J. Appl. Biol. Chem. **51**(5), 285-291 (2008) **Article**

Characterization of Potential Probiotics *Bacillus subtilis* CS90 from Soybean Paste (*Doenjang*) and Its Antimicrobial Activity against Food-borne Pathogens

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Received September 20, 2008; Accepted November 14, 2008

A potential probiotics bacterial strain, CS90, was isolated from Korean soybean paste (*doenjang*). The strain CS90 showed antimicrobial activity against food-borne pathogenic bacteria including *Salmonella enterica, Salmonella enteritids, Salmonella typhymurium, Bacillus cereus, Listeria ivanovii, Listeria. monocytogenes, Sthaphylococcus aureus,* and *Sthaphylococcus epidermidis* and showed a significant survival rate of 35.7 to 57.8% under the artificial gastric acidic condition (pH 2 to 3). The strain CS90 was classified as *Bacillus subtilis* based on morphological, physiological, chemotaxonomic features and phylogenetic analysis based on 16S rDNA sequence and designated as *B. subtilis* CS90. *B. subtilis* CS90 can be used as a potential probiotics.

Key words: *probiotics, food-borne pathogenic bacteria, antimicrobial activity, phylogenetic analysis,* Bacillus subtilis *CS90*

Recently, interest on the probiotics has been increasing [Hong *et al.*, 2005]. Probiotics are generally defined as live microbial feed supplements that can benefit the host by improving its intestinal balance [Fuller, 1989] through immunomodulation, competitive exclusion of gastrointestinal pathogens, and secretion of antimicrobial compounds, which suppress the growth of harmful bacteria [Due Ie *et al.*, 1989; Fuller, 1989]. Although studies have demonstrated a direct probiotics effect of *Bacillus* spores, preliminary studies with the poultry provided evidence of a competitive exclusion of *Escherichia coli* O78:K80 by *Bacillus subtilis* [La Ragion *et al.*, 2001], and a number of studies have demonstrated that harmful bacteria are suppressed by various *Bacillus* spore formers [Due Ie *et al.*, 1989; Vaseeharan *et al.*, 2003; Barbosa *et al.*, 2005]. Pinchuk *et al.* [2001] reported the characterization of an antibiotic produced by the *B. subtilis* strain found in the commercial product Biosporin, known to inhibit the growth of *Helicobacter pylori*. In addition, a large number of *Bacillus* products are used as 'novel foods' or as dietary supplements with claims of 'enhancing' the well-being of the user [Hong *et al.*, 2005].

Soybeans and soy-containing foods are excellent and inexpensive sources of dietary proteins, carbohydrates, vitamins, and minerals such as isoflavone, soyasaponin, γ-aminobutyric acid, trypsin inhibitor, and phytic acid [Anderson *et al.*, 1995; Sung *et al.*, 2004]. In particular, fermented soybean foods, one of a major source of protein of Koreans, such as *doenjang* (soybean paste), *kanjang* (soybean source), and *cheonggukjang* (soybean cook) have served as condiments for thousands of years [Choi *et al.*, 2002]. In the production of Korean traditional fermented soybean foods, *Bacillus* species notably *B. subtilis, B. licheniformis*, and *B. pumilus* are predominantly used [Yoo *et al.*, 1999; Yun *et al.*, 2003; Kim *et al.*, 2004; Paik *et al.*, 2004; Kang *et al.*, 2005; Joo *et al.*, 2007].

In the present study, the isolation of potential probiotics and the classification of the isolate were performed by the morphological, physiological, chemotaxonomic features, and phylogenetic analyses.

Materials and Methods

Isolation of *Bacillus* sp. Korean traditional fermented soybean foods, *cheonggukjang*, *deonjang*, and *kanjang*,

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Abbreviations: TSA, tryptic soy agar; TSB, tryptic soy broth; MIC, minimal inhibitory concentration; GI, gastrointestinal

doi:10.3839/jabc.2008.044

were collected from a local market (Jinju, Korea). Collected samples were diluted with 0.85% NaCl, and then 0.1 mL of the diluted suspension was plated on the TSA (Difco, Detroit, MI, USA) plates. After the plates were incubated at 37°C for 48 h, a single colony was selected as a pure isolate on each TSA plate. Isolates were stored at −80°C with 20% sterile glycerol until needed.

Antibacterial activity. Twelve food-borne pathogenic bacteria including *Escherichia coli* KCTC 1682, *Salmonella enterica* KCTC 12456, *Salmonella enteritids* KCTC 12400, *Salmonella typhimurium* KCTC 1925, *Shigella flexineri* KCTC 2008, *Shigella sonnei* KCTC 2518, *Bacillus cereus* KCTC 1012, *Listeria innocula* KCTC 3586, *L. ivanovii* KCTC 3444, *Listeria monocytogenes* KCTC 3569, *Staphylococcus aureus* KCTC 1621, and *Staphylococcus epidermidis* KCTC 3958 were used as test organisms. These test organisms were grown on the TSA plates at 37°C.

The isolated *Bacillus* strains were cultured in 250 mL Erlenmeyer flasks containing 40 mL of TSB (Difco) at 30°C for 48 h in a gyratory shaking incubator at 150 rpm. The cultured cells were removed from the broth by centrifugation at 15,000 rpm for 10 min, and the resultant supernatants were sterilized by filtration through a 0.20-μm non-pyrogenic membrane (Minisart, Sartorius, Goettingen, Germany). Fifty micro-liters of the sterilized supernatant was applied on to the 8-mm sterile paper disk, and the disk was placed on the TSA plate which was spread with a test organism for antibacterial activity assay. After 48 h of incubation at 37°C, the antibacterial activity was measured as the diameter of the clear zone formed.

Determination of tolerances of acid, artificial gastric acid, and bile. The method of Hyronimus *et al.* [2000] was modified to determine the tolerances to acid, artificial gastric acid, and bile acid. For determination of acid tolerance, the isolated *Bacillus* strains were grown in TSB at 30°C for 48 h, and diluted to 10^6 CFU mL^{-1} in fresh TSB adjusted to different pH values (2, 2.5, and 3) with hydrochloric acid (3 M). Diluted cells were incubated for 3 and 6 h at 37°C. After incubation, the cells were serially diluted in a phosphate buffer (0.1 M, pH 6.2) to neutralize the medium acidity, and the viable cells on the TSA plate were counted after 48 h of incubation at 30°C. The survival rate was calculated as the percentage of *Bacillus* sp. colonies grown on the TSA plate compared to the initial bacterial concentration.

For determination of the artificial gastric acid tolerance, an artificial gastric juice was created by adding 1% pepsin (Sigma, St. Louis, MO, USA) to the TSB at different pH values (2, 2.5, and 3). Cells grown in TSB at 30°C for 48 h was diluted to 10^6 CFU mL^{-1} in fresh artificial gastric acids adjusted to different pH values (2, 2.5, and 3). Diluted cells were incubated for 3 and 6 h at 37°C. After incubation, the cells were serially diluted in phosphate buffer (0.1 M, p H 6.2) to neutralize the medium acidity. The viable cells were then incubated on the TSA plate for 48 h at 30°C and counted. The survival rate was calculated as the percentage of *Bacillus* sp. colonies grown on the TSA plate compared to the initial bacterial concentration.

For determination of bile tolerance, 15 μL of condiment the cells grown in TSB at 30°C for 48 h (equivalent to 10^6 CFU m^{-1}) was spotted onto the TSA plates containing oxgall bile (Sigma) at different concentration (0.1-1% w/v). The plates were incubated at 30°C for 5 days. The MIC of the bile for *Bacillus* strain was determined as the lowest concentration totally inhibiting the cell growth at the spots as judged from visual examination of the spots.

Morphological and physiological characteristics. Cell morphology was examined by light microscopy after Gram staining. Flagellum type was determined by transmission electron microscopy (JEM 1010, JEOL, Tokyo, Japan) using preparations negatively stained with 1% phosphotungstic acid. Phenotypic characterization was carried out by standard methods using API50CHB kits (bioMérieux, Montalieu Vercieu, France). The methods described by Cowan *et al.* [1965] were used for the following physiological tests: catalase and oxidase activities, and hydrolyses of gelatin, casein and starch. Growths, in response to various concentrations of NaCl and temperature range, were determined in TSB as a basal medium.

Chemotaxonomy and DNA base composition. The biomass for cellular fatty acid analysis was prepared from a culture grown on a TSA plate for 24 h at 37°C. Fatty acid methyl esters were prepared using the method described in the manual of the MIDI Microbial Identification System. The resultant esters were separated using a gas chromatograph fitted with a phenylmethyl silicone-fused silica capillary column (25 m×0.2 mm; Hewlett Packard, Palo Alto, CA, USA). DNA base composition was measured by reversed-phase high pressure liquid chromatography after the DNA was hydrolyzed into nucleosides [Tamaoka *et al.*, 1984].

Sequencing of the 16S rDNA and phylogenetic tree analysis. The 16S rDNA of strain CS90 was amplified by PCR. PCR amplification was performed as follows: 30 cycles of denaturation at 90°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The PCR primers used to amplify 16S rDNA fragments were the bacterial-specific, 5'-CGGAGAGTTTGATCCTGG-3' (1BF, forward) and 5'-TACGGCTACCTTGTTACGAC-3' (2BR, reverse) [Cho *et al.*, 2007]. Amplified 16S rDNA fragments were used as the sequencing templates.

Nucleotide sequences were determined by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer, Norwalk, CT, USA). Assembly of the nucleotide sequences was performed with the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada). All reference sequences were obtained from the National Center for Biotechnology Information (NCBI) and Ribosomal Database Project (RDP) databases. The 16S rDNA similarity values were determined based on the alignments, and the evolutionary distances were calculated. Phylogenetic analysis was performed using neighbor-joining methods [Saito *et al.*, 1987]. Bootstrap analysis was performed using data re-sampled 1,000 times using the DNAMAN analysis system (Lynnon Biosoft). Nucleotide sequence data reported for the 16S rDNA of strain CS90 are available in the GenBank database under the accession number FJ210720.

Results and Discussion

Antimicrobial activity against food-borne pathogenic bacteria. A total of 26 samples were collected from soybean fermented foods, *cheonggukjang*, *deonjang*, and *kanjang*. Approximately 150 strains of *Bacillus* sp. were isolated from these samples. Antibacterial activities of the isolated *Bacillus* strains against twelve food-borne pathogenic bacteria were tested. Five strains, designated as *Bacillus* sp. CS9-4, CS45, CS62, CS72, and CS90, showed a broad range of antibacterial activities (Table 1). The *Bacillus* sp. CS9-4, CS62, and CS90 were isolated from *deonjang* and *Bacillus* sp. 45 and *Bacillus* 72 were obtained from the *kanjang*. There were many reports that *Bacillus* sp. has plays important roles in the Korean traditional fermented soybean foods [Yoo *et al.*, 1999; Yun *et al.*, 2003; Kim *et al.*, 2004; Paik *et al.*, 2004; Kang *et al.*, 2005; Joo *et al.*, 2007; Ryu *et al.*, 2007].

All five *Bacillus* strains showed antibacterial activities against *S. enteritids*, *S. typhymurium*, and *S. aureus*. *Bacillus* sp. CS9-4 and CS62 showed inhibitory activities against Gram-negative bacteria such as *E. coli*, *S. enterica*, *S. enteritids*, *S. typhymurium*, *S. flexineri*, and *S. sonnei*, known as the main pathogens causing diarrhea in human [Li *et al.*, 2005; Gast *et al.*, 2006]. The production of an antimicrobial agent is considered to be one of the major functions of the probiotics. Therefore, this function may be one of the principal criteria applied for the screening of potential probiotics strain [Chang *et al.*, 2001; Hong *et al.*, 2005; Guo *et al.*, 2006].

Several *Bacillus* species secrete antibiotics, some of which are lipopeptide derivatives functioning as a surface-active agent [Das *et al.*, 2008]. These lipopeptides including iturin [Maget-Dana *et al.*, 1994], surfactin [Cho *et al.*, 2003], fengycin [Vanittanakom *et al.*, 1986],

Table 1. Antibacterial activity of isolated strains from the different soybean fermented foods when incubated with twelve food-borne pathogenic bacteria

Food-borne pathogenic microorganisms	Inhibitory zone[1] (mm)				
	Isolated strain				
	CS9-4	CS45	CS62	CS72	CS90
Gram-negative bacteria					
E. coli KCTC[2] 1682	12.4	-[3]	21.3	9.7	-
S. enterica KCTC 12456	19.2	9.8	24.5	-	9.6
S. enteritids KCTC 12400	28.4	13.2	29.1	24.7	12.7
S. typhymurium KCTC 1925	27.8	22.4	28.6	25.6	13.4
S. flexineri KCTC 2008	24.1	12.8	23.9	12.9	-
S. sonnei KCTC 2518	26.2	14.4	27.0	25.9	-
Gram-positive bacteria					
B. cereus KCTC 1012	30.1	25.6	29.8	-	26.1
L. innocula KCTC 3586	14.3	-	-	-	-
L. ivanovii KCTC 3444	24.8	11.8	23.1	-	23.9
L. monocytogenes KCTC 3589	25.7	21.9	14.3	-	25.6
S. aureus KCTC 1621	22.6	12.7	23.2	13.6	14.3
S. epidermidis KCTC 3958	-	11.6	22.7	11.7	12.8

[1]The antifungal activity was estimated by measuring the diameter of the clear zone (including paper disks, 8 mm diameter) of growth inhibition.
[2]KCTC, Korea Collection for Type Culture
[3]-, no inhibition

Table 2. Survival rate of isolated strains from different soybean fermented foods under acidic condition after 3 and 6 h of incubation[1)]

Isolated strain	Incubation time (h)	Survival rate (%)		
		pH		
		2	2.5	3
CS9-4	3	5.2	11.2	14.1
	6	0.3	5.2	5.2
CS45	3	11.6	25.0	23.0
	6	5.0	16.6	20.0
CS62	3	16.8	25.7	29.7
	6	8.9	16.8	24.8
CS72	3	16.9	27.6	30.7
	6	6.1	10.0	23.4
CS90	3	42.5	55.3	59.5
	6	23.4	38.3	46.8

[1)]Each isolated *Bacillus* sp. was tested in triplicate for its tolerance in acidified TSB.

Table 3. Survival rate of isolated strains from different soybean fermented foods under artificial gastric acidic condition after 3 and 6 h of incubation[1)]

Isolated strain	Incubation time (h)	Survival rate (%)		
		pH		
		2	2.5	3
CS9-4	3	2.4	6.1	9.1
	6	0	0	3.1
CS45	3	8.6	14.9	17.3
	6	0	1.8	4.7
CS62	3	12.1	18.1	23.6
	6	1.1	1.8	4.5
CS72	3	11.8	19.2	25.6
	6	1.0	2.1	4.2
CS90	3	35.7	42.8	57.8
	6	14.3	21.4	28.5

[1)]Each isolated *Bacillus* sp. was tested in triplicate for its tolerance in artificial gastric acidified TSB.

plipastatin [Tasuge *et al.*, 1996] lichenysin [Yakimov *et al.*, 1995], pumilacidin [Naruse *et al.*, 1990] and di- and tripeptides such as bacilysin [Walker *et al.*, 1970] synthesize non-ribosomally. In particular, *B. subtilis* produces the antimicrobial lipopeptides such as surfactin, fengycin, iturin, bacillomycins, and mycosubtilins [Vater *et al.*, 2002; Kim *et al.*, 2007].

Survival rates of *Bacillus* sp. under gastrointestinal tract conditions. Survival rates of several *Bacillus* strains under acidic conditions are shown in Table 2. In general, survival rates of the isolated *Bacillus* sp. decrease with the lapse of time under acidic condition. One of the tested *Bacillus* sp., CS90, showed a significantly high survival rate. Survival rates of the strain CS90 were 35.7 (at pH 2), 42.8 (at pH 2.5), and 57.8% (at pH 3), after 3 h of incubation. Other *Bacillus* sp. such as CS9-4, CS45, CS62, and CS72 showed somewhat lower survival rates.

Survival rates of the isolated *Bacillus* strains under artificial gastric conditions are shown in Table 3. A slightly lower degree of survival rates were observed under the artificial gastric condition compared with the acidic condition (Table 2). Low survival rates were observed from *Bacillus* strains CS9-4, CS45, CS62, and CS72: 2.4 to 12.1 after 3 h and 0 to 1.1 after 6 h at pH 2; 6.1 to 19.2 after 3 h and 0 to 2.1 after 6 h at pH 2.5; and 9.1 to 25.6 after 3 h and 3.1 to 4.2% after 6 h at pH 3, respectively. On the other hand, *Bacillus* sp. CS90 showed the significantly higher survival rates of 35.7 at pH 2.0, 42.8 at pH 2.5, and 57.8% at pH 3.0 after 3 h. All strains were able to withstand bile concentration higher than 1.0% (data not shown).

Bacillus probiotics differ in many characteristics from those based on lactic acid bacteria [Chang *et al.*, 2001; Kim, 2005]. Whereas lactobacilli represent a normal resident GI microflora of humans, the saprophytic GI bacteria of *Bacillus* genera belong only to the transitory GI bacteria. Thus, the use of *Bacillus* products raised a number of questions, including their safety. Over the past three decades, this genus has expanded to accommodate more than 100 species [Sorokulova *et al.*, 2008]. However, only a few of these species are used as probiotics for human: *B. subtilis, B. licheniformis, B. calusii, B. coagulans, B. cereus, B. pumilus, Bacillus laterosporus*, as well as some invalid species named as *B. toyoi* and *B. polyfermenticus* [Due Ie *et al.*, 2004; Urdaci *et al.*, 2004]. On the contrary, Sorokulova *et al.* [2008] recently reported that *B. subtilis* strain may be considered as non-pathogenic and safe for human consumption.

Identification of potential probiotic *Bacillus* sp. CS90. The morphological, biochemical, and physiological characteristics of the strain CS90 were analyzed (Table 4). The cells, incubated for 24 h at 37°C, were Gram-positive and rod-shaped, measuring 0.6 to 0.8×2 to 3 μm. The strain was facultatively anaerobic and grew at 10 to 50°C with an optimum growth temperature of 30 to 37°C. The strain grew in the presence of 0 to 15%(w/v) NaCl and had catalase and oxidase activities, but no urease activity. Skim milk, gelatin, cellulose, and starch were hydrolyzed by CS90. Reactions for the oxidation and fermentation of carbohydrates as the sole carbon sources are shown in Table 4. The cellular fatty acid profile of strain CS90 revealed the presence of large amounts of saturated and branched fatty acids, and the majority of the fatty acids had the iso-$C_{15:0}$ (25.44%) and anteiso-$C_{15:0}$

Table 4. Phenotypic characteristics of *Bacillus subtillis* CS90

Characteristics	Reaction	Characteristics	Reaction
Morphology		Rhamnose	-
Shape	Rod	Dulcitol	-
Gram stain	+	Inositol	+
Cell dimension (μm)	0.6 to 0.8×2 to 3	Mannitol	+
Flagellation	+	Sorbitol	+
Swarming on soft TS agar	+	α methyl-D-mannoside	-
Endospore formation	+	α methyl-D-glucoside	+
Physiological properties		*N* acetyl glucosamine	-
Aerobic growth	+	Amygdaline	+
Growth at temperature (°C)	10 to 50	Arbutine	+
Growth in NaCl (%)	<15	Esuline	+
Biochemical characteristics		Salicine	+
Oxidase activity	+	Cellobiose	+
Catalase activity	+	Maltose	+
Urease activity	-	Lactose	-
Hydrolysis of		Melibiose	+
Skim milk	+	Saccharose	+
Casein	-	Trehalose	+
Gelatin	+	Inuline	-
Cellulose	+	Melezit ose	-
Starch	+	D-raffinose	+
Carbohyrates		Amidon	+
Glycerol	+	Glycogen	+
Ertythritol	-	Xylitol	-
D-arabinose	-	β gentiobiose	+
L-arabinose	+	D-turanose	-
Ribose	+	D-lyxose	-
D-xylose	+	D-tagatose	-
L-xylose	-	D-fucose	-
Adonitol	-	L-fucose	-
β methyl-xyloside	-	D-arabitol	-
Galactose	-	L-arabitol	-
D-glucose	+	Gluconate	-
D-fructose	+	2 ceto-gluconate	-
D-mannose	+	2 ceto-gluconate	-
L-sorbose	-	DNA G+C content (mol%)	53.5

[1)]Symbol: +, positive reaction; -, negative reaction.

(39.84%) (data not shown). The G+C content of the DNA was 53.5 mol%. Morphological, biochemical, and physiological analyses clearly demonstrated that strain CS90 is a member of the genus *Bacillus*. The chemotaxonomic data, i.e. G+C content of DNA and the anteiso-$C_{15:0}$ as the major cellular fatty acid also fall within the ranges found in the *Bacillus* species. In addition, results of the physiological properties indicated strain CS90 differs from the other species of *Bacillus* in the carbohydrate utilization pattern. Not enough properties were examined in this study to compare the bacterium in more detail with the description available in the Bergey's Manual.

A complete 16S rDNA sequence of strain CS90 (1,517 bp; accession number; FJ210720) was determined. A phylogenetic tree constructed using the sequence data showed that strain CS90 grouped within the evolutionary radiation, encompassing the genus *Bacillus* and occupying a distinct phylogenetic position within this genus. The level of 16S rRNA similarity between strain GS01 and the *Bacillus* species ranged from 91.4 to 99.6%. The highest 16S rDNA sequence similarity (99.7%) was

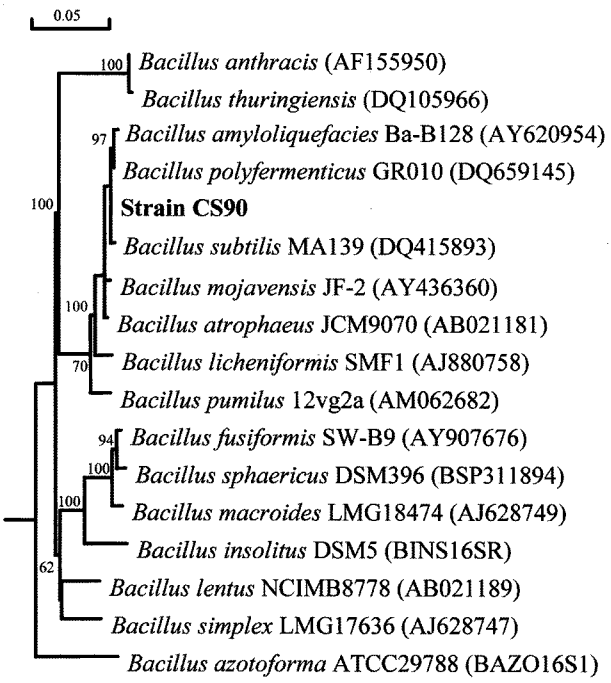

Fig. 1. Phylogenetic relationships of strain CS90 and other closely related *Bacillus* sp. based on 16S rDNA. Number above each node us the confidence level (%) generated from 1,000 bootrap trees. The scale bar is in fixed nucleotide substitutions per sequences position.

observed between strain CS90 and *B. subtillis* MA139 (Fig. 1). Broad-range amplification and sequencing of 16S rDNA templates are useful for the characterization of many species. There are several advantages to using the ribosomal gene sequences for taxonomic purposes. DNA is more easily isolated and manipulated than RNA and can be readily sequenced, thus making it a more appropriate choice for routine analysis. Regions of the nucleotide conservation allow the selection of a broad-range or universal oligonucleotide primers for PCR amplification of virtually all prokaryotic organisms, assuming that they contain similar nucleotide sequences at the corresponding primer-binding sites [Cho *et al.*, 2007]. The present phylogenetic study clearly established that strain CS90 is closely related to *B. subtillis*. To the best of our knowledge, this is the first report on the isolation of a potential probiotic *Bacillus subtilis* from *doenjang*.

In conclusion, the present study identified the probiotic activity of *B. subtilis* CS90 against the food-borne pathogenic bacteria such as *S. enteric, S. enteritids, S. typhymurium, B. cereus, L. ivanovii, L. monocytogenes, S. aureus,* and *S. epidermidis. B. subtilis* CS90 can be used as a probiont against the food-borne pathogenic bacteria.

Acknowledgments

This work was supported by a grant (No. 08-C2-15-002) from the Academic-industrial Cooperative Research Program of the Small & Medium Business Administration (SMBA), Republic of Korea. This work was supported by 2008 Post Doctoral Course Program of Sunchon National University, Republic of Korea.

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