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Isolation, Identification, and Characterization of *Bacillus* strains from the Traditional Korean Soybean-fermented Food, *Chungkookjang*

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A total of 45 bacterial strains were isolated from the traditional Korean soybean-fermented food, *Chungkookjang*. Among these strains, seven strains were selected and identified based on morphological, physiological, and biochemical characteristics, as well as phylogenetic analysis using 16S rDNA sequences. All strains were Gram-positive, aerobic, motile, oxidase-positive, rod-shaped, and endospore-forming bacteria, and produced extracellular enzymes such as amylase, cellulase, lipase, protease, and xylanase. The isolates were grown in the presence of 0-11% (w/v) NaCl. Growth was optimal at pH 6-9 and at temperatures of 30-45°C. According to VITEK automicrobic system tests and supplementary tests, the isolates were similar to several species of the genus *Bacillus*. The phylogenetic analysis of seven bacterial strains based on comparisons of 16S rDNA sequences, revealed that the strains were closely related to *Bacillus* species. The identification of strains that produced surfactin was also carried out, based on PCR screening of the *sfp* gene. Among the seven isolated strains, six yielded a surfactin-positive result with PCR.

Key words: Bacillus, Chungkookjang, *extracellular enzyme, identification, surfactin*

Chungkookjang is a traditional Korean food made of cooked whole soybeans fermented with *Bacillus* strains such as *B. subtilis*, *B. pumilus*, *B. amyloliquefaciens*, and *B. licheniformis* [Kim *et al.*, 2003b; Kwon *et al.*, 2004; Kim *et al.*, 2003a; Lee *et al.*, 2005b]. The quality of *Chungkookjang* varies considerably with the soybean variety, fermenting microorganisms, fermentation process, and the ratio of additional ingredients [Lee *et al.*, 2005b]. During fermentation, *Bacillus*-derived enzymes hydrolyze soybean proteins, oligosaccharides, and lipids into easily digestible free amino acids, free sugars, and fatty acids, respectively [Ashiuchi *et al.*, 2001; Lee and Kim, 2004]. *Chungkookjang* is also known to have various functional properties such as fibrinolytic activity, antimicrobial activity, immunohistochemical activity, and hypocholesterolemic effects [Ko *et al.*, 2004; Kim *et al.*, 2004; Kang *et al.*, 2003; Lee *et al.*, 2005a]. Thus, *Chungkookjang* has not only a nutritional role, but also various physiological effects that are beneficial to human health.

Bacillus species are good secretors of proteins and metabolites. Most species of *Bacillus* strains have a high capacity to secrete a variety of extracellular enzymes such as amylase, arabinase, cellulase, lipase, protease, and xylanase, and these enzymes play important roles in many biotechnological processes [Sinchaikul *et al.*, 2002; Cherry and Fidantsef, 2003]. However, the properties of their enzymes vary from strain to strain.

In particular, various *Bacillus* strains produce one of the most potent lipopeptide biosurfactants, surfactin which shows high surface activity and therapeutic potential [Besson and Michel, 1992; Peypoux *et al.*, 1999]. Surfactin demonstrates antimicrobial, antiviral, antitumor, and hemolytic activities [Hwang, 2005; Kim *et al.*, 1998].

Biosurfactants are biological surface-active compounds produced by certain bacteria, yeasts, and fungi, with a wide variety of chemical structures such as lipopeptides, glycolipids, fatty acids, and polymer types [Desai and Banat, 1997; Rosenberg and Ron, 1999]. They are amphiphilic molecules consisting of both hydrophilic and hydrophobic regions, allowing them to aggregate at

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interfaces between different fluid phases such as oil/water or water/air interfaces [Karanth *et al.*, 1999]. Biosurfactants have important advantages over chemically synthesized surfactants, such as their biodegradability, low toxicity, activity under a variety of conditions (extremes of pH, temperature, and salinity), ecological acceptability, structural diversity, and ability to be produced from renewable and cheaper substrates. Due to their activity features, biosurfactants have a broad range of industrial and commercial applications such as enhanced oil recovery, crude oil drilling, lubrication, and the bioremediation of pollutants, as well as the cosmetic, health care and food processing industries [Banat *et al.*, 2000; Mulligan, 2005].

In this study, we performed an investigation for *Bacillus* strains that are biotechnologically interesting for industrial application, by isolating and identifying seven strains from *Chungkookjang* that produced extracellular enzymes, and then, among them, we detected surfactin-producing strains using blood agar plates and PCR methods.

Materials and Methods

Preparation of *Chungkookjang*. To prepare the *Chungkookjang*, carefully selected soybeans were washed and then soaked in a 3-fold amount of water at room temperature for 12 h. Subsequently, the water was decanted, and the soybeans were autoclaved at 121°C for 30 min, and cooled to 50°C. The cooled soybeans were fermented for 48 h at 45°C in a *Chungkookjang* incubator (Wooho Oriental Medical Center, Busan, Korea).

Culture media and growth conditions. For all experiments, the microbial strains were routinely cultivated at 37°C in Luria-Bertani (LB) medium composed of 1% NaCl, 1% tryptone, and 0.5% yeast extract. To observe the growth profiles, a single colony of an isolate was pre-inoculated in LB broth and incubated at 37°C under shaking conditions until the stationary phase. The freshly grown cells were inoculated (1%) into LB broth and cultured under various temperature (between 25°C and 60°C with an interval of 5°C) and pH (between pH 4 and pH 13 with an interval of 1) conditions. Salt tolerance experiments were performed on LB broth with NaCl, in various concentrations [between 0% and 13% (w/v) with an interval of 1%] at 37°C. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (BioPhotometer 6131, Eppendorf, Hamburg, Germany).

Isolation of bacterial strains from *Chungkookjang*. For bacterial isolation, 90 mL of sterilized saline solution (NaCl 0.85%, w/v) was added to 10 g of *Chungkookjang* sample and homogenized for 2 min in a blender (Happy dream DMP-900, Dreamtech, Bucheon, Korea). This sample solution was diluted serially ten-fold with 0.85% NaCl (10^1-10^8), spread onto LB agar plates, and incubated for 16 h at 37°C. The bacterial colonies were isolated according to their different morphological characteristics. Individual colonies were selected randomly and purified by single colony isolation after triple re-streaking on LB agar medium.

Identification of selected bacterial isolates. Identification of the *Chungkookjang* bacterial isolates was performed based on their morphological, physiological, and biochemical characteristics, as described in Bergey's Manual of Systematic Bacteriology [Claus and Berkeley, 1986]. The morphological characteristics of the isolates were observed with a phase-contrast microscope (Olympus biological microscope, high-class system BX51) using cells grown on LB media. The VITEK system (VITEK 2 Compact 60, bioMerieux Inc., Hazelwood, MO, USA) was used to further characterize the *Bacillus* isolates via enzymatic, acidification, alkalinization, assimilation, inhibition, and precipitation tests. The experiments were performed in duplicate with an inoculum subcultured at least once under the same test conditions.

Extraction of DNA. To determine the 16S rDNA sequences and detect the surfactin-producing strains, each strain's genomic DNA was extracted with the GeneAll Genomic DNA Extraction Kit (GeneAll™ GenEx™ Genomic Sx, GeneAll Biotechnology, Korea) according to the manufacturer's directions.

PCR amplification and sequencing of 16S rDNA. To confirm the identities of the isolates, PCR amplification and sequencing of the 16S rRNA gene were performed. The 16S rRNA genes were PCR-amplified from the genomic DNA using the bacterial universal primer set of 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGC TAC CTT GTT ACG ACT T-3'), which were also used for sequencing [Lane, 1991; Weisburg *et al.*, 1991]. The PCR reaction mixture consisted of 5 μL of 10× PCR reaction buffer (100mM Tris-HCl pH 8.3, 500 mM KCl, 11 mM $MgCl_2$, and 0.1% gelatin), 1 μL of 10 mM dNTP mix, 1 μL of 10 pM primers, 2.5 μL of genomic DNA template, and 2.5 U REDTaq™ DNA Polymerase (Sigma) prepared in a final 50 μL reaction volume. Amplification was carried out with the Program Temp Control System (model PC708, ASTEC, Japan). The thermal cycling program was as follows: initial denaturation at 95°C for 5 min, which was followed by 30 cycles of denaturation at 95°C for 30 sec, primer annealing at 56°C for 30 sec, extension at 72°C for 90 sec, and a final extension at 72°C for 10 min. The amplified PCR products were analyzed by 0.8% (w/v) agarose gel

electrophoresis and purified using Wizard^{R} SV Gel and the PCR Clean-Up System (Promega), according to the manufacturer instructions. Sequencing of the amplified DNA fragments was performed using the services of Solgent Co., Ltd. (Korea, http://www.solgent.co.kr).

Phylogenetic analysis. The acquired sequences were used for a gene homology search, with the 16S rDNA sequences available in the public databases from BLAST (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD, USA), and were identified to the generic level [Altschul *et al.*, 1997; Benson *et al.*, 1999]. Using the CLUSTAL-X Multiple Sequence Alignment Program (Strasburg, France), the 16S rDNA sequences of the isolated strains were aligned with sequences of related organisms obtained from GenBank [Thompson *et al.*, 1997]. Phylogenetic analysis was performed with PHYLIP [Felsenstein, 1985], and a phylogenetic trees was constructed via the neighbor-joining method using the TreeView program [Saitou and Nei, 1987]. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed.

GenBank accession number. The partial 16S rDNA sequences of the isolated strains JK-1, JKA-25, JKB-8, JKC-13, JKC-14, JKC-15, and JKC-16 were deposited in GenBank under accession numbers DQ846632, EF427891, EF517122, EF517121, EF517120, EF517119, and EF427892, respectively.

Detection of extracellular enzyme production. The extracellular enzyme production of the isolates was observed via direct agar plate assay. The assay allows for enzymatic activity detection by measuring the diameter of the transparent, or coloured halozone that is produced in the culture medium. Protease activity was observed by the direct appearance of hydrolysis halos surrounding the colonies after growth on 1% (w/v) skim milk-LB agar plates [Ali Amoozegar *et al.*, 2006]. Cellulase and xylanase activities were detected after growth on either 1% (w/v) carboxymethyl cellulose (CMC)-LB agar plates or 1% (w/v) oat spelt xylan-LB agar plates, followed by a Congo red test [Teather and Wood, 1982]. Amylase activity was detected after growth on 2% (w/v) soluble starch-LB agar plates by a hydrolysis zone around the colony, using iodine staining [Agrawal *et al.*, 2005]. Lipase producing strains were detected under UV illumination using the MUF-staining technique [Diaz *et al.*, 1999].

Detection of biosurfactant activity on blood agar plates. The surfactin production of the isolated strains was assayed on blood agar plates using the method of Nakano *et al.* (1988) and Feignier *et al.* (1995), which was modified as follows. Colonies from the seven isolated strains were transferred onto sheep blood agar plates (Asan Pharmaceutical, Korea) and incubated for 48-72 h at 37°C. The surfactin producers produced a hemolysis zone around the colony.

PCR method for detecting surfactin-producing strains. The *sfp* gene is responsible for the ability of *B. subtilis* to produce the lipopeptide biosurfactant, surfactin. Therefore, *Bacillus* spp. surfactin-producing strains can be rapidly, simply, and efficiently detected by PCR methods [Nakano *et al.*, 1992; Hsieh *et al.*, 2004]. In this study, PCR, with primer pairs specific to the *sfp* gene, was used to detect the isolated strains. The *sfp* 675 bp fragment, corresponding to the *B. subtilis sfp* gene (GenBank accession no. X63158) at positions 167-841, was amplified from the genomic DNA by PCR, using two oligonucleotide primers: *sfp*-f (5'-ATG AAG ATT TAC GGA ATT TA-3') and *sfp*-r (5'-TTA TAA AAG CTC TTC GTA CG-3'). Twenty-five cycles of PCR were run at 94°C for 1 min, 46°C for 30 sec, and 72°C for 1 min. The PCR products were then analyzed by 2% agarose gel electrophoresis.

Results and Discussion

Isolation of bacterial strains from *Chungkookjang*. A total of 45 bacterial strains were isolated from the traditional Korean soybean-fermented food *Chungkookjang*. We finally selected seven bacterial isolates based on their morphological features, and extracellular enzyme-producing abilities. The seven strains were designated as JK-1, JKA-25, JKB-8, JKC-13, JKC-14, JKC-15, and JKC-16, and used for further experiments.

Morphological characteristics of the isolated strains. All of these isolates were found to be aerobic, Gram-positive, motile, rod-shaped, and endospore-forming bacteria, in overnight culture on LB medium at 37°C. The shapes of the colonies were also examined on the plates. After incubation for 16 h at 37°C on LB agar medium, the strain colonies were white-colored, circular, and flat in appearance. Most of the isolates were wavy, opaque, and non-glistening, but strain JKA-25 had a smooth to wavy edge, was opaque to clear, and glistening.

Microscopic observations. The morphological features were confirmed using a phase-contrast microscope. Under microscopy, the morphology of the isolated strains was revealed as rod-shaped cells (data not shown). Micrograph images indicated that strains JK-1, JKA-25, JKC-13, and JKC-16 had a similar long (0.7-0.9 × 2.0-2.3 μm) and straight rod shape; while strain JKC-15 was shortest (0.8-0.9 × 1.8-2.0 μm), and strains JKB-8 and JKC-14 were longest (0.8-1.0 × 2.5-2.8 μm).

Cultural characteristics of the isolated strains. The optimum growth conditions of the strains were also

Table 1. Cultural characteristics of the isolated strains

Characteristics \ Strain No.	JK-1	JKA-25	JKB-8	JKC-13	JKC-14	JKC-15	JKC-16
Temperature (°C) for:							
Optimum growth	35-40	45	35	35	35-45	30-40	40
Growth range	25-50	25-55	25-50	25-50	25-50	25-50	25-50
pH for optimum:							
Optimum growth	6-9	9	8	8-9	7-8	6-8	8
Growth range	5-9	6-12	6-10	6-10	6-11	5-10	6-10
Growth in NaCl at (%, w/v):							
0.0	+	+	+	+	+	+	+
1.0	+	+	+	+	+	+	+
3.0	+	+	+	+	+	+	+
5.0	+	+	+	+	+	+	+
7.0	+	+	+	+	+	+	+
9.0	+	+	+	+	+	+	+
10.0	+	+	+	+	+	+	+
11.0	+	+	+	+	+	+	+
12.0	-	+	-	-	+	-	+
13.0	-	-	-	-	+	-	+
Anaerobic growth:	-	-	-	-	-	-	-

[a]+, positive; [b]-, negative.

investigated. Most of the strains grew between 25°C and 50°C, with optimal growth at approximately 30-40°C (Table 1). In particular, strains JKA-25 and JKC-14 grew well at temperatures of 45°C and 35-45°C, respectively. The initial pH range for cell growth was measured from pH 4 to pH 13. The isolates were able to grow at pH ranging from 5 to 12, with optimum growth occurring at the initial pH values of 6-9. The JKA-25 strain was relatively stable in a wide range of pH values, from 6-12. The optimal pH values were 6-9 for JK-1, and 9 for JKA-25. As shown in Table 1, all isolates grew well at the various concentrations of NaCl, ranging from 1 to 11% (w/v). Strains JKC-14 and JKC-16 were able to grow in the presence of up to 13% (w/v) NaCl.

Physiological and biochemical characteristics of the isolated strains. The physiological and biochemical characteristics of the isolated strains are indicated in Table 2. All the tested strains had both oxidase and catalase activity. The biochemical characteristics, which were investigated with the help of a VITEK system, varied within the strains tested.

Among the strains, JKA-25 was positive in the acidification test for amygdalin, cyclodextrine, N-acetyl-glucosamine, and salicin; the enzymatic test for alkalin phophatase, L-leucine arylamidase, and β-mannosidase; and the inhibition test for polymyxin-β resistance. JKA-25 was negative in the acidification test for inulin; the enzymatic test for α-galactosidase; the assimilation test for D-gluconate, D-melibiose, D-raffinose, and D-turanose; and was weakly active for putrescine, which was different from the other six strains. On the other hand, ala-phe-pro-arylamidase was produced only by strain JKC-16, L-aspartate arylamidase was produced only by strain JKB-8, assimilation of D-galactose and novobiocin resistance were observed only by strain JK-1, and resistance to kanamycin and oleandomycin were only determined in strain JKC-13.

The isolates were further characterized and identified by the VITEK system. The biochemical data obtained using the VITEK system were similar to those of *B. subtilis* (JK-1 exhibited a 94% matching level), *B. licheniformis* (JKA-25 exhibited a 95% matching level), and *B. subtilis*/*B. amyloliquefaciens* (JKB-8, JKC-13, JKC-14, JKC-15, and JKC-16 exhibited 86%, 86%, 95%, 93%, and 86% matching levels, respectively). Based on their morphological, cultural, physiological, and biochemical characteristics, all strains were similar to several species of the genus *Bacillus*.

16S rDNA sequence similarity and phylogenetic analysis. Molecular identification of the isolated strains was carried out based on 16S rDNA sequence analysis. The 1.4 kb sequences obtained from the strains were aligned with all the presently available 16S rDNA sequences in the GenBank database. As a result, a phylogenetic tree was constructed using the neighbor-joining method, and is shown in Fig. 1. Phylogenetic

Table 2. Physiological and biochemical characteristics of the isolated strains

Characteristics \ Strain No.	JK-1	JKA-25	JKB-8	JKC-13	JKC-14	JKC-15	JKC-16		JK-1	JKA-25	JKB-8	JKC-13	JKC-14	JKC-15	JKC-16
Physiological characteristics								γ-glutamyl transferase	+	+	-	-	+	+	+
oxidase activity	+	+	+	+	+	+	+	Alkalinisation test:							
catalase activity	+	+	+	+	+	+	+	argin dihydrolase	+	+	+	+	+	+	-
Biochemical characteristics[a]								arginine	+	+	+	+	+	+	+
Acidification test:								lactate	-	+	+	+	+	+	-
amygdalin	-	+	-	-	-	-	-	pyruvate	+	(+)	+	-	+	+	+
cyclodextrine	-	+	-	-	-	-	-	urease	+	+	+	+	+	+	-
D-galactose	-	-	-	-	-	-	-	Assimilation test:							
D-glucose	+	+	+	+	+	+	+	2-keto-D-gluconate	+	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	+	-	acetate	+	+	+	+	+	+	+
D-mannose	-	+	+	+	-	-	-	amygdalin	+	+	-	-	-	-	-
D-melezitose	-	-	-	-	-	-	-	arbutine	+	+	-	-	-	-	(-)
D-ribose	+	-	(+)	-	+	+	+	citrate	+	-	+	+	+	+	-
D-tagatose	-	-	-	-	-	-	-	D-cellobiose	+	+	-	-	-	-	-
D-trehalose	-	+	+	+	-	+	+	D-galactose	+	-	-	-	-	-	-
glycogene	-	(-)	-	-	-	-	(-)	D-galacturonate	+	+	+	+	+	+	+
inulin	+	-	+	+	+	+	+	D-gluconate	+	-	+	+	+	+	+
lactose	-	-	-	-	-	-	-	D-glucose	+	+	+	+	+	+	+
L-rhamnose	-	-	-	-	-	-	-	D L-lactate	-	-	-	-	-	-	-
maltose	+	+	+	+	-	-	-	D-mannose	-	+	+	+	-	-	-
maltotriose	-	+	+	+	-	-	-	D-melezitose	-	-	-	-	-	-	-
methyl-D-xyloside	-	-	-	-	-	-	-	D-melibiose	+	-	+	+	+	+	+
methyl-β-D-glucopyranoside	+	+	+	+	+	+	-	D-raffinose	+	-	+	+	+	+	+
methyl-β-D-glucopyranoside	-	+	+	+	-	-	-	D-sorbitol	+	+	+	+	+	+	+
myo-inositol	+	+	+	+	+	+	+	D-trehalose	+	+	+	+	+	+	+
N-acetyl-glucosamine	-	+	-	-	-	-	-	D-turanose	+	-	+	+	+	+	+
palatinose	+	+	+	+	+	+	(+)	D-xylose	(+)	+	-	-	(+)	(+)	-
pullulan	-	-	-	-	-	-	-	erythritol	-	-	-	-	-	-	-
raffinose	+	-	+	+	-	-	-	gentobiose	+	+	-	-	+	-	+
salicin	-	+	-	-	-	-	-	glucuronate	+	+	+	+	+	+	+
sorbitol	+	-	+	+	+	-	+	glycerol	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	lactose	-	-	-	-	-	-	-
xylose	-	-	-	-	-	-	-	L-arabinose	+	+	+	+	+	+	+
Enzymatic test:								L-glutamate	+	+	+	+	+	+	+
alanine arylamidase	+	-	-	+	+	(+)	+	L-malate	+	+	+	+	+	+	+
ala-phe-pro-arylamidase	-	-	-	-	-	-	+	L-proline	-	-	-	-	-	-	-
alkalin phosphatase	(-)	+	-	-	-	-	-	L-rhamnose	-	-	-	-	-	-	-
ellman	+	+	+	+	+	+	+	L-sorbose	-	-	-	-	-	-	-
glycine arylamidase	(-)	+	-	(-)	+	-	-	methyl-α-D-glucopyranosidase	+	+	+	+	+	+	(-)
L-aspartate arylamidae	(-)	-	+	(-)	(-)	-	(-)	N-acetyl-glucosamine	+	(-)	+	+	+	+	-
L-leucine arylamidase	-	+	-	-	-	-	-	nitrate	-	+	+	+	-	-	+
L-lysine arylamidase	-	-	-	-	-	-	-	putrescine	-	(+)	-	-	-	-	-
L-proline arylamidase	-	-	-	-	-	-	-	sucrose	+	+	+	+	+	+	+
L-pyroglutamic acid arylamidase (pyrase)	+	+	+	+	+	+	+	xylitol	-	-	-	-	-	-	-
L-pyrrolydonyl arylamidase	+	+	+	+	+	+	+	Precipitation test:							
phenylalanine arylamidase	+	+	+	+	+	+	+	esculin hydrolysis	+	+	+	+	+	-	+
phosphatidylinositol phospholipase C	-	-	-	-	-	-	-	tetrazolium RED	-	+	-	(-)	+	+	(-)
phosphoryl cholin	-	-	-	-	-	-	-	Inhibition test:							
PNP-N-acetyl-β-D-galactosaminidase 1	-	-	-	-	-	-	-	bacitracin resistance	+	+	+	+	+	+	-
tyrosine arylamidase	+	+	+	+	+	+	+	growth in 6.5% NaCl	+	+	+	+	+	+	+
α-galactosidase	+	-	+	+	+	+	+	kanamycin resistance	-	-	-	+	-	-	-
α-glucosidase	+	+	+	+	+	+	+	novobiocin resistance	+	-	-	-	-	-	-
α-mannosidase	-	-	-	-	-	-	-	oleandomycin resistance	-	-	-	+	-	-	-
β-mannosidase	-	+	-	-	-	-	-	optochin resistance	+	+	+	+	+	+	+
β-N-acetyl-glucosaminidase	-	-	-	-	-	-	-	plomixin-β resistance	-	+	-	+	-	-	-
β-xylosidase	+	-	-	-	+	+	-	polymyxin-β resistance	-	+	-	-	-	-	-

[a]VITEK system was used; [b]+, positive; [c]-, negative; [d](+), weakly positive; [e](-), weakly negative.

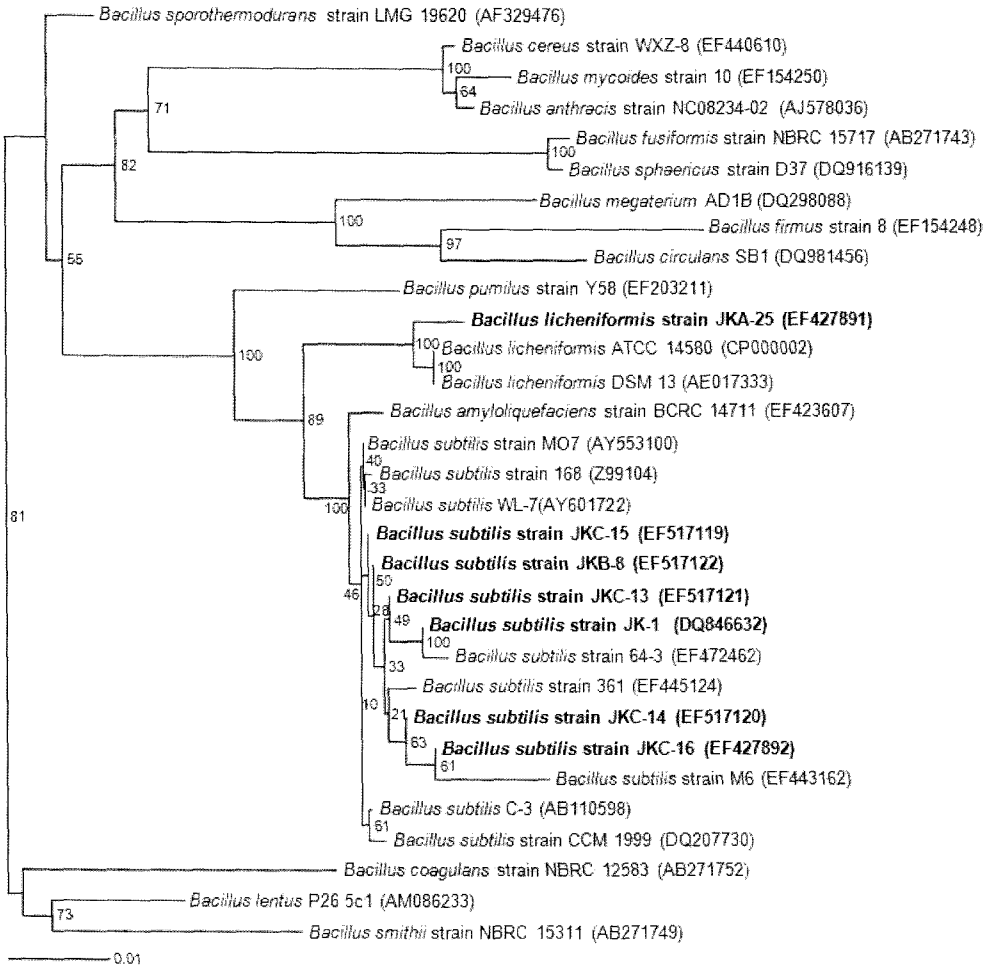

Fig. 1. Phylogenetic tree based on partial 16S rDNA sequences, showing the relationship between the isolated strains and other species belong to the genus *Bacillus*. The accession numbers are in parentheses. The tree was constructed using the CLUSTAL-X and neighbour-joining method. Scale bar corresponds to 0.01 subscriptions per nucleotide position. Numbers at nodes indicate levels of bootstrap support (%) determined from 100 resampled data.

analysis using the 16S rDNA sequences indicated that all the bacterial isolates belonged to the genus *Bacillus*. From the branching pattern of the tree, six isolates (JK-1, JKB-8, JKC-13, JKC-14, JKC-15, and JKC-16) were determined to be close relatives to *B. subtilis*, and they had 99% 16S rDNA sequence-similarity with the following strains: *B. subtilis* strain WL-7 (Genbank accession number AY601722), *B. subtilis* No. 66 (Genbank accession number AB110598), *B. subtilis* subsp. *subtilis* str. 168 (Genbank accession number Z99104), and *B. subtilis* strain CCM 1999 (Genbank accession number DQ207730). The partial 16S rDNA sequence of strain JKA-25 showed 99% identity with the sequences of *B. licheniformis* ATCC14580 (Genbank accession number CP000002) and *B. licheniformis* DSM13 (Genbank accession number AE017333). In the phylogenetic tree (Fig. 1), strain JKA-25 was most closely related to *B. licheniformis*.

Therefore, the isolated strains were identified as *Bacillus* species based on their morphological, cultural, physiological, and biochemical characteristics, as well as 16S rDNA sequence analyses. Accordingly, we named the isolated strains *B. subtilis* strain JK-1, *B. licheniformis* strain JKA-25, *B. subtilis* strain JKB-8, *B. subtilis* strain JKC-13, *B. subtilis* strain JKC-14, *B. subtilis* strain JKC-15, and *B. subtilis* strain JKC-16. The results revealed that *B. subtilis* and *B. licheniformis* were the main fermenting microorganisms in this *Chungkookjang*.

Extracellular enzyme production of the isolated strains. We also determined whether the seven isolated strains produced various extracellular enzymes on agar plates with selection medium. The results of extracellular enzyme production are presented in Fig. 2. Most of strains exhibited halozones around their colonies on LB agar plates containing starch, CMC, tributyrin, skim milk, or oat spelt xylan, indicating that they produced extracellular enzymes such as amylase, cellulase, lipase, protease, or xylanase. JKA-25 was the only isolate

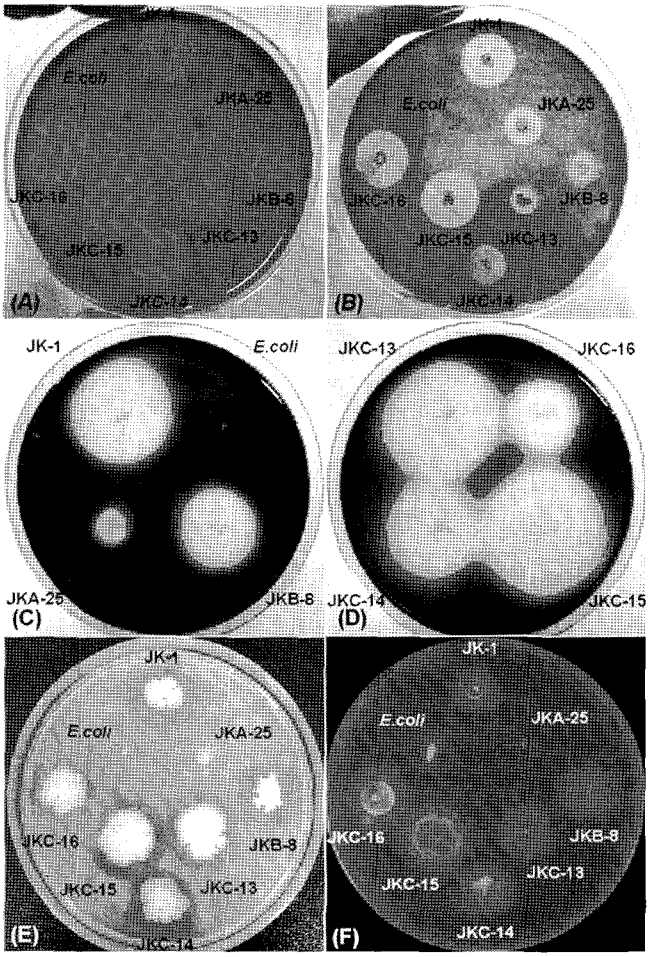

Fig. 2. Degradation of various substrates in agar plates by extracellular enzyme released from the isolated strains. The cells were grown on LB agar plates supplemented with various substrates [2% (w/v) soluble starch, 1% (w/v) CMC, 3% (w/v) tributyrin, 1% (w/v) skim milk, 0.5% (w/v) oat spelt xylan] at 37°C for 16 h. *Escherichia coli* is shown as controls. (A) xylanase activity (B) cellulase activity (C), (D) amylase activity (E) protease activity (F) lipase activity.

without the ability to degrade xylan, skim milk, or tributyrin.

Detection of biosurfactant-producing strains on blood agar plates. Carrillo *et al.* (1996) reported an association between hemolysis and biosurfactant-production, and recommended a simple and easy method to test for biosurfactant activity. Thus, we detected the biosurfactant-producing isolates using a blood agar lysis method. Of the seven strains tested, six strains (JK-1, JKB-8, JKC-13, JKC-14, JKC-15, and JKC-16) had a blood hemolysis zone, but strain JKA-25 did not produce any clear zones around its colonies on blood agar plates, as can be seen in Fig. 3. Hsieh *et al.* (2004) reported that red blood cell lysis is related to the production of a highly surface-active compound by *Bacillus* spp. strains.

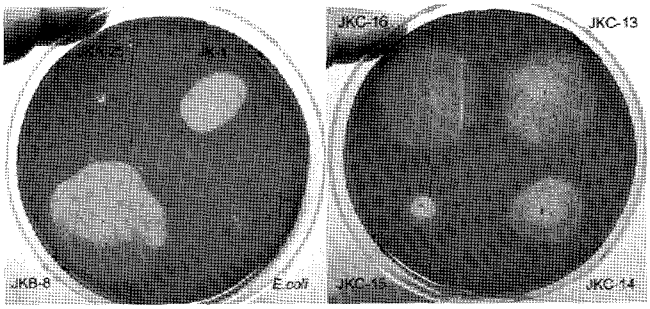

Fig. 3. Surfactin production assayed on sheep blood agar plates. Surfactin production was detected by a hemolysis zone around the colony. *Escherichia coli* is shown as a control.

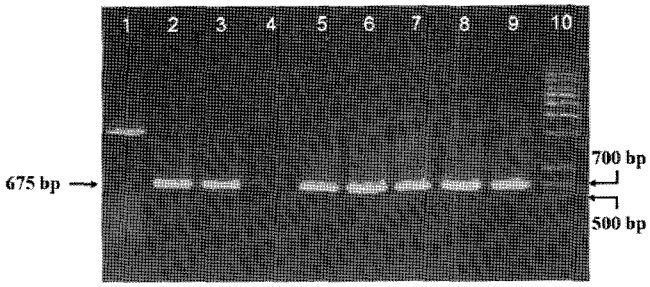

Fig. 4. PCR product profiles of the strains tested. 1, 50 bp molecular weight marker (Promega); 2, *Bacillus subtilis* subsp. *subtilis* strain 168; 3, *B. subtilis* strain JK-1; 4, *B. licheniformis* strain JKA-25; 5, *B. subtilis* strain JKB-8; 6, *B. subtilis* strain JKC-13; 7, *B. subtilis* strain JKC-14; 8, *B. subtilis* strain JKC-15; 9, *B. subtilis* strain JKC-16; 10, molecular weight marker (Vivagen).

Detection of surfactin-producing strains based on PCR screening. The *sfp* gene is an essential component of peptide synthesis systems, and also plays a role in the regulation of surfactin biosynthesis gene expression [Nakano *et al.*, 1992]. Besides strain JKA-25, six other strains were positive to specific primers of the *sfp* gene (Fig. 4) and were evaluated for surfactin production. All strains had the same hemolysis zone pattern (Fig. 3) and PCR results (Fig. 4).

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References

Agrawal M, Pradeep S, Chandraraj K, and Gummadi SN (2005) Hydrolysis of starch by amylase from *Bacillus* sp. KCA102: a statistical approach. *Process Biochem* **40**, 2499-2507.

Ali Amoozegar M, Zahra Fatemi A, Reza Karbalaei-heidari H, and Reza Razavi M (2006) Production of an extracellular alkaline metalloprotease from a newly isolated, moderately halophile, *Salinivibrio* sp. strain AF-2004.

Microbiol Res (Article) 1-9.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389-3402.

Ashiuchi M, Kamei T, Baek DH, Shin SY, Sung MH, Soda K, Yagi T, and Misono H (2001) Isolation of *Bacillus subtilis* (*chungkookjang*), a poly-γ-glutamate procedure with high genetic competence. *Appl Microbiol Biotechnol* **57**, 764-769.

Banat IM, Makkar RS, and Cameotra SS (2000) Potential commercial applications of microbial surfactants. *Appl Microbiol Biotechnol* **53**, 495-508.

Benson DA, Boguski MS, Lipman DJ, Ostell J, Oullette BFF, Rapp BA, and Wheeler DL (1999) GenBank. *Nucleic Acids Res* **27**, 12-17.

Besson F and Michel G (1992) Biosynthesis of iturin and surfactin by *Bacillus subtilis*: Evidence for amino acid activating enzymes. *Biotechnol Lett* **14**, 1013-1018.

Carrillo PG, Mardaraz C, Pitta-Alvarez SI, and Giulietti AM (1996) Isolation and selection of biosurfactant-producing bacteria. *World J Microbiol Biotechnol* **12**, 82-84.

Cherry JR and Fidantsef AL (2003) Directed evolution of industrial enzymes: an update. *Curr Opin Biotechnol* **14**, 438-443.

Claus D and Berkeley RCW (1986) Genus *Bacillus*. In *Bergey's Manual of Systematic Bacteriology*, pp. 1105-1139, Williams & Wilkins, Baltimore.

Desai JD and Banat IM (1997) Microbial production of surfactants and their commercial potential. *Microbiol Mol Biol Rev* **61**, 47-64.

Diaz P, Prim N, and Pastor FIJ (1999) Direct fluorescence-based lipase activity assay. *Biotechniques* **27**, 697-699.

Feignier C, Besson F, and Michel G (1995) Studies on lipopeptide biosynthesis by *Bacillus subtilis*: isolation and characterization of iturin-, surfactin + mutants. *FEMS Microbiol Lett* **127**, 11-15.

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

Hsieh FC, Li MC, Lin TC, and Kao SS (2004) Rapid detection and characterization of surfactin-producing *Bacillus subtilis* and closely related species based on PCR. *Curr Microbiol* **49**, 186-191.

Hwang MH, Lim JH, Yun HI, Rhee MH, Cho JY, Hsu WH, and Park SC (2005) Surfactin C inhibits the lipopolysaccharide-induced transcription of interleukin-1 and inducible nitric oxide synthase and nitric oxide production in murine RAW 264.7 cells. *Biotechol Lett* **27**, 1605-1608.

Kang MJ, Kim JI, and Kwon TW (2003) Effect of *Chongkukjang* on blood glucose and lipid profile in neonatal streptozotocin-induced diabetic rats. *Food Sci Biotechnol* **12**, 544-547.

Karanth NGK, Deo PG, and Veenanadig NK (1999) Microbial production of biosurfactants and their importance. *Curr Sci* **77**, 116-126.

Kim K, Jung SY, Lee DK, Jung JK, Park JK, Kim DK, and Lee CH (1998) Supression of inflammatory responses by surfactin, a selective inhibitor of platelet cytosolic phopholipase A_2. *Biochem Pharmacol* **55**, 975-985.

Kim SS, Lee JH, Ahn YS, Kim JH, and Kang DK (2003a) A fibrinolytic enzyme from *Bacillus amyloliquefaciens* D4-7 isolated from *Chungkook-jang*: It's characterization and influence of additive on thermostability. *Korean J Microbiol Biotechnol* **31**, 271-276.

Kim Y, Cho JY, Kuk JH, Moon JH, Cho JI, Kim YC, and Park KH (2004) Identification and antimicrobial activity of phenylacetic acid produced by *Bacillus licheniformis* isolated from fermented soybean, *Chungkook-Jang*. *Curr Microbiol* **48**, 312-317.

Kim YS, Jung HJ, Park YS, and Yu TS (2003b) Characteristics of flavor and functionality of *Bacillus subtilis* K-20 *Chungkukjang*. *Korean J Food Sci Technol* **35**, 475-478.

Ko JH, Yan JP, Zhu L, and Qi YP (2004) Identification of two novel fibrinolytic enzymes from *Bacillus subtilis* QK02. *Comp Biochem Physiol C Toxicol Pharmacol* **137**, 65-74.

Kwon HY, Kim YS, Kwon GS, Kwon CS, and Sohn HY (2004) Isolation of immuno-stimulating strain *Bacillus pumilus* JB-1 from *Chungkukjang* and fermentational characteristics of JB-1. *Korean J Microbiol Biotechnol* **32**, 291-296.

Lane DJ (1991) 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115-175, John Wiley & Sons, New York.

Lee CH, Yang EI, Song GS, Chai OH, and Kim YS (2005a) *Chongkukjang* mucilage stimulates immunohistochemical activities of gastrointestinal tract in rats. *Food Sci Biotechnol* **14**, 813-817.

Lee EJ and Kim JK (2004) Characteristics of taste components of *Chongkukjang* fermented with *Bacillus subtilis*. *Food Sci Biotechnol* **13**, 572-575.

Lee MY, Park SY, Jung KO, Park KY, and Kim SD (2005b) Quality and functional characteristics of *Chungkukjang* prepared with various *Bacillus* sp. isolated from traditional *Chungkukjang*. *J Food Sci* **70**, M191-196.

Mulligan CN (2005) Environmental applications for biosurfactants. *Environ Pollut* **133**, 183-198.

Nakano MM, Corbell N, Besson J, and Zuber P (1992) Isolation and characterization of sfp: A gene that functions in the production of the lipopeptide biosurfactant, surfactin, in *Bacillus subtilis*. *Mol Gen Genet* **232**, 313-321.

Nakano MM, Marahiel MA, and Zuber P (1988) Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. *J Bacteriol* **170**, 5662-5668.

Peypoux F, Bonmatin JM, and Wallacb J (1999) Recent trends in the biochemistry of surfactin. *Appl Microbial Biotechnol* **51**, 553-563.

Rosenberg E and Ron EZ (1999) High- and low-molecular-mass microbial surfactants. *Appl Microbiol Biotechnol* **52**, 154-162.

Saitou N and Nei M (1987) The neighbor-joining method: a

new method for reconstructing phylogenetic tree. *Mol Biol Evol* **4**, 406-425.

Sinchaikul S, Sookkheo B, Topanuruk S, Juan HF, Phutrakul S, and Chen ST (2002) Bioinformatics, functional genomics, and proteomics study of *Bacillus* sp. *J Chromatogr B Analyt Technol Biomed Life Sci* **771**, 261-287.

Teather RM and Wood PJ (1982) Use of congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl Environ Microbiol* **43**, 777-780.

Thompson, JD, Gibson TJ, Plewniak F, Jeanmougin F, and Higgins DG (1997) The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876-4882.

Weisburg WG, Barns SM, Pelletier DA, and Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697-703.