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ARTICLE

Isolation and Characterization of a Protease-Producing Bacterium, *Bacillus amyloliquefaciens* P27 from Meju as a Probiotic Starter for Fermented Meat Products

Mi-Sun Lee, Na-Kyoung Lee, Kyung-Hoon Chang, Shin-Yang Choi[1], Chi-Kwang Song[2], and Hyun-Dong Paik[1,*]

Division of Animal Life Science and Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea
[1]Korea Food Research Institute, Seongnam 463-746, Korea
[2]Food R&D, CJ CheilJedang Corp., Seoul 152-050, Korea

Abstract

This study was performed to select protease-producing *Bacillus* sp. as a potential probiotic starter for fermented meat products. In order to isolate protease-producing bacterium from *meju*, measured the diameter of the clear zone on agar plate (TSA, 1% (w/v) skim milk) and analyzed for intracellular protease activity, then 10 *Bacillus*-like strains were isolated. Three *Bacillus*-like strains (P19, P27, and P33) among 10 strains were able to tolerate in acidic condition (TSB, pH 2.5, 2 h incubation). These 3 strains were showed antimicrobial activity against food-borne pathogenic bacteria. These vegetative cells of 3 strains were showed a survival rate of 0.04% to 0.08% under the artificial gastric acidic condition (TSB, pH 2.5 with 1% (w/v) pepsin), but spore-forming cells were 56.29% to 84.77%. Vegetative cells of 3 strains were the least bile-resistant, while spore-forming cells of 3 strains showed higher survival rate more than 76% under artificial bile condition (TSB, 0.1% (w/v) oxgall bile). In these strains, P27 strain was finally selected as a good probiotic strain. P27 strain was tentatively identified as *Bacillus amyloliquefaciens* by API CHB kit and 16S rDNA sequence analysis. The results of this study suggest that *B. amyloliquefaciens* P27 can be used as a potential probiotic starter for fermented meat product.

Key words: *Bacillus amyloliquefaciens*, probiotic starter, protease-producing strain, meju, fermented meat product

Introduction

Recently, interest on the probiotics has been increasing and methods developed for human medicine and agriculture for which the mechanisms by which probiotics operate have been well defined. Probiotics are generally defined as live microbial feed supplements that can benefit the host by improving its intestinal balance (Chang and Liu, 2002; Fuller *et al*., 1989; Zhou *et al*., 2010). The presence of an adequate number of live probiotic cells in a food product at the time of consumption represents the first challenge for the development of a probiotic product, taking the recommended dose of at least 10^8 cells/day into account (Hammes and Haller, 1998). Secondly, the probiotic cultures have ability to survive passage through the upper part of the gastrointestinal tract. High tolerance to low pH and bile salts corresponding to the conditions of the human gastrointestinal tract (GIT) has therefore been considered as important selection criteria (Tuomola *et al.,* 2001).

Probiotic bacteria are mostly the lactic acid bacteria, such as the lactobacilli and bifidobacteria, partly spore-forming *Bacillus* species, or yeasts like *Saccharomyces boulardii* (Hoa *et al*., 2000). Many studies have demonstrated direct probiotics effects of *Bacillus* spores (Casula and Cutting, 2005; Sánchez *et al*., 2009). In addition, a large of *Bacillus* products are used as 'novel foods' or as dietary supplements with claim of 'enhancing' the well-being of the consumer (Hong *et al*., 2005).

Fermented foods are important part of traditional diets around the world especially, type of traditional fermented soybean food in Asian countries. *Meju* is a fermented rectangular block of crushed cooked soybeans and to make *doenjang* and *kochujang*, one of important traditional fermented foods in Korea. Healthful physiological effects of Korean fermented foods have received attention from public and industry (Kim *et al*., 2009b). Soybean and soy-

*Corresponding author: Hyun-Dong Paik, Division of Animal Life Science and Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea. Tel: 82-2-2049-6011, Fax: 82-2-455-1044, E-mail: hdpaik@konkuk.ac.kr

containing foods are excellent and inexpensive suppliers of dietary proteins, carbohydrates, vitamins, and minerals (Anderson *et al*., 1995; Cho, 2008; Sung *et al*., 2004). One of Korean soybean fermented soybean foods, *meju* is major source of protein of Korean for thousands of years. *Meju* is necessary to produce all kind of bacteria for fermentation of *doenjang* and the majority of species is *Bacillus* sp. (*B. subtilis, B. licheniformis, B. amyloliquefaciens,* and *B. pumilus*). The producing-protease bacteria help digestion because their protease decomposes protein into amino acid.

Microbial proteases from bacteria, yeast and molds have also been developed for meat tenderization (Diaz *et al*., 1997; Kim *et al*., 2008). In particular, protease from *Bacillus* sp. have become the most important industrial enzymes based on the world-wide enzyme sales (Park *et al*., 2005). The crude protease(s) from *B. polyfermenticus* SCD treated jerky was good affected on quality than those of control (Kim *et al*., 2008). For *B. subtilis*, safety in humans is already supported by the fact that *B. subtilis* is a long history of beneficial properties assigned such as *natto* or as food supplements (as in Lactipan Plus in Italy) (Hong *et al*., 2008). Therefore, *Bacillus* sp. has probiotic properties could be used as starter culture for fermented meat products instead of lactic acid bacteria such as the traditional Japanese staple.

Through this study, protease-producing bacteria from *meju* was screened and characterized probiotic characteristics of selected *Bacillus*-like strains that including their resistances to artificial gastric juice and artificial bile acid, and antimicrobial activity. In next, *B. amyloliquefacies* P27 will be used as a probiotic and starter culture for fermented meat products.

Materials and Methods

Preparation of *meju* and isolation of *Bacillus*-like strains

Meju were obtained from local market and *Bacillus*-like strains were isolated from these products. Each sample (10 g) was dissolved in 90 mL of 0.85% (w/v) NaCl. After dilution, 0.1 mL of the diluted suspension was spread on the plate count agar (PCA, Difco, Benton Dickinson, Sparks, MD, USA) plates followed by incubation at 37°C for 24 h. Colonies showing *Bacillus*-like strains were selected as a pure isolate on each TSA plate. Each strain was stored at -70°C as a stock solution in 30% (v/v) glycerol during the experiments.

Screening of protease-producing bacteria

Selection of the strain with protease activity was performed by TSA with 1% (w/v) skim milk. Each strain was spotted on TSA plates by the toothpick method (Yun *et al*., 2003). After incubation at 30°C for 16 h, the diameter of the clear zone on agar plate was measured and isolated colonies that over 25 mm clear zone size were used for 2nd screening.

Each of the isolated colonies from 1st screening were inoculated in 7 mL TSB at 30°C for 16 h. Supernatant of TSB culture solution was obtained by centrifuging at 22,000 *g* for 20 min and 1 mL of the supernatant were used as enzyme solution to measure protease activity (Jung *et al*., 2008). This enzyme reaction was carried out by adding 0.5 mL of enzyme solution to 1.5 mL of 2% casein solution, 1 mL of Mcllavain buffer (pH 6.0) at 38°C for 1 h. The reaction was stopped by adding 3 mL of 0.4 M trichloroacetic acid (TCA), and the solution was allowed to stand at 38°C for 15 min. After that, the mixture was centrifuged at 3,000 *g* for 20 min, and 1.0 mL of the supernatant was used for next measurement. One mL of the supernatant was mixed with 5 mL of 0.4 M Na_2CO_3 solution and 1 mL of 1 N Folin reagent, and the mixture was stood at 38°C for 30 min. The absorbance of the above mixture was measured at 660 nm using a spectrophotometer (JASCO V-530, Tokyo, Japan), which was equivalent to the amount of converted tyrosine based on a standard curve. One unit of protease activity was defined as liberation of 1 μg of tyrosine per minute.

Tolerance to acidic condition on agar plate

To determine the tolerance to acidic condition, the method of Hyronimus *et al.* (2000) was modified as follows. The isolated *Bacillus* strains were grown in TSB at 30°C for 16 h, and diluted to 10^6 CFU/mL in fresh TSB adjusted to different pH values (2, 3, and 7) with 3 M hydrochloric acid. After incubation at 37°C for 2 h, each culture were streaked on the TSA plates (pH 7.0) and then incubated at 30°C for 16 h.

Antimicrobial activity of strains and supernatant concentrates

Eighteen food-borne pathogenic bacteria were used as test organisms: *Escherichia coli* ATCC 9637, *E. coli* ATCC 10536, *E. coli* ATCC 25922, *E. coli* O157, *Listeria monocytogenes* ATCC 15313, *Salmonella* Enteritidis KCCM 12021, *Salmonella* Typhimurium P99, *S.* Typhimurium ATCC 14028, *Staphylococcus aureus* ATCC 14458, *S. aureus* ATCC 25923, *S. aureus* KCCM 32395,

S. aureus KCCM 40510, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus agalactiae* ATCC 13813, *S. agalactiae* ATCC 14364, *Pseudomonas aeruginosa* ATCC 15522, *Bacillus cereus* ATCC 9634, *B. cereus* ATCC 11778. These test organisms were grown on the TSA plates at 37°C for 24 h.

The isolated *Bacillus* strains were cultured in 100 mL TSB at 30°C for 12 h in shaking incubator 150 rpm. Antimicrobial activity was measured as two methods. The first one was flip method. The isolated *Bacillus* strain streaked on TSA plates after that incubated at 30°C for 16 h. Then agars which grown the *Bacillus* strain flipped to top of plates and overlaid 5 mL of each soft agar (TSA, 0.8% (w/v) agar) inoculated 100 μL of indicator strains. After incubation at 37°C for 24 h, the antimicrobial activity was measured the clear zone around the isolated *Bacillus* strains. The second one was paper disc method. That is, the *Bacillus* cultured cells were removed from the medium by centrifugation at 22,000 g for 20 min, and the supernatant filtered through a 0.45 μm syringe filter (Toyo Roshi Kaisha, Tokyo, Japan). After filtration, 20 mL of the supernatant was lyophilized for concentration. The TSA plates were prepared for antimicrobial activity which was spread with a test organism, and put on 8-mm paper disc. For preparing 10 times concentrates, lyophilized supernatant was dissolved in 2 mL sterile phosphate buffered saline (PBS, pH 7.0) before using. Fifty microliter of 10 times concentrates was applied on to the 8-mm paper disc. After incubation at 37°C for 24 h, the antimicrobial activity was measured as the diameter (mm) of the clear zone.

Tolerance to artificial gastric juice and artificial bile acid

The artificial gastric juice and artificial bile acid tolerance of *Bacillus* sp. were studied on 2 types of vegetative cells and spore-forming cells. Spores were formed by heating at 80 for 30 min after incubation at 30°C for 48 h.

To investigate survival of *Bacillus* sp. cultures under artificial gastric juice condition, strain culture inoculated (1%) into TS broth that had been acidified to pH 2.5 (using HCl) containing 1% of pepsin and incubated at 37°C for 2 h. After that to estimate bile tolerance of *Bacillus* sp. cultures, each strain cultures inoculated (1%) into TS broth containing 0.1% oxgall bile and incubated at 37°C for 24 h. Then, the number of viable *Bacillus* sp. cells was determined by serial dilution with 0.85% NaCl and spread on TSA plates. Plates were incubated at 30°C for 12 h, and the survival rate was calculated as the percentage of *Bacillus* sp. grown on the TSA plates.

Microbial identification of probiotic strains

Cell morphology, gram staining, carbohydrate usability, and 16 rDNA sequencing were analyzed for the tentative microbial identification of probiotic strains. Test culture was grown on TSA plates at 30°C for 16 h, and then inoculated onto test media for biochemical tests with commercial API 50 CHB kit (ATB System, bioMerieux, Marcy l'Etoile, France). The colonies were suspended in API 50 CHB medium and the strips were inoculated then, incubated at 37°C and observed at 24 and 48 h. Among these strains, P27 strain was additionally identified by using the 16 rDNA sequence performed by Macrogen (Seoul, Korea), the sequence was determined by BLAST program of GENEBANK data library.

Results and Discussion

Screening of protease-producing bacteria from *meju*

Thirteen kinds of Korean traditional *meju* were collected from local market and 471 *Bacillus*-like strains were isolated from these products. The presence of various *Bacillus* sp. in *meju* and other soy fermented foods (*doenjang* and *kochujang*) was reported (Cho, 2008; Jung *et al*., 2008; Mo *et al*., 2010; Yun *et al*., 2003). Sixty-nine *Bacillus*-like strains with protease activity were isolated using TSA plate with 1% (w/v) skim milk using the toothpick method. A clear zone around strains suggested production of the protease by the *Bacillus*-like strains.

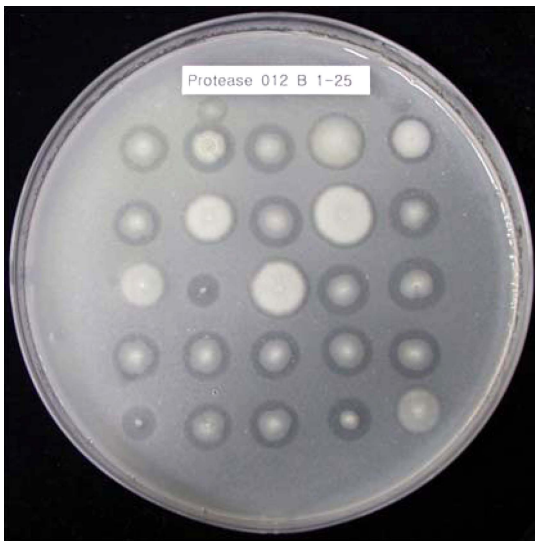

Fig. 1. Example of isolation of protease-producing *Bacillus*-like strains.

These 69 isolated strains which exhibited higher protease activities with over 25 mm a clear zone (Fig. 1), then strains were analyzed for intracellular protease activity.

For intracellular protease activities of the 69 isolated strains, the strain was cultivated in TSB medium at 30°C for 16 h. After determination of protease activity, 10 strains showing high protease activities were selected for determining their acidic tolerance. The protease activity of P19, P27, and P33 strains was 0.98±0.20, 1.42±0.15, and 0.84±0.03 U/mL, respectively.

Tolerance to acidic condition on agar plate

Ten selected strains were using to confirm their tolerance to acidic condition. Seven strains inoculated TS broth at pH 2 and 3 showed lower growth on TSA plate than the other 3 strains. Three strains of P19, P27, and P33 showed growth on TSA plates as control. Therefore, three strains were selected for further study.

Table 1. Antimicrobial activities of protease-producing strains isolated from *meju* against food-borne pathogenic bacteria

Indicator Strains	Size of inhibitory clear zone by flip method			Diameter (mm) of inhibitory clear zone by disc method		
	P19	P27	P33	P19	P27	P33
E. coli ATCC 9637	-	-	-	-	-	-
E. coli ATCC 10536	-[1)]	-	-	-	-	-
E. coli ATCC 25922	-	-	-	-	-	-
E. coli O157	-	-	-	-	-	-
L. monocytogenes ATCC 15313	++	++	++	12.5[5)]	15.0	-
S. Enteritidis KCCM 12021	-	-	-	-	-	-
S. Typhimurium P99	-	-	-	-	-	-
S. Typhimurium ATCC 14028	-	-	-	-	-	-
S. aureus ATCC 14458	++	++	++	-	-	-
S. aureus ATCC 25923	+[2)]	+	+	-	-	-
S. aureus KCCM 32395	++	++	++	11.0	10.5	-
S. aureus KCCM 40510	+[3)]	+	+	-	-	-
S. epidermidis ATCC 12228	++[4)]	++	++	14.5	15.0	-
S. agalactiae ATCC 13813	++	++	++	-	12.0	-
S. agalactiae ATCC 14364	+	+	+	-	11.0	-
P. aeruginosa ATCC 15522	-	-	-	-	-	-
B. cereus ATCC 9634	+	++	++	11.5	11.5	10.5
B. cereus ATCC 11778	+	+	+	11.0	11.5	10.5

[1)]No inhibition, size of inhibition zone is [2)]0.5 mm, [3)]0.5-2.0 mm, [4)]>2.0 mm, and [5)]diameter of the inhibition zone (including paper disk, 8 mm diameter).

Antimicrobial activity of strains and supernatant concentrations

Antimicrobial activities of the selected 3 strains were tested against 18 food-borne pathogenic bacteria in this study. Flip method showed a broad range of antimicrobial activities against food-borne pathogenic bacteria than paper disc method (Table 1).

Using Flip method, isolated 3 strains showed antimicrobial activity against food-borne pathogenic bacteria including *L. monocytogenes* ATCC 15313, *S. aureus* ATCC 14458, *S. aureus* ATCC 25923, *S. aureus* KCCM 32395, *S. epidermidis* ATCC 12228, *S. agalactiae* ATCC 13813, *S. agalactiae* ATCC 14364, *B. cereus* ATCC 9634, and *B. cereus* ATCC 11778.

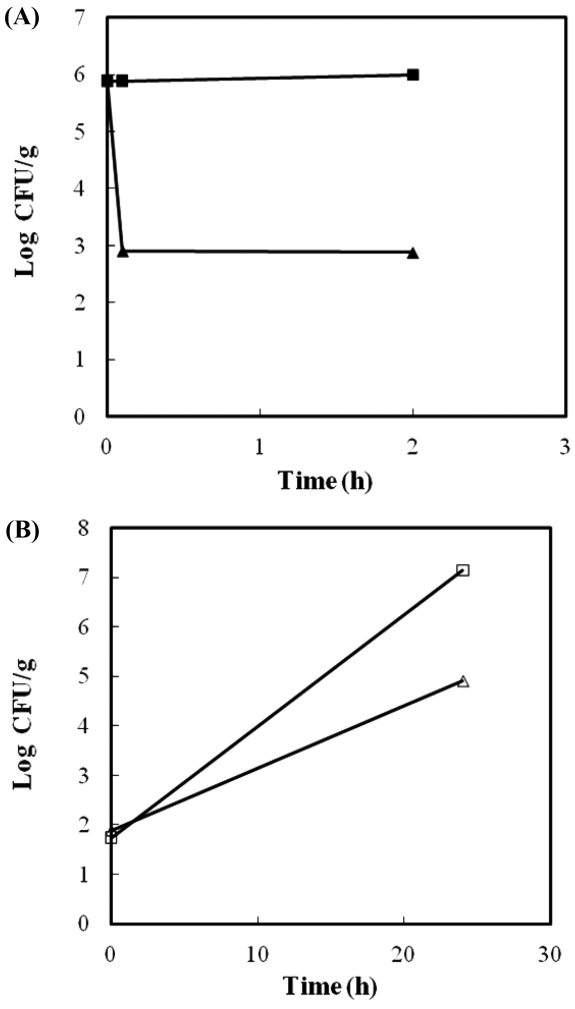

Fig. 2. Survival of vegetative cells of strain P27 in (a) artificial gastric juice, (b) treated with artificial bile acid after artificial gastric juice treatment (pH 2.5) at 37°C for 2 h. ■, no artificial gastric juice treatment (control); ▲, treated with artificial gastric juice (pH 2.5); □, no bile acid added after treatment with artificial gastric juice (control); △, treated with artificial gastric juice (pH 2.5) and bile acid.

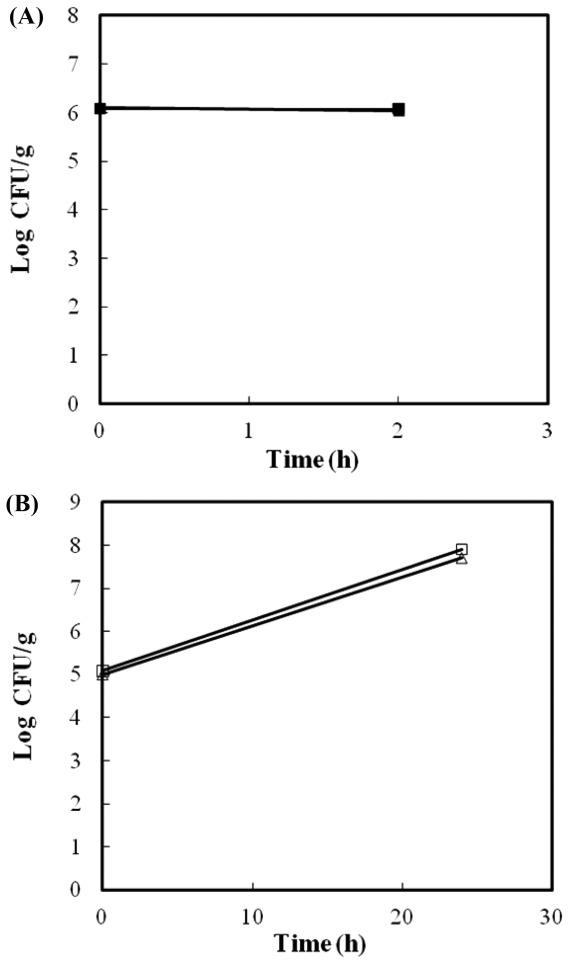

Fig. 3. Survival of spore-forming cells of strain P27 in (a) artificial gastric juice, (b) treated with artificial bile acid after artificial gastric juice treatment (pH 2.5) at 37°C for 2 h. ■, no artificial gastric juice treatment (control); ▲, treated with artificial gastric juice (pH 2.5); □, no bile acid added after treatment with artificial gastric juice (control); △, treated with artificial gastric juice (pH 2.5) and bile acid.

However, using paper disc method, inoculated 10 times concentrates showed different results between each strain. P27 strain showed relatively wide antimicrobial spectrum against *L. monocytogenes* ATCC 15313, *S. aureus* KCCM 32395, *S. epidermidis* ATCC 12228, *S. agalactiae* ATCC 13813, *S. agalactiae* ATCC 14364, *B. cereus* ATCC 9634, and *B. cereus* ATCC 11778.

Several *Bacillus* sp. is reported as the production of antimcirobial substances including organic acid, hydrogen peroxide, ethanol, and bacteriocin against food-borne pathogens (Cho, 2008; Kim *et al*., 2009a; Wilson *et al*., 2005). In this study, the concentrates of P27 strain has antimicrobial effect against several food-borne pathogens, this result represents the possibility of usable biocontrol substances.

Tolerance to artificial gastric juice and artificial bile acid

The important characteristics of probiotics is resistant to gastric and bile acids, as they pass through the duodenum in order to reach the small intestinal tract (Mainville *et al.*, 2005). Our study was investigated as 2 types of vegetative cells and spore-forming cells for the tolerance to artificial gastric juice and artificial bile acid. Survivals in artificial gastric juice are shown in Table 2 and survivals in artificial bile acid are shown in Table 3. Vegetative cells of 3 strains were showed a low survival rate of 0.04 to 0.08%, whereas spore-forming cells were showed a high survival rate of 56.29 to 84.77% under the artificial gastric acidic condition. The vegetative cells of 3 strains were the least bile-resistant (Table 2). However, spore-forming cells of 3 strains showed higher survival rate more than 76% in artificial bile acid shown as Table 3. In

Table 2. Viability of vegetative cells and spore-forming cells of protease-producing strains isolated from *meju* in artificial gastric juice

Strain	Vegetative cell (CFU/mL)			Spore forming cell (CFU/mL)		
	Control	1% Pepsin (pH 2.5)	Viability (%)	Control	1% Pepsin (pH 2.5)	Viability (%)
P19	2.1×10^{6}	8.0×10^{2}	0.04	4.1×10^{5}	3.5×10^{5}	84.77
P27	9.9×10^{5}	7.5×10^{2}	0.08	1.2×10^{6}	1.1×10^{6}	84.62
P33	6.3×10^{5}	4.5×10^{2}	0.07	5.4×10^{5}	3.0×10^{5}	56.29

Table 3. Viability of vegetative cells and spore-forming cells of protease-producing strains isolated from *meju* in artificial bile acid after artificial gastric juice treatment

Strain	Vegetative cell (CFU/mL)			Spore forming cell (CFU/mL)		
	Control	0.1% Oxgall	Viability (%)	Control	0.1% Oxgall	Viability (%)
P19	5.4×10^{6}	5.0×10^{0}	0.00	4.9×10^{7}	5.2×10^{7}	106.46
P27	1.4×10^{7}	5.9×10^{4}	0.42	6.7×10^{7}	5.1×10^{7}	76.50
P33	1.1×10^{8}	4.9×10^{4}	0.04	1.6×10^{7}	1.3×10^{7}	83.46

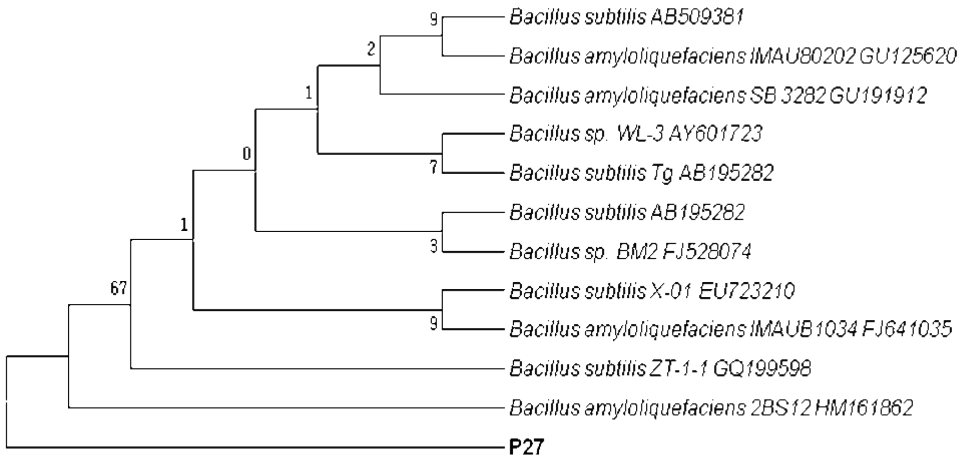

Fig. 4. Phylogenetic tree based on 16S rDNA sequences showing the position of strain P27 and representatives of some related taxa.

most study using spore-forming cells of *Bacillus*, their data showed high survival in artificial gastric juice and artificial bile acid (Cho, 2008; Patel *et al*., 2009).

Microbial identification of probiotic strains

Identification using API 50 CHB kit showed strain P19 to have 77.1% similarity to *Bacillus subtilis/Bacillus amyloliquefaciens*, strain P27 to have 98.5% similarity to *Bacillus subtilis/Bacillus amyloliquefaciens,* and strain P33 to have 97.5% similarity to *Bacillus subtilis/Bacillus amyloliquefaciens.*

P27 strain showed the best probiotic characteristics among 3 strains studied. Therefore, for the specific identification, P27 strain was identified through the 16S rDNA sequence analysis. The result of 16S rDNA sequence showed 97% identities with *Bacillus amyloliquefaciens* 16s rDNA sequence of GENBANK (Fig. 4).

In conclusion, this study showed that protease-producing bacterium was screened from *meju* and investigated probiotic characteristics. Among these isolates, *B. amyloliquefaciens* P27 has a highest protease, high survival in artificial gastric juice and artificial bile acid, and wide range of antimicrobial activity. On the basis of these results, *B. amyloliquefaciens* P27 could be a good possible probiotics and a fermentation starter for fermented meat product in next study.

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