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Characterization of *Bacillus polyfermenticus* KJS-2 as a Probiotic

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The identification and characterization of *Bacillus polyfermenticus* KJS-2 (*B. polyfermenticus* KJS-2) was conducted using TEM, an API 50CHB kit, 16S rDNA sequencing, a phylogenetic tree, and catalase and oxidase testing. The conversion rate of glucose to lactic acid by *B. polyfermenticus* KJS-2 was found to be 60.71±4.9%. In addition, treatment of *B. polyfermenticus* KJS-2 with artificial gastric juice (pH 2.0) and bile acid (pH 6.5) for 4 h resulted in a final viability of 140±7.9% and 108±3.5%, respectively. Finally, the results of adhesion experiments using Caco-2 cells revealed that the adherence of *B. polyfermenticus* KJS-2 to Caco-2 cells was approximately 65±0.6%.

Keywords: *Bacillus polyfermenticus* KJS-2, probiotic, characterization, industrial application

Probiotics are defined as live microbial food ingredients that have a beneficial effect on human health [25]. Lactobacilli, streptococci, bifidobacteria, enterococci, and bacilli species are the bacteria most often used in the production of probiotics. At the very least, a good probiotic should posses the following 5 characteristics: (1) the probiotic should promote growth or increased resistance to disease; (2) the probiotic should be non-toxic and non-pathogenic; (3) the probiotic should be present as viable cells, preferably in large numbers, although there is no known minimum effective dose; (4) the probiotic should be capable of surviving and metabolizing in the gut environment; and (5) the probiotic should be stable and capable of remaining viable for long periods of time under storage and field conditions [8]. Appropriate applications of probiotics have been shown to improve intestinal microbial balance, thereby leading to improved nutritional absorption and reduced pathogenic problems in the gastrointestinal tract [5, 8, 9, 20, 23]. Probiotics have also been applied to a wide range of aquatic organisms, including salmon and shrimp infected with pathogenic bacteria [2, 7, 26].

In 1933, Terakado isolated several endospore-forming rods from the air, four of which were used to make the commercial mixed strain product known as Bispan [18]. These four strains have other morphology. Bispan strains are described in the Japanese Pharmacopoeia as amylolytic bacilli, together with *Bacillus subtilis* (*B. subtilis*) and *Bacillus mesentericus* [18]. However, Bispan strains are distinct from *B. subtilis* strains because they are capable of producing a larger amount of acetic and lactic acids from glucose and lactose, respectively [14, 15, 18]. Bispan has long been used for the treatment of chronic intestinal disorders, since the live strains in the form of active endospores can successfully reach the target intestine in humans and animals [13].

Although *B. polyfermenticus* KJS-2 is one of the Bispan strains, there is currently little information regarding this organism. In this study, *B. polyfermenticus* KJS-2 was isolated from Bispan and then characterized by 16S rDNA sequencing, phylogenetic analysis, catalase and oxidase testing, and TEM observation. In addition, it was evaluated for its ability to metabolize lactic acid, its stability in artificial gastric juice and bile acid, and by a Caco-2 cell *in vitro* adhesion assay. Furthermore, *B. polyfermenticus* KJS-2 was evaluated using an API 50CHB kit.

The producer strain, *B. polyfermenticus* KJS-2 KCCM10769P, was maintained at −70°C in tryptic soy broth (TSB, Difco) containing 20% (v/v) glycerol. Working cultures were propagated in TSB with shaking at 37°C. All reagents were purchased from Sigma (St. Louis, MO, U.S.A.). All microorganisms were obtained from the Korea Culture Center of Microorganisms (KCCM) or the Korean Collection for Type Cultures (KCTC).

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The total genomic DNA of *B. polyfermenticus* KJS-2 was prepared using exponential-phase cells that were cultured in TSB using a salting out procedure for bacterial genomic DNA preparation [16]. Oligonucleotide primers described in Thomas [28] were used to amplify the gene encoding 16S rDNA. PCR amplification was performed in a master mix with a final reaction volume of 50 μl that contained 10 μl of mixed deoxynucleoside triphosphate (2 mM), 5 μl of 10× n*Taq*-Tenuto reaction buffer (Enzynomics Co., Korea), 3 μl of DMSO (dimethyl sulfoxide; Sigma), 1 μl of chromosomal DNA (100 ng/μl), 1 μl of each primer (20 pmol/μl), 28 μl of ddH_2O that had been sterilized by autoclaving, and 1 μl of n*Taq*-Tenuto DNA polymerase (5 U/μl; Enzynomics Co., Korea). The total mixture was overlaid with mineral oil and then subjected to the following conditions: denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. The purified PCR products were then cloned into the pGEM T-easy vector (Promega, U.S.A.), after which 16S rDNA sequencing was performed by Genotec Co. (South Korea) using an ABI Prism 377 DNA sequencer. Nucleotide sequence similarities were then determined using the 16S bacterial cultures Blast Server [1]. A neighbor-joining phylogenetic tree was constructed using MEGA version 2.1 [17].

Catalase and oxidase tests were conducted using the method described by Hanker and Robin [11]. The strains used for the catalase test were *Staphylococcus aureus* ATCC 25923 (positive control), *Streptococcus pyogenes* ATCC 12344 (negative control), and *B. polyfermenticus* KJS-2. Briefly, a loopfull of 16 h-old-culture grown on agar was transferred to a glass test tube containing 0.5 ml of distilled water and mixed thoroughly. Hydrogen peroxide (3%) solution (0.5 ml) was then added and the presence of bubbles was taken to indicate the presence of catalase.

The strains used for oxidase test were *Pseudomonas aeruginosa* ATCC 27853 (positive control), *Escherichia coli* ATCC 25922 (negative control), and *B. polyfermenticus* KJS-2. For the oxidase test, the organisms were grown on media, after which two-to-three drops of the reagent *N,N,N',N'*-tetramethyl-*p*-phenylenediamine were added to the surface of each organism. A positive test was indicated by a change in color to pink, maroon, and then black within 10–30 sec. A negative test was indicated by a light pink coloration or the absence of coloration.

The carbohydrate metabolism of all presumptive *B. polyfermenticus* KJS-2 isolates was determined using API 50CHB strips (bioMérieux S.A., Marcy-l'Étoile, France). Briefly, isolates were grown on tryptic soy agar (TSA) at 30°C for 18–24 h. The colonies were then suspended in 2 ml of sterile 0.85% saline solution with a concentration sufficient to correspond to McFarland No. 2. Next, 0.1 ml of this suspension was diluted in 10 ml of API 50 CHB medium. The strips were then inoculated, incubated for 24 h at 30°C, and read after 24 h. The results were scored according to the manufacturer's instructions and the emerging biochemical profile was identified using the APILAB software, Version 4.0, 2007 (bioMérieux S.A., Marcy-l'Étoile, France) [27].

To evaluate the lactic acid production, *B. polyfermenticus* SCD and *B. polyfermenticus* KJS-2 were grown on TSA at 37°C for 16 h. Precultures were then prepared in medium containing 4 ml of PSG medium (10 g polypeptone S, 10 g yeast extract, 20 g glucose, 35 g K_2HPO_4, per liter) [22]. Two ml of this culture was then used to inoculate a 500-ml screw-capped shake flask containing 200 ml of PSG production medium under anaerobic conditions at 200 rpm. The amount of lactic acid was determined by HPLC (Agilent Technologies, 1100 series, U.S.A.) in conjunction with a refractive index detector under the following conditions: column, MetaCarb 87 H (MetaChem Technologies Inc., Torrance, CA, U.S.A.); column temperature, 25°C; solvent for elution, 0.1 N H_2SO_4 solution; flow rate, 0.5 ml/min. Prior to measuring the optical purity, the samples were filtered through a 0.2-μm pore size polytetrafluoroethylene (PTFE) membrane (JP020, Advantec, Tokyo) to remove extra molecules.

B. polyfermenticus KJS-2 was plated onto the agar medium to induce spore formation. The cells were then suspended in sterile distilled water, followed by centrifugation at 6,000 rpm for 10 min at 4°C. The cells were then washed once with sterile distilled water, after which the pellets were fixed with 0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde at 4°C for 2 to 4 h. The specimens were then postfixed in 1% osmium tetroxide for 2 h, dehydrated in a graded alcohol series, treated with propylene oxide, and embedded in Epon 812. The resultant blocks were then cut using an LKB ultramicrotome (Nova, Sweden), after which thin-sectioned specimens were mounted on 200 mesh copper grids and stained with uranyl acetate plus lead citrate. The prepared specimens were then observed through a transmission electron microscope (JEM 1200EX II; JEOL, Japan).

Approximately 10^8 CFU/g of spored *B. polyfermenticus* KJS-2 was suspended in an equal volume of (i) TSB with a pH that had been adjusted to final 2.0 using 1 M HCl (for acid challenge) or (ii) TSB (final pH 6.5) containing 0.3% (w/v) Oxgall. The suspended cells were then incubated aerobically at 37°C for 4 h. At each hour during the incubation period, aliquots of the samples were plated on TSA with an inoculation level of 1% (v/v). The samples were then incubated aerobically at 37°C for 18 h [3].

Caco-2 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco, Invitrogen Corporation, U.S.A.) containing 25 mM glucose, 1.0 mM sodium pyruvate, 10% heat inactivated fetal bovine serum (Gibco, Invitrogen Corporation, U.S.A.), 1% nonessential amino acids solutions, and antibiotics (100 U of penicillin

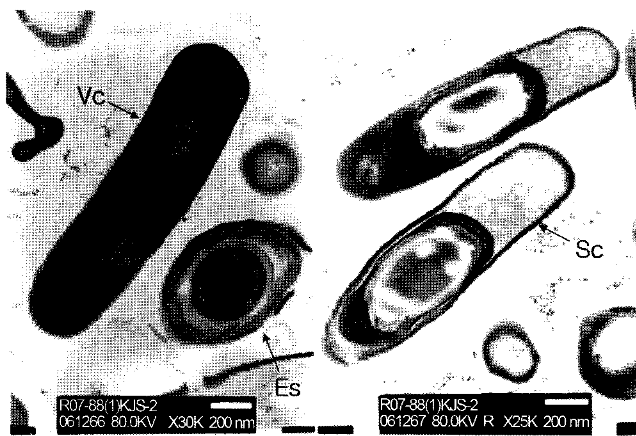

Fig. 1. Transmission electron microscopy observation of *B. polyfermenticus* KJS-2. VC, vegetative cell; SC, spore coat; ES, endospore.

G per ml and 100 μg of streptomycin sulfate per ml). The Caco-2 cells were grown under standard conditions (37°C, 5% CO_2) and the medium was replaced every two days. Monolayers of Caco-2 cells, which were used in the adherence assay, were prepared by seeding 6-well tissue culture dishes (Falcon type 3046; Becton Dickinson Labware, Oxnard, CA, U.S.A.) with 9.5×10^4 cells per 4 ml of culture medium. Next, 3.1×10^6 CFU/ml and 2.2×10^6 CFU/ml of *B. polyfermenticus* KJS-2 and *B. polyfermenticus* SCD were added to the Caco-2 cells in the culture dishes. The samples were then incubated for 2 h and then washed 3 times with sterile PBS (pH 7.4), after which the number of adhered *B. polyfermenticus* KJS-2 was determined by plating the diluted *B. polyfermenticus* KJS-2 suspensions on TSA [10].

B. polyfermenticus KJS-2 was isolated from Bispan. The colony shape of *B. polyfermenticus* KJS-2 grown on TSA plates was visibly distinguished from those of *B. polyfermenticus* SCD and other *Bacillus*. Specifically, *B. polyfermenticus* KJS-2 produced opaque, dark-yellow colonies with a round and flat shape.

Transmission electron microscopy revealed that *B. polyfermenticus* KJS-2 was characterized as a rod bacterium with a length that ranged from 0.5 to 2 μm that formed endospores ranging in size from 0.4 to 0.6 μm (Fig. 1). The border of the exosporium, the cortex, the sporederm, and the spore coat of the organism was clear and smooth with no breakage [12].

Table 1 shows the catalase and oxidase activities of the bacteria. Note the correspondence between effervescence and color formation. Although the lowest catalase activity was measured after 16 h of cultivation, the catalase activity was obviously correlated with the accumulation of its specific substrate (H_2O_2) [11]. In addition, *B. polyfermenticus* KJS-2 was found to be oxidase positive.

The sequence of 16S rDNA from *B. polyfermenticus* KJS-2 differed from that of *B. polyfermenticus* SCD

Table 1. Direct visual estimation of bacterial catalase and oxidase activities.

Organisms	Effervescence[a]	Color[b]
Positive control		
Staphyloccus aureus ATCC 25923	+++	
Pseudomonas aeruginosa ATCC 27853		+++
Negative control		
Streptococcus pyogenes ATCC 12344	–	
Escherichia coli ATCC 25922		–
Test strain		
Bacillus polyfermenticus KJS-2	+	+++

[a]Catalase activity, decomposition of H_2O_2.
[b]Oxidase activity, oxidation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine reagent.

(Accession No. AY149473, GenBank) and from *Bacillus subtilis* 168 (*B. subtilis* 168, Accession No. NC000964, GenBank) by three nucleotides out of 1,550. *B. polyfermenticus* KJS-2 occupied the position of *Bacillus amyloliquefaciens* (Accession No. CP000560, GenBank) in the phylogenetic tree (>99%).

Biochemical characterization of *B. polyfermenticus* KJS-2 was conducted using an API 50CHB kit. *B. polyfermenticus* KJS-2 utilized glucose, fructose, mannose, mannitol, sorbitol, α-methyl-D-glucoside, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, and sucrose as carbon sources. The percentage of positive results for the carbohydrate tests of our *B. polyfermenticus* KJS-2 isolates was within the range reported by others [21]. However, the number of carbon sources utilized by *B. polyfermenticus* KJS-2 was relatively smaller than the number of carbon sources utilized by *B. subtilis* 168 and *B. polyfermenticus* SCD (Table 2). The API 50CHB strips were analyzed using the Apiweb software to identify corresponding *Bacillus* species. *B. polyfermenticus* KJS-2 was found to be 62% homologous to *B. subtilis* and *Bacillus amyloliquefaciens*, whereas it was found to be 97.4% and 99.9% homologous to *B. subtilis* 168 and *B. polyfermenticus* SCD, respectively. In the case of carbohydrate utilities obtained from the API 50CHB strips, *B. polyfermenticus* KJS-2 could be clearly distinguished from *B. subtilis* 168 and *B. polyfermenticus* SCD (Table 2). Therefore, *B. polyfermenticus* KJS-2 was identified as a new strain isolated from Bispan and deposited in the KCCM under Accession No. KCCM10769P.

In the anaerobic culture, the conversion rate of glucose to lactic acid by *B. polyfermenticus* KJS-2 and *B. polyfermenticus* SCD was 60.71±4.9% and 58.57±5.9% after 24 h, respectively. If the pH, dissolved oxygen, and nitrogen source are controlled, the efficiency of lactic acid production may be increased by increasing the fermentation time [22]. Use of *Bacillus* to produce lactic acid in simple media has previously been reported [6]. The production of lactic acid is expected to induce antimicrobial activity against pathogenic bacteria *via* a reduction in pH. Therefore,

Table 2. Identification of carbon sources utilized by *B. polyfermenticus* KJS-2 and two other *Bacillus* strains with the API 50CHB kit.

No	Substrates	*B. polyfermenticus* KJS-2	*B. polyfermenticus* SCD	*B. subtilis* 168
0	Control	–	–	–
1	Glycerol	–	+	+
2	Erythritol	–	–	–
3	D-Arabinose	–	–	–
4	L-Arabinose	–	+	+
5	Ribose	–	+	–
6	D-Xylose	–	–	–
7	L-Xylose	–	–	–
8	Adonitol	–	–	–
9	β-Methyl-D-xyloside	–	–	–
10	Galactose	–	–	–
11	Glucose	+	+	+
12	Fructose	+	+	+
13	Mannose	+	+	+
14	Sorbose	–	+	–
15	Rhamnose	–	–	–
16	Dulcitol	–	–	–
17	Inositol	–	+	+
18	Mannitol	+	+	+
19	Sorbitol	+	+	+
20	α-Methyl-D-mannoside	–	–	–
21	α-Methyl-D-glucoside	+	+	+
22	*N*-Acetyl glucosamine	–	+	+
23	Amygdalin	+	+	+
24	Arbutin	+	+	+
25	Esculin	+	+	+
26	Salicin	+	+	+
27	Cellobiose	+	+	+
28	Maltose	+	+	+
29	Lactose	–	+	–
30	Melibiose	–	+	–
31	Sucrose	+	+	–
32	Trehalose	–	+	+
33	Inulin	–	–	–
34	Melezitose	–	–	–
35	Raffinose	–	–	–
36	Starch	–	–	–
37	Glycogen	–	–	+
38	Xylitol	–	–	–
39	Gentiobiose	–	+	+
40	D-Turanose	–	–	–
41	D-Lyxose	–	–	–
42	D-Tagatose	–	–	–
43	D-Fucose	–	–	–
44	L-Fucose	–	–	–
45	D-Arabitol	–	–	–
46	L-Arabitol	–	–	–
47	Gluconate	–	–	–
48	2 Keto-gluconate	–	–	–
49	5 Keto-gluconate	–	–	–

+: utilized; –: not utilized.

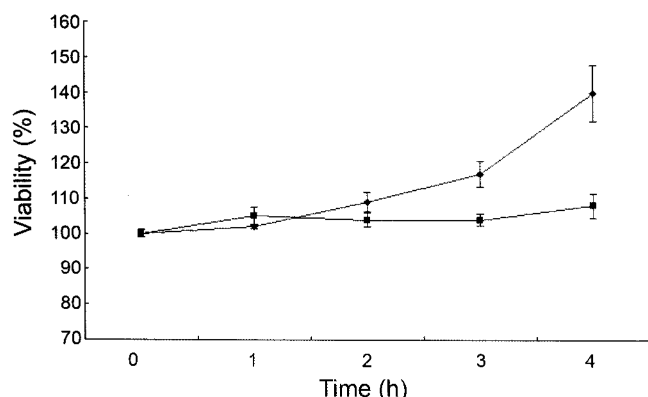

Fig. 2. Comparison of viability of *B. polyfermenticus* KJS-2 with artificial gastric juice (pH 2.0) and artificial bile acid (pH 6.5). ◆, pH 2.0; ■, pH 6.5.

lactic acid producing *B. polyfermenticus* KJS-2 can be used as a raw material for the production of biodegradable polymers with applications in medical, pharmaceutical, and food industries.

Cellular stress begins in the stomach, which has a pH as low as 2.0. To determine the effect of the acidic pH of the stomach on the survival of *B. polyfermenticus* KJS-2, an *in vitro* system with a pH of 2.0 was utilized (Fig. 2). When *B. polyfermenticus* KJS-2 was exposed to pH 2.0, it survived for 4 h and its concentration increased. These results indicate that *B. polyfermenticus* KJS-2 is not adversely affected by strongly acidic conditions. After the microorganisms pass through the stomach, they enter the upper intestinal tract, where bile salt is secreted into the stomach [4]. Therefore, it is necessary for probiotic lactic acid bacteria to be resistant to bile. Accordingly, we evaluated the sensitivity of *B. polyfermenticus* KJS-2 to bile by culturing it in TSB containing 0.3% bile (Fig. 2). The results revealed that the level of *B. polyfermenticus* KJS-2 was maintained in the presence of bile, which indicates that it is relatively resistant to bile. The viability of *B. polyfermenticus* KJS-2 in artificial gastric juice and bile salt was rarely intended to increase correspondence to *B. polyfermenticus* SCD and other commercially beneficial bacteria [14].

We investigated the adherence of *B. polyfermenticus* KJS-2 to Caco-2 cells. The human Caco-2 cell line is one of the best model systems for evaluating the interactions between bacterial and intestinal epithelial cells [24]. In this study, *B. polyfermenticus* KJS-2 and *B. polyfermenticus* SCD were adherent to approximately 65±0.6% and 54±1.4% of Caco-2 cells, respectively. *B. polyfermenticus* KJS-2 is used as a probiotic in the normal intestinal microbiota to counteract invasion by pathogenic bacteria. Therefore, the ability of *B. polyfermenticus* KJS-2 to inhibit the adhesion of pathogenic bacteria is expected to be highly specific and depends on both probiotic and pathogenic bacteria [19].

In conclusion, *B. polyfermenticus* KJS-2 produced lactic acid, remained stable in artificial gastric juice and bile acid, and adhered to Caco-2 intestinal epithelial cells. Taken together, these characteristics indicate that *B. polyfermenticus* KJS-2 is suitable for industrial use as a probiotic.

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