Journal of Hill Research
15(2) : 56-62, 2002

Phylogenetic Analysis of *Bacillus* Strains Isolated from Fermented Soybean Foods of Asia: Kinema, Chungkokjang and Natto

Jyoti Prakash Tamang[a*], Saroj Thapa[a], Sailendra Dewan[a], Yasuko Jojima[b], Ryosuke Fudou[b] and Shigeru Yamanaka[bc]

[a]*Food Microbiology Laboratory, Department of Botany, Sikkim Government College, Gangtok, Sikkim 737 102, India*

[b]*Central Research Laboratories, Ajinomoto Co Inc., 1-1, Suzuki-cho, Kawasaki-ku 210-8681, Japan*

[c]*Faculty of Textile Science and Technology, Shinshu University, Ueda 386-8567, Japan*

(Received 19 August 2002; revised version received 27 November 2002; accepted 30 November 2002)

ABSTRACT

Kinema, chungkokjang and natto are sticky, non-salty, flavouresome fermented soybean foods of Asia. A total of 38 strains of dominant endospore-forming and rod-shaped bacteria were isolated from these fermented soybean foods and studied phenotypically. On the basis of selected criteria and API 50 CHB tests, all endospore-forming rods were identified as Bacillus subtilis. *The identification was confirmed by phylogenetic analyses carried out on six randomly selected strains of* Bacillus subtilis *by sequencing of the 16S rRNA gene.*

INTRODUCTION

Preparation and consumption of fermented soybean foods is a traditional art of the people of South-East Asia. Kinema is a whole-soybean non-salty fermented food with sticky texture, gray tan in colour and a characteristic flavour. It is eaten as side-dish in the Eastern Himalayan regions of Nepal, the Darjeeling hills and Sikkim in India and in Bhutan (Tamang, 2001). Chungkokjang is a fermented soybean paste, consumed as soup with boiled rice in Korea (Kim *et al.*, 2000). Natto a highly sticky fermented soybean food is consumed in Japan (Nikkuni, 1997).

Conventional methods for identification are not sufficient to analyse the diversity among species of bacteria. Hara *et al.* (1995) reported that the plasmid of *Bacillus subtilis* (*natto*) strain resembles that of *Bacillus subtilis* strain, isolated from kinema in the partial nucleotide sequences. Sarkar *et al.*

* *Corresponding Author*

(2002) studied the genomic diversity of *Bacillus* species particularly *B. subtilis* isolated from kinema and soumbala, fermented locust bean of Africa by RAPD-PCR technique. It was the purpose of the present study identify predominant *Bacillus* spp. and to analyse the phylogenetic relationship on the basis of sequencing of the 16S rDNA gene. Isolates from kinema, chungkokjang and natto were included.

MATERIALS AND METHODS

Sample collection

Six samples of kinema were purchased from Darjeeling and Gangtok markets in India. Six natto samples were purchased from supermarkets in Kawasaki in Japan. The samples were collected in sterile bags, transportedto the laboratory immediately and analysed. Four dried samples of chungkokjang were collected from Kanghwa and Anshan regions of South Korea and were refrigerated prior to analysis.

Reference strain

Type strain *Bacillus subtilis* JCM 1465 was obtained from Japan Collection of Microorganisms (JCM, RIKEN, Wako-shi, Japan).

Microbial analysis

Ten g of well-mixed sample were blended with 90 ml sterile physiological saline (0.85 %) in Stomacher lab-blender 400 (Seward Medical, London, UK). One ml dilution was mixed with 9 ml sterile physiological saline, and heated for 2 min in continuously boiling water for spore counts (Tamang and Nikkuni, 1996). Decimal dilutions were prepared in sterile diluents and 1 ml of appropriate dilutions were mixed with molten nutrient agar (M001, HiMedia Laboratories, Mumbai, India) and incubated at 37° C for 18 h for enumeration of spores. After purification of representative isolates they were kept on nutrient agar in cryotubes at -20° C for further analyses.

Phenotypic characterization

Gram staining was performed accordingly to Bartholomew (1962). Cell morphology and motility test were observed in a phase contrast microscope (Olympus CH3-BH-PC, Tokyo, Japan) following a standard method (Harrigan, 1998). Catalase activity was tested with 10 % (w/v) H_2O_2. Cultures were grown on sucrose glutamate agar plates containing (g/L): sucrose, 50; monosodium glutamate, 15; potassium dihydrogen phosphate, 2.7; disodium hydrogen phosphate, 4.2; sodium chloride, 0.5; magnesium sulphate, 0.5; agar, 20 and 0.1 µg biotin/ ml. Growth was observed in presence or absence of biotin (Hara and Ueda, 1982). For observation of stickiness, cultures grown on phytone agar (Nagai *et al.*, 1994) at 37° C for 24 h were pulled by touching with an inoculating needle. All other phenotypic characteristics were carried out following the methods described by Claus and Berkeley (1986) and Schillinger and Lücke (1987). Ability of the isolates to ferment carbohydrates was studied using API 50 CHB (BioMérieux, Lyon, Marcy-l' Etoile, France) system.

Bacterial strains and DNA isolation

A total of six strains of *Bacillus*, KD:B1 and KG:B1 isolated from kinema, CA:B1 and CK:B1 from chungkokjang and JN-1 and JA-1 from natto, were selected randomly, and were grown aerobically at 37° C in Luria-Bertani (LB) medium (1 % tryptone, 05 % yeast extract, 1 % sodium chloride; w/v, pH 7.0). Cells were harvested by centrifugation of the cultures at the mid-exponential phase of growth, washed with 0.9 % NaCl solution, and resuspended in pure water. The chromosomal DNA was prepared by the method of Saito and Miura (1963).

16S rRNA gene sequencing and phylogenetic analysis

The 16S rDNA fragments that corresponded to positions 8-1543 of *Escherichia coli* 16S rRNA (Brosius *et al.*, 1978) were amplified by the PCR directly from cell lysates which were prepared by

treating cell suspensions with proteinase K (Hiraishi, 1992; Hiraishi *et al.*, 1994). The PCR products were sequenced directly with a SequiTherm Long-Read Cycle sequencing kit (Epicentre Technologies, Madison, WI, USA) and fluorescent primers and analyzed with a DNA sequencer (Pharmacia LKB Biotechnology, Uppsala, Sweden). The nucleotide sequences of were revealed using the Genetyx-Mac programme version 10 (Software Development Co. Ltd., Tokyo, Japan) and preliminary searches in the DDBJ (DNA Data Bank of Japan, Mishima, Japan) database were performed with the programme BLAST (Altschul *et al.*, 1997). Sequences of the close relatives were retrieved from DDBJ. Multiple alignment of sequence, calculation of the corrected evolutionary distance based on nucleotide substitution rates (K_{nuc} values) (Kimura, 1980) and construction of a phylogenetic tree by the neighbour-joining method (Saitou and Nei, 1987) was performed using the Clustal W programme (Thompson *et al.*, 1994). Branching patterns of trees were evaluated by bootstrapping with 1000 resamplings (Felsenstein, 1985). Alignment positions with gaps and unidentified bases were excluded for the calculations. The tree was illustrated by using the Treeview programme (Page, 1997). The 16S rDNA sequences of six strains CA: B1, CK: B1, KD: B1, KG: B1, JN-1 and JA-1 have been deposited in the DDBJ database with accession numbers AB072569, AB072570, AB072571, AB072572, AB072573 and AB072574, respectively.

RESULTS AND DISCUSSION

Morphology and cultural characteristics

Six samples of kinema, four samples of chungkokjang and six samples of natto were collected from different places of India, South Korea and Japan, respectively and analysed for microbial load. Average load of endospore-forming bacteria of the samples was 10^8 cfu/g (Table 1). A total of 38 strains of aerobic, rod-shaped, Gram-positive endospore-forming bacteria isolated from kinema, chungkokjang natto and were characterized (Table 2). All these endospore-formers were identified as *Bacillus subtilis* (Ehrenberg) Cohn according to the criteria laid down by Claus and Berkeley (1986) as well as sugar-profile in API system. With respect to spore position and reduction of nitrate, *Bacillus subtilis* strains isolated from kinema and chungkokjang differed from *Bacillus subtilis* (*natto*) strains and type strain *Bacillus subtilis* JCM 1465. All strains of *Bacillus* isolated from kinema and

Table 1. Microbial load of spores of *Bacillus* isolated from kinema, chungkokjang and natto

Sample with sample code	Place	Country	Log cfu/g[e]
Kinema (KD:B)[a]	Darjeeling	India	8.3
Kinema (KG:B)[a]	Gangtok	India	8.5
Chungkokjang (CA:B)[b]	Anshan	South Korea	7.6
Chungkokjang (CK:B)[b]	Kanghwa	South Korea	8.1
Natto (JN)[a]	[c]Naruse Co.	Japan	8.8
Natto (JA)[a]	[d]Kawasaki	Japan	8.5

[a]Data represent the means of 5 samples from each source.
[b]Data represent the means of 3 samples.
[c]Commercial natto starter
[d]Asamshi Taro Amada Food Co.
[e]cfu, colony forming unit

Table 2. Differentiation characteristics of *Bacillus* strains isolated from fermented soybeans foods of Asia

Characteristic	Kinema		Chungkokjang		Natto		*Bacillus subtilis* JCM 1465
	KD:B	KG:B	CA:B	CK:B	JN	JA	
	(n = 8)[1]	(n = 10)	(n = 4)	(n = 6)	(n = 4)	(n = 6)	
Cell width (μm)	0.7-1.0	0.5-1.0	0.5-0.8	0.5-0.8	0.8-0.9	0.8-0.9	0.8-0.9
Cell length (μm)	2.0-4.0	1.9-4.0	1.7-3.0	1.8-3.0	2.0-3.0	2.0-3.0	2.0-3.0
Spore position	c/cp[2]	c/cp	c/cp	c/cp	c	c	c
NO_3 reduction	-(6)[3], +(2)[4]	-(7), +(3)	-(2), +(2)	-(2), +(4)	+	+	+
Biotin requirement	+	+	+	+	+	+	-
Stickiness on phytone agar	+	+	+	+	+	+	-

[1]n = number of strain
[2]c/pc, central/para-central
[3]Number of strains showing negative result
[4]Number of strains showing positive result.

All strains were rods, Gram-stained, catalase positive, aerobic, showed motility, produced acid from D-glucose, L-arabinose and D-mannitol but no gas from D-glucose; hydrolysed casein and starch; grew well at pH 5.7 and 6.8, at 7 % NaCl, no growth at 55-60° C.

chungkokjang showed central to paracentral position of spores with few strains showing negative nitrate reduction test, whereas natto strains showed central position of spores and all reduced nitrate (Table 2). However, all strains of *Bacillus subtilis* isolated from kinema, chungkokjang and natto showed stickiness on phytone agar and cooked soybean, which are characteristics property of non-sa[illegible] fermented soybean foods of Asia, and their requirement of biotin from growth (Tamang and Nikkuni, 1996). These characters differed with type strain of *B. subtilis*.

Phylogenetic analysis

In order to investigate the phylogenetic relationship of isolates to other bacteria, the sequence of a 16S rRNA gene PCR product was determined and then analysed. A comparative analysis of the 16S rRNA gene sequences revealed that strains CA:B1 and CK:B2 isolated from chungkokjang, KD:B1 and KG:B1 isolated from kinema and JN-1 isolated from natto had identical sequences except that of JA-1 (natto) which had one ambiguous nucleotide. The evolutionary distance between four strains CK:B1, KD:B1, JN-1 and JA-1, and *Bacillus subtilis* was 0.002 K_{nuc} as calculated by the ratio of nucleotide substitution per nucleotide site, indicating 99 % homology with *Bacillus subtilis* type strain. However, the evolutionary distance between the strains CA:B1 and KG:B1, and *Bacillus subtilis* was 0.005 K_{nuc}, showing approximately 99.5 % homology

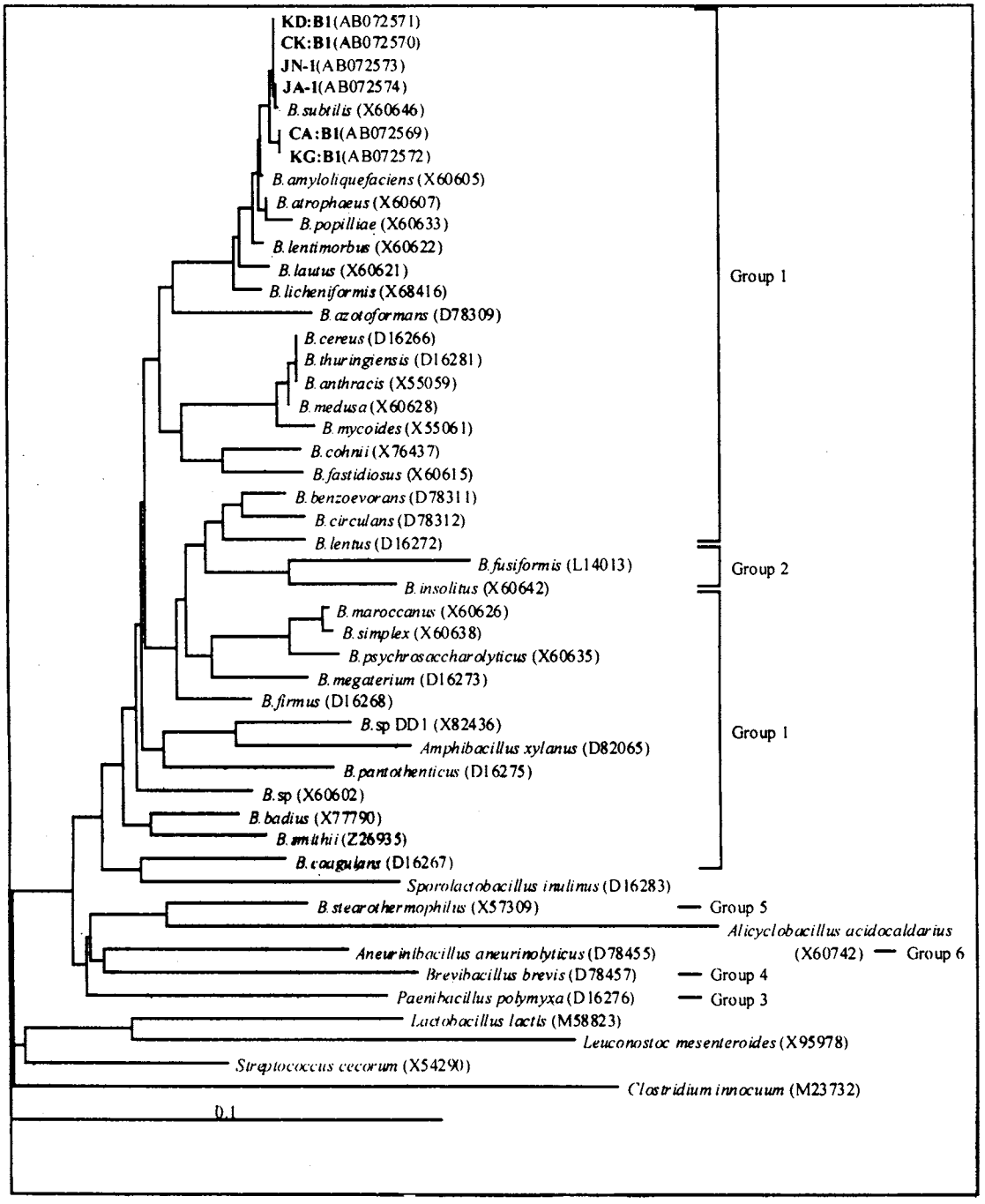

Fig. 1. Phylogenetic tree showing the relationships of *Bacillus subtilis* strains to other strains of the genus *Bacillus* and related genera based on partial sequence of 16S rRNA gene

with type strain. The phylogenetic analyses revealed that all six strains belonged to *Bacillus subtilis* (Fig 1). This is the first report to describe the phylogeny of *Bacillus subtilis* isolated from similar non-salty fermented sticky soybean foods of Asia. Diversity of *Bacillus subtilis*-dominating fermented soybeans foods of Asia from the Eastern Himalayan regions to far-east Asia needs to be studied in details to trace the antiquity and similarity in the food culture of Asia.

ACKNOWLEDGEMENT

The first author is grateful to Department of Biotechnology, Government of India for research project. The authors would like to thank Prof. Michio Kozaki of Japan for helpful discussion and late Jayant Prakash Tamang for sending the samples of chungkokjang from Korea.

REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Bartholomew, J. W. (1962) Variables influencing results and the precise definition of the steps in Gram staining as a means of standardizing the results obtained. *Stain Technology* **37**: 139-155.

Brosius, J., Palmer, J. L., Kennedy, J. P. and Noller, H. F. (1978) Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *The Proceedings of National Academy of Sciences USA* **75**: 4801-4805.

Claus, D. and Berkeley, R. C. W. (1986) Genus *Bacillus* Cohn 1872, 174. In: *Bergey's Manual of Systematic Bacteriology* (Eds: Sneath, P. H. A., Mair, N. S., Sharpe, M. E. and Holt, J. G.), Vol. 2, pp. 1105-1139. Williams and Wilkins, Baltimore, MD.

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

Hara, T. and Ueda, S. (1982) Regulation of polyglutamate production in *Bacillus subtilis (natto)*: transformation of high PGA productivity. *Agricultural and Biological Chemistry* **46**: 2275-2281.

Hara, T., Hiroyuki, S., Nobuhide, I. and Shinji, K. (1995) Plasmid analysis in polyglutamate-producing *Bacillus* strain isolated from non-salty fermented soybean food, "kinema", in Nepal. *Journal of General and Applied Microbiology* **41**: 3-9.

Harrigan, W. F. (1998) Laboratory Methods in Food Microbiology, 3rd Edition, 532 pp. Academic Press, London.

Hiraishi, A. (1992) Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. *Letters in Applied Microbiology* **15**: 210-213.

Hiraishi, A., Shin, Y. K., Ueda, Y. and Sugiyama, J. (1994) Automated sequencing of PCR-amplified 16S rDNA on 'Hydrolink' gels. *Journal of Microbiological Methods* **19**: 145-154.

Kim, J. I., Han, J. H., Kang, M. J. and Kwon, T. W. (2000) The hypoglycemic effect of Chongkukjang in normal rats. In: *The Proceedings of The Third International Soybean Processing and Utilization Conference* (Ed: Saio, K.), pp. 171-172. The Japanese Society for Food Science and Technology and ISPUC-III, Tsukuba, October 15-20, 2000, Japan.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. *Journal of Molecular Biology* **16**: 111-120.

Nagai, T., Nishimura, K., Suzuki, H. Banba, Y., Sasaki, H. and Kiuchi, K. (1994) Isolation and char-

acterization of *Bacillus subtilis* strain producing natto with strong Umami-taste and high viscosity. *Nippon Shokuhin Kogyo Gakkaishi* **41**: 123-128.

Nikkuni, S. (1997) Natto, kinema and thua-nao: traditional non-salted fermented soybean foods in Asia. *Farming Japan* **31 (4)**: 27-36.

Page, R. D. M. (1997) TREEVIEW program version 1.5.1. University of Glasgow, UK.

Saito, H. and Miura, K. (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochemica et Biophysica Acta* **72**: 619-629.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic tree. *Molecular Biology and Evolution* **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 beans (African Soumbala). *International Journal of Food Microbiology* **77**: 175-186.

Schillinger, U. and Lücke, F. K. (1987) Identification of lactobacilli from meat and meat products. *Food Microbiology* **4**: 199-208.

Tamang, J. P. (2001) Kinema. *Food Culture* **3**: 11-14.

Tamang, J. P. and Nikkuni, S. (1996) Selection of starter culture for production of kinema, fermented soybean food of the Himalaya. *World Journal of Microbiology and Biotechnology* **12 (6)**: 629-635.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.