J. Gen. Appl. Microbiol., **53**, 315–323 (2007)

Full Paper

Phylogeny of γ-polyglutamic acid-producing *Bacillus* strains isolated from fermented soybean foods manufactured in Asian countries

Jomkhwan Meerak,[1] Haruna Iida,[1] Yusuke Watanabe,[1] Mika Miyashita,[2] Hajime Sato,[2] Yasuyoshi Nakagawa,[2] and Yasutaka Tahara[1,*]

[1] *Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka 422–8529, Japan*
[2] *Biological Resource Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu 292–0818, Japan*

(Received June 18, 2007; Accepted September 18, 2007)

Natto-like fermented soybean products are manufactured and consumed in many Asian countries. In this study, we isolated thirty-four *Bacillus* strains capable of producing γ-polyglutamic acid (PGA) from natto in mountainous areas of South Asia and Southeast Asia and from soils in Japan. To elucidate the phylogeny of these PGA-producing strains, phylogenetic trees based on sequences of 16S rDNA, housekeeping genes of *rpoB* (RNA polymerase β-subunit) and *fus* (elongation factor G) were constructed. A phylogenetic tree based on 16S rDNA sequences showed that twenty-one isolates were clustered in the same group of *B. subtilis*. The other thirteen isolates were located in the cluster of *B. amyloliquefaciens*. Phylogenetic trees based on the partial sequences of *rpoB* and *fus* genes were similar to the phylogeny based on 16S rDNA sequences. The results of the present study indicate that PGA-producing strains isolated from local natto in Asian countries and soil in Japan can be divided into two species, *B. subtilis* and *B. amyloliquefaciens*.

Key Words——*Bacillus*; fermented soybean foods; phylogeny; γ-polyglutamic acid; *rpoB* and *fus* sequences; 16S rDNA sequences

* Address reprint requests to: Dr. Yasutaka Tahara, Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422–8529, Japan.

E-mail: acytaha1@agr.shizuoka.ac.jp

Abbreviations: NBRC, Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, Japan; BCC, BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, Thailand; BCRC, Bioresource Collection and Research Center, Food Industry Research and Development Institute, Taiwan; ATCC, American Type Culture Collection, USA; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, USA; NCIB, National Collection of Industrial, Food and Marine Bacteria, NCIMB, Ltd., UK; IFO, Institute for Fermentation, Osaka, Japan; NCDO, National Collection of Dairy Organisms, UK; JCM, Japan Collection of Microorganisms, Japan.

Introduction

Natto, a sticky fermented food made from steamed soybeans, is produced and eaten nationwide in Japan. Natto-like fermented soybean foods are also manufactured in other Asian countries, including eastern Nepal, northern West Bengal and Sikkim provinces in India, Bhutan, northern Myanmar, northern Thailand, and Yunnan province in China, which places overlapped the “laurel forest zone in South Asia and Southeast Asia” cited by ethnologist Sasuke Nakao (1966). Japanese commercial natto is produced in modern facilities using a pure culture of *Bacillus subtilis*, but natto products in other Asian countries are manufactured in rural mountainous areas through a natural fermentation process in which steamed soybeans are

wrapped in plant leaves and then fermented by natural organisms at ambient temperature (Chantawannakul et al., 2002; Chunhachart et al., 2006b; Sarkar et al., 2002; Tamang, 1999). Natto and natto-like fermented foods are distinct from other soybean fermented foods due to their viscosity, and the sticky material is mainly composed of γ-polyglutamic acid (PGA), a polymer that consists of D- and L-glutamic acid polymerized through γ-glutamyl bonds. Natto-producing bacteria all have the capability to produce PGA and have been classified as *B. subtilis* or *Bacillus* species. Although there have been a few reports on identification of bacteria involved in production of local natto (Chantawannakul et al., 2002; Inatsu et al., 2006; Sarkar et al., 2002), there has been little investigation of the molecular taxonomy of isolates from Asian local natto.

This paper describes the phylogeny of thirty-four *Bacillus* strains isolated as PGA-producing bacteria from Asian local natto and soils in Japan based on sequences of 16S rDNA and housekeeping genes of *rpoB* (RNA polymerase β-subunit) and *fus* (elongation factor G).

Materials and Methods

Isolation of bacterial strains. *Bacillus* strains were isolated from local natto purchased at local markets in different countries of South Asia and Southeast Asia and from soils collected in Japan. The sites of isolation and strain numbers are listed in Table 1. One gram of an isolation source was homogenized in 99 ml of 0.85% NaCl, and a portion of the homogenized suspension was incubated at 80°C for 20 min to destroy all vegetative cells. The suspension was diluted with 0.85% NaCl and spread onto a nutrient-agar plate, which was then incubated at 37°C for 24 h. Isolated bacterial colonies were randomly selected and restreaked onto a fresh nutrient-agar plate until purified colonies were obtained. Mucoid colony production was tested by incubating isolated strains on LB agar containing 1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar (pH 7.5) at 37°C for 24 h.

Preparation of genomic DNA. Bacterial cells were grown with shaking at 37°C overnight in LB medium and then collected by centrifugation. Genomic DNA was extracted and purified by the method of Saito and Miura (1963).

16S rDNA sequencing. Almost complete 16S rDNA was amplified by PCR with the primers 9F (5′-GAGTTTGATCCTGGCTCAG-3′, positions 9 to 27 on 16S rDNA by the *Escherichia coli* numbering system; Brosius et al., 1981) and 1510R (5′-AAGGAGGTGATCCAGCC-3′, positions 1510 to 1495 on 16S rDNA). The 1.5-kb amplified 16S rDNA fragment was purified by using a GFX PCR DNA and Gel Brand purification kit (Amersham Biosciences, Piscataway, NJ, USA) to remove primers and free nucleotides. The PCR products obtained were directly sequenced by using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). PCR purification and sequencing were done according to the instructions of the manufacturers. Oligonucleotide primers used to sequence the amplified 16S rDNA fragments were the same as those used in the PCR reaction and an additional two primers, 785F (5′-GGATTAGATACCCTGGTAGTA-3′, positions 785 to 805) and 802R (5′-TACCAGGGTATCTAATCC-3′, positions 802 to 785). The sequences of 16S rDNA were analyzed by an automated DNA sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

rpoB and fus sequences. *rpoB* (RNA polymerase β-subunit) and *fus* (elongation factor G) were amplified by using a nested PCR and sequenced according to the method of Sato (2006). Forward primers rpoB1909F (5′-GCNYTNATGGGNRCNAA-3′) and fus265F (5′-CCNGGNCAYGTNGAYTTYAC-3′) and reverse primers rpoB2324R (5′-AYNGCRTCYTCRWARTT-3′) and fus821R (5′-ACNCCYTTRTTYTTRAANGC-3′) were selected to amplify *rpoB* and *fus* fragments of *Bacillus* RNA polymerase and elongation factor G gene, respectively. For nested PCR, each primary PCR product was added to the reaction mixture as a template. Primers rpoB1927FM13 (5′-GTAAAACGACGGCCAGTATGCARMGNCARGCNGTNCC-3′), rpoB2306RM13R (5′-GGAAACAGCTATGACCATGTANCCNTBCCANGKCAT-3′), fus367FM13 (5′-GTAAAACGACGGCCAGTGARACNGTNTGGCGNCARGC-3′) and fus821RM13 (5′-GGAAACAGCTATGACCATGACNCCYTTRTTYTTRAANGC-3′) were used as nested primers to amplify partial sequences of *rpoB* (379 bp) and *fus* (454 bp), respectively. The nested PCR products were purified and directly sequenced as mentioned before. Primers used for sequencing were M13 (5′-GTAAAACGACGGCCAGT-3′) and M13R (5′-GGAAACAGCTATGACCATG-3′).

Phylogenetic analysis. All sequences were assembled using the AutoAssembler program (Applied Biosystems). Multiple alignment of the sequences de-

Table 1. *Bacillus* strains used in this study.

Species and strain	Other designation	Source of isolation and country of origin	Identified as
B. amyloliquefaciens			
NBRC 14141		soil; Japan	
NBRC 15535^T	ATCC 23350; NRRL B-14393	soil; Japan	
B. atrophaeus			
JCM 9070^T	ATCC 49337; NRRL NRS-213; NBRC 15539	soil; Colorado, USA	
B. licheniformis			
NCDO 1772^T	ATCC 14580; NCIB 9375; NRRL NRS-1264; NBRC 12200		
B. mojavensis			
BCRC 17124			
B. subtilis subsp. *spizizenii*			
NBRC 101239^T	NRRL B-23049	soil; Sahara desert, Tunisia	
B. subtilis subsp. *spizizenii*			
NBRC 101240	NRRL B-14697	soil; Rosamond, California, USA	
B. subtilis subsp. *subtilis*			
NBRC 101245	NRRL B-23068		
B. subtilis subsp. *subtilis*			
NBRC 101247	NRRL B-23074		
B. subtilis subsp. *subtilis*			
NBRC 13719^T	ATCC 6051; NCIB 3610; NBRC 16412; IFO 16412; NRRL B-4219; NRRL NRS-1315		
ID1	BCC 23066	Kinema; Darjeeling, India	*B. subtilis*
ID2	BCC 23067	Kinema; Darjeeling, India	*B. subtilis*
ID3	BCC 23068	Kinema; Darjeeling, India	*B. subtilis*
ID5	BCC 23069	Kinema; Darjeeling, India	*B. subtilis*
ID6	BCC 23070	Kinema; Darjeeling, India	*B. subtilis*
NDN3	BCC 23076	Kinema; Dharan, Nepal	*B. subtilis*
TM1	BCC 23082	Thua-nao; Maehongsorn, Thailand	*B. subtilis*
TM2	BCC 23083	Thua-nao; Maehongsorn, Thailand	*B. subtilis*
TM3	BCC 23084	Thua-nao; Maehongsorn, Thailand	*B. subtilis*
TM5	BCC 23085	Thua-nao; Maehongsorn, Thailand	*B. subtilis*
CR2	BCC 23063	Tan-douchi; Ruili, China	*B. subtilis*
CR3	BCC 23064	Tan-douchi; Ruili, China	*B. subtilis*
CR4	BCC 23065	Tan-douchi; Ruili, China	*B. subtilis*
KT5	BCC 23071	Chungkuk jang; Taegu, Korea	*B. subtilis*
KT9	BCC 23072	Chungkuk jang; Taegu, Korea	*B. subtilis*
W20	BCC 23086; NBRC 101584	soil; Hokkaido, Japan	*B. subtilis*
B10	BCC 23062; NBRC 101588	soil; Hokkaido, Japan	*B. subtilis*
Y6-1	BCC 23094; NBRC 101592	soil; Iwate, Japan	*B. subtilis*
Y7-1	BCC 23095; NBRC 101590	soil; Iwate, Japan	*B. subtilis*
Y16-1	BCC 23089; NBRC 101581	soil; Nagano, Japan	*B. subtilis*
Y21-1	BCC 23090; NBRC 101582	soil; Nagano, Japan	*B. subtilis*
NDN1	BCC 23075	Kinema; Dharan, Nepal	*B. amyloliquefaciens*
NDN4	BCC 23077	Kinema; Dharan, Nepal	*B. amyloliquefaciens*
ND1	BCC 23073	Kinema; Dhankuta, Nepal	*B. amyloliquefaciens*
ND4	BCC 23074	Kinema; Dhankuta, Nepal	*B. amyloliquefaciens*
TC3	BCC 23079	Thua-nao; Chiangmai, Thailand	*B. amyloliquefaciens*
TC4	BCC 23080	Thua-nao; Chiangmai, Thailand	*B. amyloliquefaciens*
TC5	BCC 23081	Thua-nao; Chiangmai, Thailand	*B. amyloliquefaciens*
W30-2	BCC 23087; NBRC 101589	soil; Hokkaido, Japan	*B. amyloliquefaciens*
Y13	BCC 23088; NBRC 101586	soil; Nagano, Japan	*B. amyloliquefaciens*
Y26	BCC 23091; NBRC 101587	soil; Gunma, Japan	*B. amyloliquefaciens*
Y35-1	BCC 23092; NBRC 101585	soil; Yamagata, Japan	*B. amyloliquefaciens*
Y42-3	BCC 23093; NBRC 101591	soil; Yamagata, Japan	*B. amyloliquefaciens*
R9	BCC 23078; NBRC 101583	soil; Tochigi, Japan	*B. amyloliquefaciens*

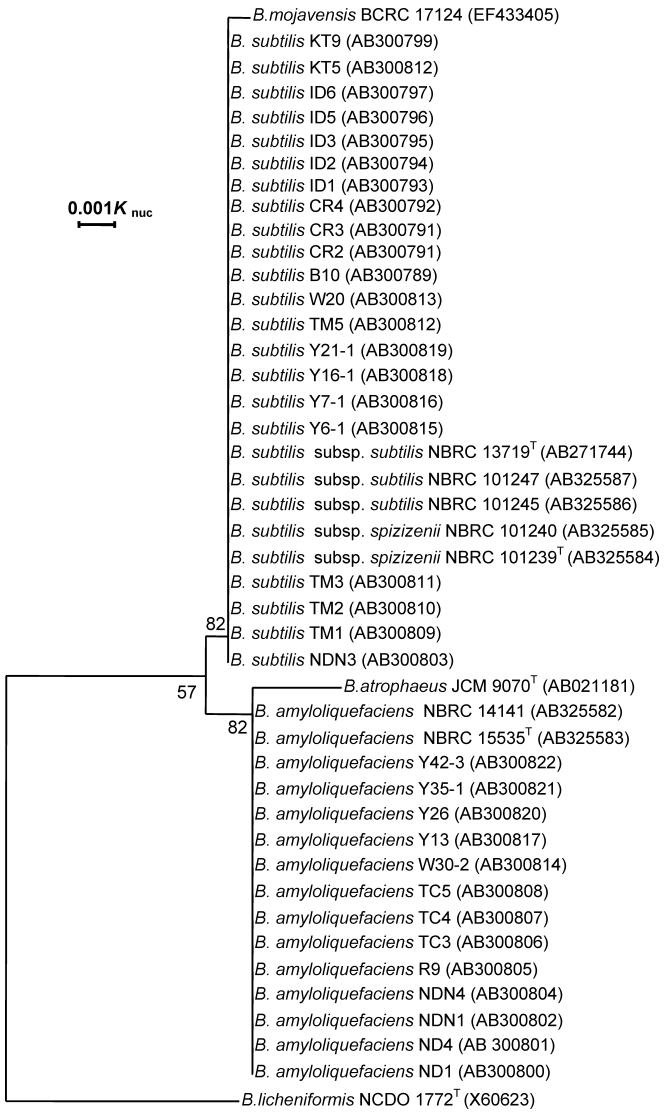

Fig. 1. A phylogenetic tree based on 16S rDNA sequences for the PGA-producing *Bacillus* strains isolated in Southeast Asia and South Asia.

The phylogenetic tree was constructed by the neighbor-joining method. The type strain of *Bacillus licheniformis* NCDO 1772^T was used for an outgroup. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications.

termined was performed with the program CLUSTAL X (version 1.81) (Thompson et al., 1997). Gaps and ambiguous bases were eliminated from the calculations. The distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura (1980). Phylogenetic trees based on 16S rDNA, *rpoB* and *fus* sequences were constructed by the neighbor-joining method (Saitou and Nei, 1987). The robustness for individual branches was estimated by bootstrapping with 1,000 replications (Felsenstein, 1985).

Reference strains. *Bacillus subtilis* subsp. *subtilis* NBRC 13719^T, NBRC 101245, and NBRC 101247, *B. subtilis* subsp. *spizizenii* NBRC 101239^T and NBRC 101240, *Bacillus amyloliquefaciens* NBRC 15535^T and NBRC 14141, *Bacillus atrophaeus* NBRC 15539^T and JCM 9070^T, *Bacillus licheniformis* NBRC 12200^T and NCDO 1772^T and *Bacillus mojavensis* BCRC 17124 were used as reference strains. 16S rDNA sequences except *Bacillus licheniformis* NCDO 1772^T and *Bacillus mojavensis* BCRC 17124, *rpoB* and *fus* sequences of reference strains were determined in this study.

Production and preparation of PGA. Bacterial cells were grown at 37°C for 20 h with shaking on a rotary shaker at 175 rpm in a 200-ml conical flask containing 100 ml of PGA medium, which consisted of 2.0% glucose, 2.0% sodium L-glutamate, 1.0% $(NH_4)_2SO_4$, 0.1% Na_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $MnCl_2 \cdot 4H_2O$, 0.005% $FeCl_3 \cdot 7H_2O$ (Kunioka and Goto, 1994), and 0.5 μg/ml of biotin (pH 7.5). The culture was centrifuged to obtain the supernatant, in which PGA was then precipitated with ethanol. The resulting precipitate was lyophilized to remove ethanol and dialyzed against distilled water. The dialyzed solution was centrifuged and the supernatant was lyophilized to give pure PGA.

SDS-PAGE and PGA determination. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of PGA was performed using 10% polyacrylamide gel by the method of Yamaguchi et al. (1996). The amount of PGA in the culture was determined by an amino acid analyzer after acid hydrolysis according to the method of Ogawa et al. (1997). The ratio of D-glutamic acid to L-glutamic acid in PGA was assayed with an L-glutamic acid assay kit (Roche Molecular Biochemicals, Basel, Switzerland) after complete acid hydrolysis of PGA as described previously (Cheng et al., 1989).

Nucleotide sequence accession numbers. The DNA sequences determined in this study were deposited in the DDBJ database under the accession numbers shown in Figs. 1 and 2.

Results

Phylogenetic analysis based on 16S rDNA sequences

A phylogenetic tree based on 16S rDNA is shown in Fig. 1. When the type strain of *B. licheniformis* was used for an outgroup, the thirty-four *Bacillus* strains

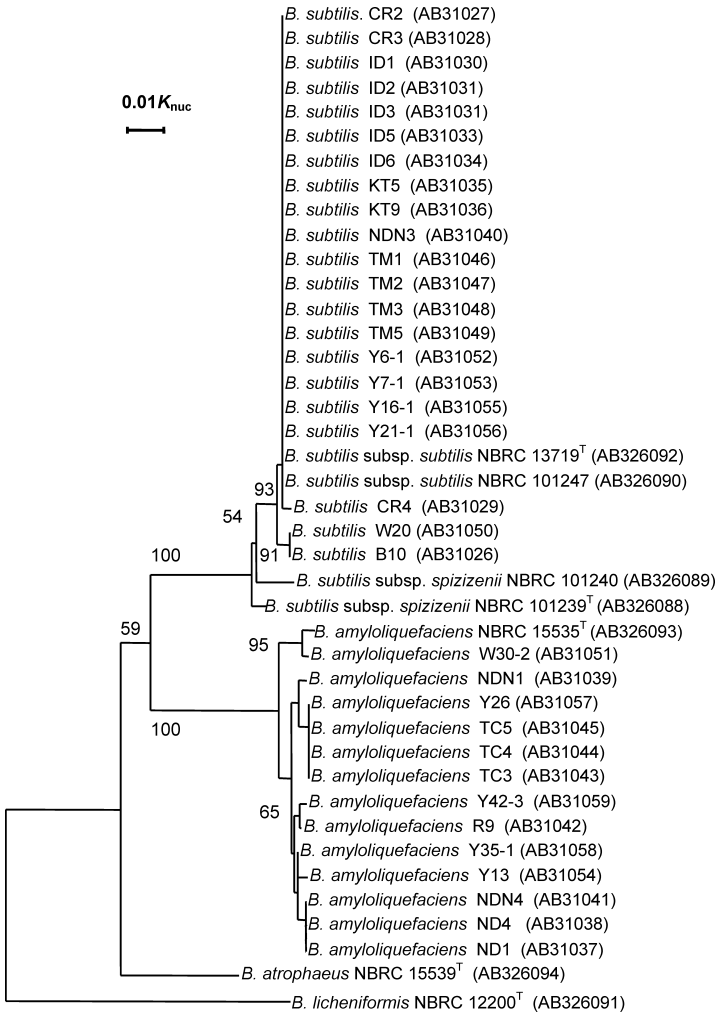

Fig. 2(a)

isolated were clearly grouped into clusters, i.e., the *B. subtilis* cluster and the *B. amyloliquefaciens* cluster. The almost complete 16S rDNA gene sequences, ranging from *E. coli* equivalent positions 28–1494 were determined and the length of sequences compared was 1,360 bp. One was composed of twenty-one *Bacillus* strains, ID1, ID2, ID3, ID5, ID6, NDN3, TM1, TM2, TM3, TM5, CR2, CR3, CR4, KT5, KT9, W20, B10, Y6-1, Y7-1, Y16-1 and Y21-1, along with *B. subtilis* NBRC 13719^T, NBRC 101239^T, NBRC 101240, NBRC 101245 and NBRC 101247. The other was composed of thirteen *Bacillus* strains, NDN1, NDN4, ND1, ND4, TC3, TC4, TC5, W30-2, Y13, Y26, Y35-1, Y42-3 and R9 along with *B. amyloliquefaciens* NBRC 15535^T and NBRC 14141. The two clusters respectively formed a monophyletic clade, and their phylogenetic distances were far from *B. atrophaeus* JCM 9070^T.

Phylogenetic analysis based on rpoB and fus sequences

In 16S rDNA analysis, each cluster of *B. subtilis* and *B. amyloliquefaciens* indicated 82% in bootstrap value. Because the figure was not very high in reliability, phylogenetic analysis was next carried out using *rpoB* and *fus* genes of which evolutional speeds are faster than those of 16S rDNA (Holmes et al., 2004). Phylogenetic trees based on *rpoB* and *fus* sequences are shown in Fig. 2. Comparison of the partial sequences of *rpoB* and *fus* were 341 bp and 411 bp, respectively. In phylogeny of *rpoB* and *fus*, each bootstrap value of the clusters of *B. subtilis* and *B. amyloliquefaciens* stood at 100%, indicating that both species are clearly discriminated. Furthermore, *B. subtilis* subsp. *spizizenii* NBRC 101240 and NBRC 101239^T were placed separately from *B. subtilis* subsp. *subtilis* NBRC 13719^T and NBRC 101247, and the isolates identified as *B.*

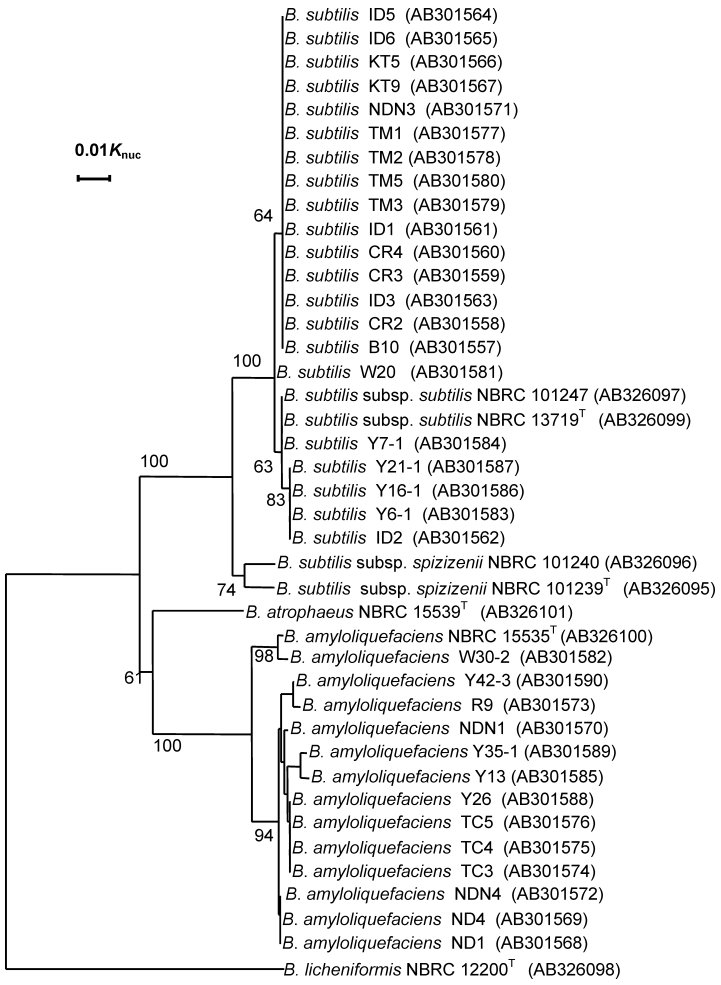

Fig. 2(b)

Fig. 2. Phylogenetic trees based on *rpoB* and *fus* gene sequences for the PGA-producing *Bacillus* strains isolated in Southeast Asia and South Asia.

The phylogenetic trees were constructed by the neighbor-joining method on the basis of *rpoB* sequences (a) and *fus* sequences (b). The type strain of *Bacillus licheniformis* NBRC 12200^T was used for an outgroup. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications.

subtilis were all included in *B. subtilis* subsp. *subtilis*.

PGA production and ratio of D*-glutamic acid to* L*-glutamic acid in PGA*

The thirty-four isolated strains and *B. subtilis* NBRC 16449 and *B. amyloliquefaciens* NBRC 15535^T and NBRC 14141 were cultured in PGA medium with shaking at 37°C for 20 h as described in MATERIALS AND METHODS. Table 2 shows PGA production and the proportion of D- and L-glutamic acid of PGA. PGA production of the isolates identified as *B. amyloliquefaciens* was almost comparable to that of the isolate identified as *B. subtilis*, although there was a difference among the strains. Amounts of PGA produced by *B. amyloliquefaciens* NBRC 15535^T and NBRC 14141 reached 5.3 and 5.2 mg/ml, respectively, and that produced by *B. subtilis* NBRC 16449 was 8.0 mg/ml. The ratio of D-glutamic acid to L-glutamic acid in PGA of *B. amyloliquefaciens* was higher on average than that in PGA of *B. subtilis*. The ratio of D- and L-glutamic acid in PGA of *B. subtilis* NBRC 16449 was 70 : 30, while the ratios of *B. amyloliquefaciens* NBRC 15535^T and NBRC 14141 were 91 : 9 and 90 : 10, suggesting that a high proportion of D-glutamic acid in PGA of *B. amyloliquefaciens* is indigenous to the species.

Discussion

Fermented soybean foods are mainly divided into two categories depending on whether brine is added or not in the process of fermentation. A salt-added fermented soybean food includes soy sauce and soy paste, and an unsalted fermented soybean food is representative of natto. Natto-like fermented foods sold at markets in other Asian countries are also produced with no addition of brine in the process of natural fermentation. These local nattos have various names, Kinema in Nepal and India and Thua-nao in Thailand.

Phylogenetic analysis indicated that the thirty-four strains isolated as PGA-producing bacteria from natto products in other Asian countries and soils in Japan can be identified as the two *Bacillus* species of *B. subtilis* and *B. amyloliquefaciens* (Figs. 1 and 2). Fifteen strains from Asian local nattos were identified as *B. subtilis*. These strains were isolated from Kinema at Darjeeling in India and at Dharan in Nepal, from Thua-nao at Maehongsorn in Thailand, from Tan-douchi at Ruili in China, and from Chungkuk jang at Taegu in Korea. On the other hand, the isolates identified as *B. amyloliquefaciens* include seven strains that were from Kinema at Dharan and Dhankuta in Nepal and from Thua-nao at Chiangmai in Thailand. From soils in Japan, six strains identified as *B. subtilis* were isolated from areas of Hokkaido, Iwate and Nagano prefectures, and another six strains identified as *B. amyloliquefaciens* were from areas of Hokkaido, Nagano, Gunma, Yamagata and Tochigi prefectures. The PGA-producing *B. subtilis* and *B. amyloliquefaciens* strains from Asian local nattos seem to be scattered, making clusters in the local natto collecting areas. For example, *B. amyloliquefaciens* was exclusively isolated from Nepali Kinema, but *B. subtilis* was isolated from Indian Kinema except for one strain from Nepal. On the other hand, both *Bacillus* strains isolated from soils in north-

Table 2. PGA production and ratio of D-glutamic acid to L-glutamic acid in PGA produced by the isolated strains, *B. subtilis* NBRC 16449, and *B. amyloliquefaciens* NBRC 15535^T and NBRC 14141.

Strain	PGA production (mg/ml)[a]	Composition of glutamic acid (%)[b]		Strain	PGA production (mg/ml)	Composition of glutamic acid (%)	
		D-isomer	L-isomer			D-isomer	L-isomer
B.subtilis NBRC 16449	8.0	70.0	30.0	*B. subtilis* Y7-1	1.5	67.0	33.0
B. subtilis ID1	0.8	67.0	33.0	*B. subtilis* Y16-1	7.5	64.5	35.5
B. subtilis ID2	3.4	83.6	16.4	*B. subtilis* Y21-1	2.9	73.4	26.6
B. subtilis ID3	2.7	85.4	14.6	*B. amyloliquefaciens* NBRC 15535^T	5.3	91.1	8.9
B. subtilis ID5	0.8	80.2	19.8	*B. amyloliquefaciens* NBRC 14141	5.2	90.0	10.0
B. subtilis ID6	1.3	65.7	34.3	*B. amyloliquefaciens* NDN1	2.7	92.4	7.6
B. subtilis NDN3	3.2	87.4	12.6	*B. amyloliquefaciens* NDN4	2.1	88.8	11.2
B. subtilis TM1	2.0	67.3	32.7	*B. amyloliquefaciens* ND1	5.7	90.3	9.7
B. subtilis TM2	1.2	77.6	22.4	*B. amyloliquefaciens* ND4	5.8	86.0	14.0
B. subtilis TM3	2.6	74.2	25.8	*B. amyloliquefaciens* TC3	2.7	87.5	12.5
B. subtilis TM5	0.7	71.1	29.0	*B. amyloliquefaciens* TC4	5.3	82.4	17.6
B. subtilis CR2	2.5	80.4	19.6	*B. amyloliquefaciens* TC5	2.5	76.9	23.1
B. subtilis CR3	0.9	70.5	29.5	*B. amyloliquefaciens* W30-2	7.5	87.8	12.2
B. subtilis CR4	3.2	76.4	23.6	*B. amyloliquefaciens* Y13	2.1	88.0	12.0
B. subtilis KT5	2.6	78.1	21.9	*B. amyloliquefaciens* Y26	6.9	89.1	10.9
B. subtilis KT9	1.4	77.0	23.0	*B. amyloliquefaciens* Y35-1	2.5	84.6	15.4
B. subtilis W20	0.3	77.8	22.2	*B. amyloliquefaciens* Y42-3	0.4	87.4	12.6
B. subtilis B10	0.5	77.8	22.2	*B. amyloliquefaciens* R9	1.1	89.3	10.7
B. subtilis Y6-1	6.5	71.0	29.0				

[a] Bacterial cells were grown in PGA medium with shaking at 37°C for 20 h as described in MATERIALS AND METHODS.

[b] Shown as an average of the triplicate.

ern Japan were widely spread regardless of where the strains are isolated. The findings and that *B. subtilis* and *B. amyloliquefaciens* are soil-borne bacteria closely related in taxonomy (O'Donnell et al., 1980) suggest that both *Bacillus* strains capable of producing PGA are ubiquitous bacteria in soil and prevail in the process of natto fermentation probably through plant leaves used for wrapping steamed soybeans in rural areas of Asian countries.

There has been no report so far that *B. amyloliquefaciens* produces PGA, and it is therefore surprising that *B. amyloliquefaciens* has been so widely isolated as a PGA-producing bacterium from Asian local nattos and soils in Japan. We also investigated *B. amyloliquefaciens* stocked at NBRC for PGA production. *B. amyloliquefaciens* NBRC 15535^T and NBRC 14141 produced 5.3 and 5.2 mg/ml of PGA, respectively (Table 2). The ratios of D-glutamic acid to L-glutamic acid in PGA were different in *B. subtilis* and *B. amyloliquefaciens* (Table 2), indicating that the ratio of D-glutamic acid to L-glutamic acid in PGA of the latter is higher than that of the former. We previously proposed the PGA structure of *B. subtilis* NBRC 16449, a natto-producing strain (Chunhachart et al., 2006a; Suzuki and Tahara, 2003). The PGA is composed of a high-molecular-mass region (F-1) with only L-glutamic acid and a 2.0-kDa low-molecular-mass region (F-2) with D- and L-glutamic acids in an 80 : 20 ratio, and F-2 consists of a D-glutamic acid cluster in the N-terminus and two or three consecutive L-glutamic acids near the C-terminus. It was found in this study that the ratio of D- and L-glutamic acids of F-2 in PGA of *B. amyloliquefaciens* NBRC 15535^T is 94 : 6 with a molecular mass of 3.1 kDa (data not shown), suggesting that the F-2 structure in PGA is different between *B. amyloliquefaciens* and *B. subtilis*. We have also cloned and purified PGA synthetase (YwsC), a crucial enzyme in PGA biosynthesis, from *B. subtilis* NBRC 16449 (Urushibata et al., 2002a, b). Therefore, it is of interest that both YwsC enzymes are compared on the characterization.

Acknowledgments

We thank Mr. T. Takahashi of Kume Quality Products Co., Ltd. for kindly providing strains isolated from soils in Japan.

References

Brosius, J., Dull, T. J., Sleeter, D. D., and Noller, H. F. (1981) Gene organization and primary structure of ribosomal RNA operon from *Escherichia coli*. *J. Mol. Biol.*, **148**, 107–127.

Chantawannakul, P., Onchareon, A., Klanbut, K., Chukeatirote, E., and Lumyong, S. (2002) Characterization of proteases of *Bacillus subtilis* strain 38 isolated from traditionally fermented soybean in northern Thailand. *Science Asia*, **28**, 241–245.

Cheng, C., Asada, Y., and Aida, T. (1989) A poly-γ-glutamate synthetic system of *Bacillus licheniformis* A35 under denitrifying conditions. *Agric. Biol. Chem.*, **53**, 2369–2375.

Chunhachart, O., Hanayama, T., Hidesaki, M., Tanimoto, H., and Tahara, Y. (2006a) Structure of hydrolysed product (F-2) released from γ-polyglutamic acid by γ-glutamyl hydrolase YwtD of *Bacillus subtilis*. *Biosci. Biotechnol. Biochem.*, **70**, 2289–2291.

Chunhachart, O., Itoh, T., Sukchotiratana, M., Tanimoto, H., and Tahara, Y. (2006b) Characterization of γ-glutamyl hydrolase produced by *Bacillus* sp. isolated from Thai Thua-nao. *Biosci. Biotechnol. Biochem.*, **70**, 2779–2782.

Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**, 783–791.

Holmes, D. E., Nevin, K. P., and Lovey, D. R. (2004) Comparison of 16S rRNA, *nifD*, *recA*, *gyrB*, *rpoB* and *fusA* genes within the family *Geobacteraceae* fam. nov. *Int. J. Syst. Evol. Microbiol.*, **54**, 1591–1599.

Inatsu, Y., Nakamura, N., Yuriko, Y., Fushimi, T., Watanasiritum, L., and Kawamoto, S. (2006) Characterization of *Bacillus subtilis* strains in Thua-nao, a traditional fermented soybean food in northern Thailand. *Lett. Appl. Microbiol.*, **40**, 1–6.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, **16**, 111–120.

Kunioka, M. and Goto, A. (1994) Biosynthesis of poly (γ-polyglutamic acid) from L-glutamic acid, citric acid, and ammonium sulfate in *Bacillus subtilis* IFO3335. *Appl. Microbiol. Biotechnol.*, **40**, 867–872.

Nakao, S. (1966) The Origin of Cultivation and Agriculture, Iwanami Shinsyo, pp. 59–75 (in Japanese).

O'Donnell, A. G., Norris, J. R., Berkeley, R. C. W., Claus, D., Kaneko, T., Logan, N. A., and Nozaki, R. (1980) Characterization of *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* by pyrolysis gas-liquid chromatography, deoxyribonucleic acid-deoxyribonucleic acid hybridization, biochemical tests, and API systems. *Int. J. Syst. Bacteriol.*, **30**, 448–459.

Ogawa, Y., Sugiura, D., Motai, H., Yuasa, K., and Tahara, Y. (1997) Efficient production of γ-polyglutamic acid by *Bacillus subtilis* (natto) in jar fermenters. *Biosci. Biotechnol. Biochem.*, **61**, 1684–1690.

Saito, H. and Miura, K. (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochem. Biophys. Acta*, **72**, 619–629.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406–425.

Sarkar, P. K., Hasenack, B., and Nout, M. J. R. (2002) Diversity and functionality of *Bacillus* and related genera isolated from spontaneously fermented soybeans (Indian Kinema) and locust bean (African Soumbala). *Int. J. Food Microbiol*., **77**, 175–186.

Sato, H. (2006) Development of methods for systematic classification of microorganisms. *In* Construction of a Genetic Resource Library of Unidentified Microbes Based on Genome Information, NEDO Report Database (http://www.tech.nedo.go.jp/index.htm), pp. 698–762 (in Japanese).

Suzuki, T. and Tahara, Y. (2003) Characterization of *Bacillus subtilis ywtD* gene, whose product is involved in γ-polyglutamic acid degradation. *J. Bacteriol*., **185**, 2379–2382.

Tamang, J. P. (1999) Development of pulverised starter cultures for Kinema production. *J. Food Sci. Technol*., **36**, 475–478.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higins, D. G. (1997) The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*., **25**, 4876–4882.

Urushibata, Y., Tokuyama, S., and Tahara, Y. (2002a) Characterization of the *Bacillus subtilis ywsC* gene, involved in γ-polyglutamic acid production. *J. Bacteriol*., **184**, 337–343.

Urushibata, Y., Tokuyama, S., and Tahara, Y. (2002b) Difference in transcription levels of *cap* genes for γ-polyglutamic acid production between *Bacillus subtilis* IFO 16449 and Marburg 168. *J. Biosci. Bioeng*., **93**, 252–254.

Yamaguchi, F., Ogawa, Y., Kikuchi, M., Yuasa, K., and Motai, H. (1996) Detection of γ-polyglutamic acid (γ-PGA) by SDS-PAGE. *Biosci. Biotechnol. Biochem*., **60**, 255–258.