS element in all the isolates by Southern-blotting. No IS was found among the strains isolated from southern to central China and from Indonesia. However, 28 other isolates (31%) carried this element (Table 2). The copy number of the IS differed among the strains and ranged

Table 2. Properties of *B. subtilis* isolates harboring IS4*Bsu*1

Strain	Source	γ-PGA[a)]	Amylase[b)]	Biotin requirement[c)]	Copy no. of IS4*Bsu*1	Sensitivity to the typed phages[d)]								
						SP50	SP10	ϕ105	SPP1	CS1	ϕ3T	S-a	BS5	ϕ29
NFRI 8334	KorB1	2	1		13									
NFRI 8336	KorB2	2	1		14									
NFRI 8306	ChiA1	2	2	R	6	VL		VL	VL	VL	VL		VL	CL
NFRI 8308	ChiA1	1	3	R	6	VL		VL		VL			VL	
NFRI 8325	ChiA2	0	3		6		CL							
NFRI 8357	ChiJ1	0	3		1									
NFRI 8358	ChiJ1	0	1		1									
NFRI 8313	LaoA3	0	1		16									
NFRI 8312	ThaA	0	3		4									
NFRI 8323	ThaA	1	2	R	17									
NFRI 8347	ThaB	1	3		15									
NFRI 8298	ThaC1	1	2		16		CL							
NFRI 8299	ThaC1	0	2	R	7									
NFRI 8300	ThaC2	0	1	R	4								VL	
NFRI 8301	ThaC2	0	3		17									
NFRI 8310	ThaC3	0	3		8									
NFRI 8311	ThaC3	0	3		3									
NFRI 8321	ThaC4	1	1	R	6									
NFRI 8322	ThaC4	1	1	R	15		CL							
NFRI 8316	MyaA2	0	3		13	CL								
NFRI 8320	MyaC2	2	2		10		CL							
NFRI 8369	MyaD	2	3		3		CL							
NFRI 8372	MalA	2	2		6									
NFRI 8290	NepD2	1	1		3	CL								
NFRI 8291	NepD4	1	1	R	2	CL	VL							
NFRI 8292	NepD5	0	1		2	CL	CL							
NFRI 8293	NepD6	1	1	R	3									
NFRI 8294	NepD7	0	1		2	CL	VL							
Miyagino	Natto	1	3	R	6		CL							
Sawamura	Natto	0	3		7		CL							
IFO13719	Type strain	0	3			VL			CL					
168		0	2			VL				VL				VL

a): 0, No γ-PGA production; 1, Production on only GSP agar; 2, Production on both GSP and TSA agar.
b): 1, Amylase formed on NB and NBG agar; 2, No amylase formed on NBG agar; 3, Amylase formed only on SMMC.
c): Strains that require biotin are indicated by R.
d): CL, Clear confluent lysis; VL, Veiled confluent lysis[1] .

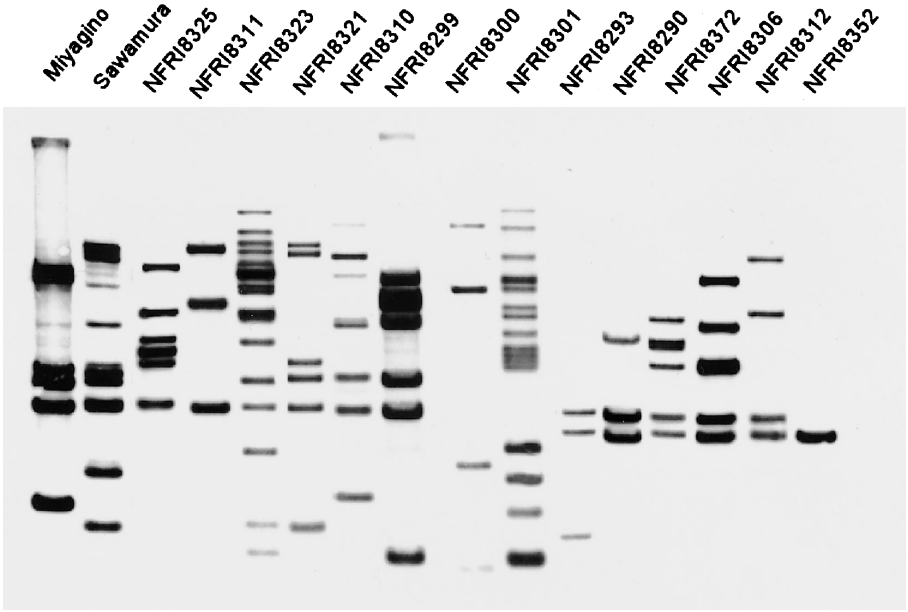

Fig. 1. Southern blots of IS4*Bsu*1 in *B. subtilis* strains isolated from fermented soybean foods
Chromosomal DNAs of indicated strains were digested with *Eco*RV and Southern-blotted using the IS4*Bsu*1 sequence of NAF4 strains[12] as the probe.

from 1 to 17 copies per chromosome. The profiles of the IS fragments in each strain were distinct (Fig. 1 and Table 2). Some of the strains isolated from the same sample showed different copy numbers and IS fragments of different sizes (Table 2). The occurrence of multiple copies of the IS indicates a high capacity for transposition, which is consistent with previous observations[12]. Only 5 strains harbored the IS among 33 isolates from salted-type fermented soybeans, while 20 out of 57 strains from non-salted type fermented soybeans had the IS. In the present study, it was also found that the IS frequency was high in fermented soybeans of Thailand (92%) and Nepal (56%), but low in those of China and Korea (17 and 20%, respectively). In contrast, Southern-blotting demonstrated that none of the 49 *B. subtilis* collections of Ackermann that were isolated from soil in Europe, Africa and other countries[1] had IS4*Bsu*1 (unpublished results). Thus, IS4*Bsu*1 appears to occur frequently in *B. subtilis*

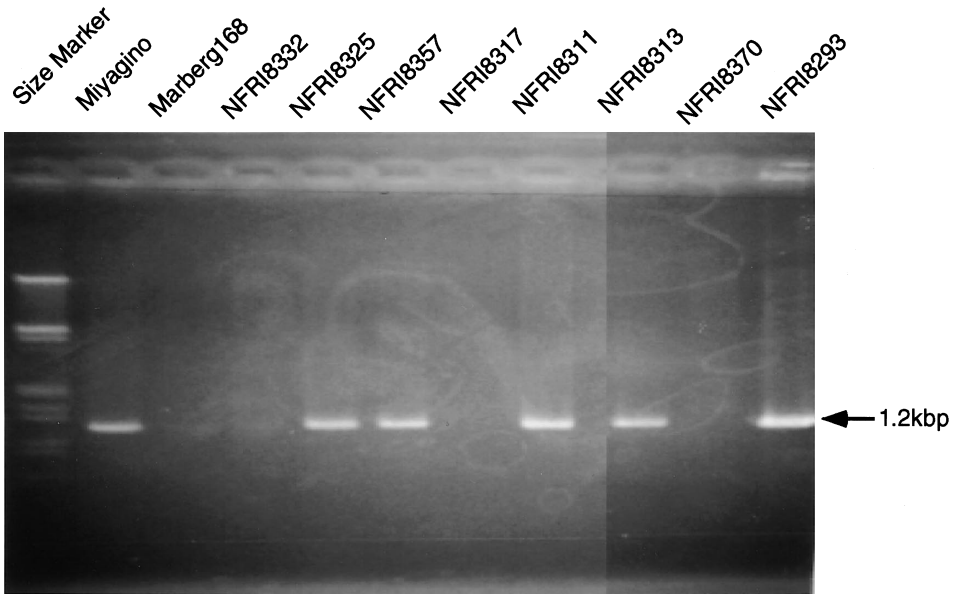

Fig. 2. Amplification of IS4*Bsu*1 sequences in *B. subtilis* strains by PCR

Table 3. Nucleotide substitutions in IS4*Bsu*1 sequences among *B. subtilis* strains

Strain	Source/Location	Nucleotide substitutions at nt			
		211	343	641	960
NAF4	Natto/ Asahikawa (Japan)	A	C	G	G
NFRI8334	Teng Jan/ Seoul (Korea)	A	C	G	G
NFRI8336	Teng Jan /Seoul (Korea)	A	C	G	G
NFRI8325	Shui Douchi/ Taipei (China)	G	C	G	G
NFRI8312	Tao Cho/ Bangkok (Thailand)	A	C	G	G
NFRI8310	Tua Nao/ Chiang Rai (Thailand)	A	C	G	G
NFRI8311	Tua Nao/ Chiang Rai (Thailand)	A	C	G	G
NFRI8347	Tua Nao/ Chiang Mai (Thailand)	A	C	G	G
NFRI8313	Mac Tua Nao/ Ruanpapan (Laos)	A	C	G	G
NFRI8316	Chine Pepoke/ Yangon (Myanmar)	A	C	G	G
NFRI8320	Chine Pepoke/ Boban (Myanmar)	G	G	T	G
NFRI8290	Kinema/ Hille (Nepal)	G	G	G	A
NFRI8292	Kinema/ Hille (Nepal)	G	G	G	G

strains in fermented soybeans, particularly of the non-salted type.

Determination of the IS sequences

Based on the intensity of the DNA bands detected by Southern-blotting (Fig. 1), the IS of the tested strains seemed to be highly homologous. To determine whether the IS elements in the strains from specific locations shared sequence similarity, we analyzed the nucleotide sequences of the IS4*Bsu*1 from 12 isolates. We amplified the 1.2 kb DNA region of the IS element by PCR and used it as the sequencing template (see Materials and methods). Fig. 2 shows that the DNA fragments were amplified only when the chromosomal DNAs from the strains harboring the IS were used as the templates (Fig. 2). The IS4*Bsu*1 sequences of the isolates from Korea, Thailand, Laos and Myanmar were identical with that of *B. subtilis* (*natto*) strain (Table 3). Thus, this sequence covers a major group and probably represents the parent sequence of the IS. The IS4*Bsu*1 sequence of strain NFRI8352 from Taiwan had one substitution, A for G at nt 211 (Table 3), whereas that of a strain from Myanmar (NFRI8320) showed 3 substitutions at nt 211, 343 and 641 (Table 3). Two strains from Nepal (NFRI8290 and NFRI8292) had 3 (at nt 211, 343 and 960) and 2 (nt 211 and 343) substitutions, respectively. Although these strains had multiple copies of the IS (Fig. 1), the sequence charts were not ambiguous, indicating that each IS copy in these strains had the same sequence. The perfect conservation of the IS4*Bsu*1 sequences among strains isolated from distantly separated countries implies that IS4*Bsu*1can distribute rapidly among *B. subtilis* strains via an unknown mechanism.

In summary, biotin requirement and amylase production are not common traits of *B. subtilis* strains isolated from fermented soybeans. Since the frequency of IS4*Bsu*1 is higher among *B. subtilis* strains from non-salted fermented soybeans, particularly those of Thailand and Nepal, this IS element has been distributed among relatively restricted groups of *B. subtilis* strains that may multiply actively in soybeans during fermentation and hence significantly contribute to soybean fermentation.

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