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Antimicrobial Activity of *Bacillus amyloliquefaciens* EMD17 Isolated from *Cheonggukjang* and Potential Use as a Starter for Fermented Soy Foods

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Abstract A *Bacillus* strain with antimicrobial activity was isolated from *Cheonggukjang* and identified as *Bacillus amyloliquefaciens.* Filtered culture supernatants of *B. amyloliquefaciens* EMD17 strongly inhibited growth of *Bacillus cereus* ATCC14579, *Listeria monocytogenes* ATCC19111, and an ochratoxin (OTA) producing *Penicillium* spp. The antimicrobial activity was not decreased by heat, pH, and proteases treatment, indicating a non-proteinous nature of the antimicrobial substance. A *B. amyloliquefaciens* EMD17 culture added to a *B. cereus* ATCC14579 culture killed *B. cereus* cells completely in 6 h, showing the bacteriocidal effect. The *srfAA* gene encoding surfactin synthetase A was detected using PCR, indicating that surfactin might be the responsible agent. Genes encoding fengycin and iturin were also detected. MS indicated production of lipopeptides, including surfactin. *Cheonggukjang* and *Doenjang* were prepared using *B. amyloliquefaciens* EMD17 as a starter and growth of spiked *B. cereus* cells was completely inhibited.

Keywords: *Bacillus amyloliquefaciens*, antimicrobial activity, lipopeptide, surfactin, starter

Introduction

Bacillus subtilis, *B. amyloliquefaciens*, and *B. licheniformis* are often isolated from fermented soy foods, including *Cheonggukjang*, *Doenjang*, and *Meju.* The quality of fermented soy foods largely depends on types of bacilli and fungi grown on soybeans during fermentation because metabolites, such as peptides, amino acids, and other compounds, that are important for food quality are produced from soybeans by proteases and amylases secreted by bacilli and fungi (1-3). Today, safety and functionality of foods are the most important consumer criteria for food selection (4-5). During fermentation, soy foods are exposed to a risk of contamination by pathogenic microorganisms, such as toxinogenic *B. cereus* and fungi, because fermentation is carried out in an open environment where pathogens often exist together with desirable organisms. For example, rice straw is used as a source of *B. subtilis* in traditional *Cheonggukjang* fermentation, but is also a source of *B. cereus* (6-7).

Often, soy foods prepared in traditional ways show poor qualities with unpleasant aromas and unattractive colors caused by organisms. Food poisoning can also occur if toxinogenic fungi or *B. cereus* are the contaminating organisms. Contamination with undesirable organisms also reduces food functionality because growth of desirable organisms is inhibited, resulting in reduced amounts of desirable metabolites in final products.

An effective method for enhancement of safety and functionality of fermented soy foods is use of *Bacillus* strains with desirable properties as starters. Desirable properties include an ability to inhibit growth of pathogenic microorganisms, an ability to produce bioactive compounds, and an ability to confer desirable organoleptic properties on fermented foods. Efforts have been made to isolate desirable *Bacillus* strains exhibiting antimicrobial activities from fermented soy foods (6-9), strains secreting fibrinolytic enzymes (10-11), and strains producing poly-γ-glutamic acid (12-13). However, only limited success has been reported regarding use of desirable strains as starters for improvement of safety and quality of fermented foods. Bacilli from fermented soy foods were isolated and strains were selected as starter candidates. *B. amyloliquefaciens* EMD17 exhibited a strong antimicrobial activity. In this study, properties of antimicrobial substances secreted by *B. amyloliquefaciens* EMD17 and potential use as a starter strain were investigated.

Materials and Methods

Isolation and identification of EMD17 from *Cheonggukjang* *Cheonggukjang* products prepared in traditional ways without starters were purchased at local markets at Jinju, Gyeongnam Province, Republic of Korea during the spring and summer of 2012. Ten g of *Cheonggukjang* was mixed with 90 mL of sterile water, homogenized using a Stomacher 80 (Seward, Worthing, UK), and serially diluted with sterile water. Diluted *Cheonggukjang* samples were spread on Luria-Bertani (LB) plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated for 48 h at 37°C. Colonies exhibiting typical *Bacillus* morphologies were examined for antimicrobial activities using the agar spot method (14). *B. cereus* ATCC14579 and an ochratoxin (OTA) producing *Penicillium* spp. were used as indicators. Bacilli were grown in LB for 48 h at 37°C and 1 μL of a culture was spotted on an LB plate that was overlaid with LB top agar (0.7%, w/v) containing 50 μL of a *B. cereus* ATCC14579 culture grown in LB for 48 h. For antifungal activity testing, potato dextrose (PD) top agar (Becton, Dickinson and Company) containing $1x10^5$ fungal spores was overlaid.

For identification of isolates, 16S rRNA and *recA* genes were amplified and nucleotide sequences were determined. Total DNA was prepared from a culture grown for 48 h in LB broth (8). The 16S rRNA gene was amplified using the primer pair 27F (5'-AGAGTTT GATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The *recA* gene was amplified using the primer pair recA-F (5'-TGAGTGATCGTCAGGCAGCCTTTAG-3') and recA-R (5'-CYTBRGATAAG ARTACCAWGMACCGC-3') (15). Sequencing was done at Cosmogenetech (Daejeon, Korea) and sequences were analyzed using the BLAST (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information, Bethesda, MD, USA). Randomly amplified polymorphic DNA-PCR (RAPD-PCR) was done for total DNA using a 10-mer (S30; Bionics, Seoul, Korea) as described previously (15).

Inhibition spectrum of *B. amyloliquefaciens* *B. amyloliquefaciens* EMD17 was grown in LB broth for 48 h at 37°C with vigorous shaking and the culture was centrifuged at 8,000×*g* for 30 min at 4°C. The supernatant was filtered using a 0.45 μm syringe filter (Minisart CE; Satorius Stedim, Aubagne, France) and the filtered supernatant (FS) was used for antimicrobial activity measurements following the agar well diffusion method (8). An agar plate (1.5%, w/v) containing a medium specific for each indicator strain was overlaid with top agar (0.7%, w/v) containing 50 μL of an indicator culture that was grown to the early exponential phase (OD_{600}=0.4). Indicators are listed in Table 1. When fungi were used as indicators, potato dextrose agar (PDA) top agar (0.7%, w/v) containing $1x10^5$ fungal spores was overlaid on an LB plate (16). Holes were made in plates using a capillary glass tube and 100 μL of FS was placed into each hole. Plates were incubated for 48 h at 37°C, and resulting inhibition zones around wells were measured.

***B. amyloliquefaciens* EMD17 production of antimicrobial substances** The effect of the growth medium on the antimicrobial activity of *B. amyloliquefaciens* EMD17 was examined using the 4 different media LB, brain heart infusion (BHI) (Becton, Dickinson and Company), tryptic soy broth (TSB) (MB Cell, Los Angeles, CA, USA), and nutrient broth (NB) (Neogen, Lansing, MI, USA). Each medium (100 mL) was inoculated at 1% (v/v) with an overnight culture in LB and incubated up to 96 h with vigorous shaking at 37°C. Aliquots were taken at time points and FS samples were obtained. FS

Table 1. Antibacterial activities of *B. amyloliquefaciens* EMD17 and other bacilli

Indicator strains	Antibacterial activity			
	B. amyloliquefaciens EMD17	*B. subtilis* W42[8]	*B. subtilis* H27[17]	*B. amyloliquefaciens* MJ1-4[16]
Bacillus cereus ATCC14579	+++	+++	++	-
Listeria monocytogenes ATCC19111	+++	+++	++	+++
Enterococcus faecalis ATCC29212	+++	+++	+	-
Pediococcus pentosaseus NRRL B-14009	-	+	++	-
Streptococcus thermophilus KFRI193	+++	+++	++	-
B. licheniformis ATCC21415	+++	-	-	+++
Leuconostoc mesenteroides ATCC9135	-	++	++	-
B. circulans ATCC4513	+	-	-	-
B. thuringiensis ATCC33679	+++	+++	-	-
Escherichia coli 0157:H7	-	-	-	-
Lactobacillus delbrueckii ssp. *lactis* ATCC4797	-	+	+	-
Lb. pentosus ATCC8041	-	-	-	-
Lb. casei ssp. *casei* ATCC4646	++	+	+	+
Lb. fermentum ATCC14931	-	+	+	-
Salmonella Typhimurium TA98	-	-	-	-
Salmonella Typhimurium TA100	-	-	-	-

*Degree of inhibition: +, 0.5 to 2 mm; ++, 2 to 4 mm; +++, more than 4 mm
Inhibition zone= (diameter of an inhibition zone in mm - diameter of a well)/2

antimicrobial activities were measured using the agar well diffusion method with *B. cereus* ATCC14579 as an indicator. Activity was expressed as an arbitrary unit (AU) per 1 mL and 1 AU was defined as the highest dilution showing a clear zone of inhibition (16).

Stability of the antimicrobial substance against enzymes, pH, and heat treatment Effects of proteases, pH, and heat treatments on the antimicrobial activity of *B. amyloliquefaciens* EMD17 were examined using the agar well diffusion method with *B. cereus* ATCC14579 as the indicator. FS samples were prepared from a *B. amyloliquefaciens* EMD17 culture grown in NB for 48 h. FS samples were incubated with proteinase K (P2308; Sigma-Aldrich, St. Louis, MO, USA), protease (P5147; Sigma-Aldrich), pepsin (P-6887; Sigma-Aldrich), or trypsin (T-8918; Sigma-Aldrich) for 2 h at 37°C at a final concentration of 1 mg/mL (17). FS samples were heated at 50, 60, 70, 80, 90, and 100°C for 30 min and 121°C for 15 min. pH values of FS samples were adjusted to 3-10 using 1 N HCl or 1 N NaOH, then FS samples were incubated for 2 h at 25°C. The remaining activity was measured using the agar well diffusion method.

Inhibition mode of antimicrobial substances *B. cereus* ATCC14579 was grown in LB broth until the cell number reached 10^5 CFU/mL. Then FS from 48 h culture in NB was added with different amount (2, 5, 10%, v/v). In a separate experiment, a *B. amyloliquefaciens* EMD17 culture was added (1%, v/v) to a *B. cereus* culture. After addition of FS samples or cultures of *B. amyloliquefaciens* EMD17, *B. cereus* cultures were further incubated for 12 h and viable *B. cereus* cells were counted every 3 h. LB agar plates were used for *B. cereus* counting when FS was added, and Pemba plates (polymyxin pyruvate egg yolk mannitol bromothymol blue agar) (MB Cell) were used for cultures when a *B. amyloliquefaciens* EMD17 culture was added. Viable cell counting was repeated in triplicate and results were reported as a mean±standard deviation (SD).

PCR amplification of lipopeptide genes from *B. amyloliquefaciens* EMD17 Total DNA was prepared from *B. amyloliquefaciens* EMD17 cells grown overnight in LB as described previously (15). Target lipopeptide genes and primers used are listed in Table 2. PCR was carried out using a MJ Mini™ PCR machine (BioRad, Hercules, CA, USA). The reaction mixture of 50 μL consisted of 1 μL of template DNA, 1 μL of each 10 μM primer, 5 μL of 0.25 mM dNTPs, and 1 μL Ex*Taq* DNA polymerase (Takara, Shiga, Japan). The cycling conditions were 95°C for 5 min, 35 cycles at 94°C for 30 s, 43-59°C for 30 s, 72°C for 1 min, and a 5 min final extension at 72°C. Amplified products were extracted from an agarose gel (2%, w/v) using a gel extraction kit and nucleotide sequences were determined using the BLAST program at NCBI.

MS analysis of culture supernatants FS samples from 48 h cultures in NB were analyzed using an ultra-performance liquid chromatography-quadrupole-time-of-flight (UPLC-Q-TOF) mass spectrometer (MS) and NB broth was used as a control. A UPLC system (Waters, Milford, MA, USA) was used and analytical samples were injected into an Acquity UPLC BEH C_{18} column (100 mm×2.1 mm×1.7 μm) (Waters) equilibrated with water containing 0.1% formic acid and eluted in a gradient with acetonitrile (ACN) containing 0.1% formic acid at a flow rate of 0.35/min for 13 min. Eluted metabolites were analyzed using Q-TOF MS (Waters) in electrospray ionization (ESI)-positive mode. The temperatures of the source and desolvation were set at 120 and 350°C, respectively, and the desolvation flow rate was 600 L/h. Voltages of the capillary and sampling cones were set at 3 kV and 30 V, respectively. MS data were collected in the *m/z*=100-1,200 range with a scan time of 0.1 s. Lock spray with leucine-enkephalin (556.2771 Da) was used at a flow rate of 5 μL/min and a frequency of 10 s to ensure accuracy and reproducibility for all analyses. Detailed chemical compositional information about cyclic lipopeptides analyzed using UPLC-Q-TOF MS was provided from elemental composition analysis and was compared with chemical structures in the ChemSpider database.

Use of *B. amyloliquefaciens* EMD17 as a starter for *Cheonggukjang* and *Doenjang* *Cheonggukjang* was prepared from soybeans (2012 crop year, Hamyang, Gyeongnam, Republic of Korea). Soybeans (300 g) were soaked in water for 16 h at room temperature, then

Table 2. PCR detection of lipopeptide biosynthesis genes from *B. amyloliquefaciens* EMD17

Lipopeptides	Gene	Primer	Primer sequences 5' to 3'	Annealing temperature (°C)	PCR product size expected	Reference
Surfactin	*srfAA*	SRFAF	TCGGGACAGAAGACATCAT	59	210	23
		SRFAR	CCACTCAAACGGATAATCCTGA			
	srf/lch	As1-F	CGCGGMTACCGVATYGAGC	43	428	24
		Ts2-R	ATBCCTTTBTWDGAATGTCCGCC			
Fengycin	*fen*	Af2-F	GAATAYMTCGGMCGTMTKGA	45	443, 452	24
		Tf1-R	GCTTTWADKGAATSBCCGCC			
Iturin	*ituD*	ITUD-F1	TTGAAYGTCAGYGCSCCTTT	55	482	25
		ITUD-R1	TGCGMAAATAATGGSGTCGT			
Iturin A	*ituA*	ITUD1F	GATGCGATCTCCTTGGATGT	55	647	25
		ITUD1R	ATCGTCATGTGCTGCTTGAG			

M, A or C; V, A, C, or G; Y, C or T; B, C, G, or T; W, A or T; D, A, G, or T; Y, C or T; K, G or T; S, C or G

autoclaved for 15 min at 121°C. Cooled soybeans were inoculated with 10^8 cells of *B. amyloliquefaciens* EMD17 and 10^7 cells of *B. cereus* ATCC 14579 per g of dry soybeans. Inoculated soybeans were fermented for 48 h at 37°C, then stored at 4°C for up to 10 days. During the fermentation and storage period, soybean samples were taken for total bacilli and *B. cereus* counts using LB and Pemba plates, respectively. *Doenjang* was prepared using whole *Meju* soybeans (Kong-Al *Meju*) as described previously (18). *B. amyloliquefaciens* EMD17 was inoculated into cooked soybeans at 10^5 cells/g of soybeans together with 10^5 cells of *Pichia farinosa* SY80 and 10^5 spores of *Rhizopus oryzae. Doenjang* was intentionally contaminated with *B. cereus* ATCC14579 at 10^4 cells/g of soybeans and fermented for 70 days at 25°C.

Results and Discussion

Isolation and identification of *B. amyloliquefaciens* EMD17 Several bacilli that inhibited growth of *B. cereus* ATCC14579 were isolated from *Cheonggukjang* products. The EMD17 isolate that showed typical *Bacillus* morphology on LB agar plates was the most inhibitory strain. EMD17 also inhibited growth of an ochratoxin (OTA) producing *Penicillium* spp. strain. The 16S rRNA and *rec*A genes were amplied from EMD17 genomic DNA using PCR and nucleotide sequences were determined. BLAST searches showed that the 1,197 bp 16S rRNA gene sequence was 100% identical with a 16S rRNA gene from *B. amyloliquefaciens* Y-32-1 (KT833128). The 710 bp *recA* sequence was 99% identical with *B. amyloliquefaciens* BEB33 (KJ009337). For species confirmation, RAPD-PCR was performed for genomic DNA from EMD17 using a 10-mer (S30) as a single primer, as described previously (15). Two 1.1 and 1.5 kb fragments were amplified (results not shown) as unique bands of *B. amyloliquefaciens* strains (15). Thus, EMD17 was positively identified as a *B. amyloliquefaciens*. Genbank accession numbers were KJ000378 and KJ000380 for partial sequences of the 16S rRNA and *recA* genes, respectively.

Inhibition spectrum of *B. amyloliquefaciens* EMD17 *B. amyloliquefaciens* EMD17 inhibited growth of 8 bacteria, including strong inhibition of the 2 pathogens *B. cereus* and *Listeria monocytogenes* (Table 1). Other *Bacillus* strains producing antimicrobial substances were tested for comparision purposes. *B. subtilis* H27 and *B. subtilis* W42 were isolated from *Cheonggukjang*. *B. subtilis* H27 secretes a 4.9 kDa bacteriocin (17) and *B. subtilis* W42 produces a 5.4 kDa bacteriocin (8). *B. amyloliquefaciens* MJ1-4, another isolate from *Meju*, produces uncharacterized non-proteinous inhibitory substance(s) (16). Unlike *B. amyloliquefaciens* MJ1-4, *B. amyloliquefaciens* EMD17 strongly inhibited *B. cereus* ATCC14579, *Enterococcus faecalis* ATCC29212, *Streptococcus thermophilus* KFRI193, and *B. thuringiensis* ATCC33679. *B. amyloliquefaciens* EMD17 strongly inhibited *B. licheniformis* ATCC21415, which was not inhibited by either *B. subtilis* H27 or *B. subtilis* W42. Thus, the inhibitory spectrum of *B. amyloliquefaciens* EMD17 was different from other bacilli previously isolated.

Five fungi were inhibited by *B. amyloliquefaciens* EMD17, including an ochratoxin (OTA) producing *Penicillium* spp. (results not shown). An *Aspergillus* spp. producing aflatoxin B1 (AFB1) was not inhibited by *B. amyloliquefaciens* EMD17, but was inhibited by *B. amyloliquefaciens* CH86-1 (19) and by *B. amyloliquefaciens* CH51 (11) (results not shown). *Penicillium roqueforti*, *Aspergillus niger*, and *Aspergillus awamori* were all strongly inhibited by *B. amyloliquefaciens* EMD17. The 4 plant pathogenic fungi also inhibited by *B. amyloliquefaciens* EMD17 were *Alternaria alternate* KACC40019 that causes alternaria leaf blight, *Botrytis cinerea* KACC40573 that causes gray mold, *Rhizoctonia solani* AG-3 KACC40136 that causes black scurf, and *Sclerotinia sclerotiorum* KACC41068 that causes sclerotinia rot. Considering the inhibition spectrum, *B. amyloliquefaciens* EMD17 is a good candidate for use as a starter for fermented soy foods such as *Cheonggukjang*, *Meju*, and *Doenjang* for inhibition of *B. cereus* and OTA producing *Penicillium* spp. growth. Also *B. amyloliquefaciens* EMD17 can be used as an agricultural biocontrol agent against plant pathogenic fungi (20).

Effect of the growth medium on growth and antimicrobial activity of *B. amyloliquefaciens* EMD17 *B. amyloliquefaciens* EMD17 grew better in LB, BHI, and TSB media than in NB medium. Culture absorbance values (OD_{600}) increased quickly, reaching 1.2-1.4 at 6 h and more than 1.6 at 36 h in LB, BHI and TSB media. However, the value was 0.7 in NB at 6 h, and the highest value of 1.4 was reached at 48 h. In BHI and TSB broth, antimicrobial activities increased in a stepwise fashion and reached the highest activity of 160 AU/mL at 48 h in BHI and 60 h in TSB. In LB medium, the highest activity was 80 AU/mL at 48 h. In NB medium, the highest activity of 160 AU/mL was observed at 48 h. Antimicrobial activities decreased after reaching highest values in all media (results not shown). No activity was detected from cultures in BHI at 96 h. Although *B. amyloliquefaciens* EMD17 showed the same level of antimicrobial activity in all 3 media, NB was used as a subsequent medium for experimentation because the highest antimicrobial activity was reached earlier and some activity still remained at 96 h.

Stability of the antimicrobial substance against enzymes, pH, and heat treatment FS samples were treated with proteases and antimicrobial activities were not affected by proteinase K, protease, trypsin, and pepsin (results not shown). Unlike inhibitory substances in FS samples, BacW42, a bacteriocin secreted by *B. subtilis* W42 and used as a control herein, was completely destroyed by proteinase K (result not shown). Antimicrobial activities of FS samples were not affected after 30 min of exposure at 50, 60, 70, 80, 90, and 100°C, even after 15 min at 121°C. The antimicrobial activity was stable at pH 3 to 10 (results not shown). Thus, the antimicrobial substance in FS samples was not a bacteriocin. Considering the high stability and antifungal activity, the substance could be a cyclic lipopeptide, such

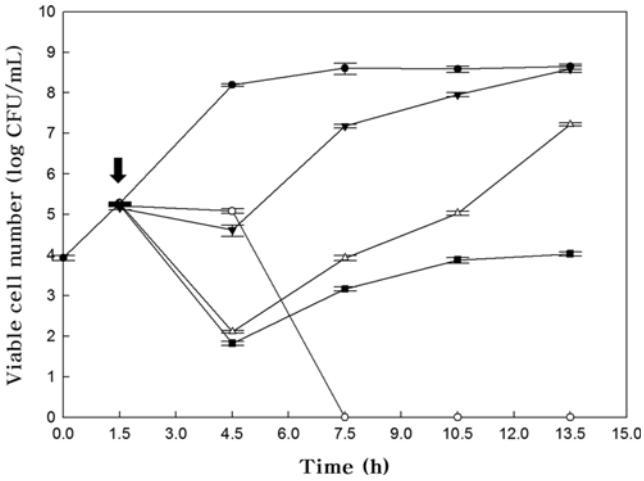

Fig. 1. Growth inhibition of *B. cereus* ATCC14579 using FS samples or cultures of *B. amyloliquefaciens* EMD17. -●-, *B. cereus* culture (control); -○-, *B. cereus* culture with an added *B. amyloliquefaciens* EMD17 culture (1%, v/v); -▼-, *B. cereus* culture with added FS samples from *B. amyloliquefaciens* EMD17 (2%, v/v); -△-, *B. cereus* culture with added FS samples (5%, v/v); -■-, *B. cereus* culture with added FS samples (10%, v/v). A vertical line indicates the time point of FS sample or culture addition.

as surfactin, fengycin, or iturin (21,22), compounds used in biotechnological and pharmaceutical processes due to surface-interface and antimicrobial properties (21).

Inhibition mode of the antimicrobial substance The number of *B. cereus* ATCC14579 cells increased quickly in LB broth for the first 4 h, then increased gradually, reaching a maximum number of $4.4x10^8$ CFU/mL at approximately 8 h. When a 1% *B. amyloliquefaciens* EMD17 culture was added to a *B. cereus* culture, *B. cereus* cells were killed rapidly and were not detected after 6 h when counted using Pemba *B. cereus* selective agar plates (Fig. 1). Addition of 2, 5, 10% (v/v) FS samples reduced viable counts of *B. cereus* for the first 3 h, then after 3 h viable counts increased gradually for the next 12 h, after which viable counts were $3.8x10^8$, $1.6x10^8$, and $1.1x10^4$ CFU/mL for cultures in which 2, 5, and 10% FS samples were added, respectively. The viable count of a *B. cereus* only control was $4.4x10^8$ CFU/mL. Addition of up to 10% FS samples failed to kill *B. cereus* cells completely, perhaps due to a low concentration of the inhibiting substance in FS samples. The inhibitory substance in FS samples was bacteriocidal based on reduction of viable *B. cereus* cell counts.

PCR amplification of lipopeptide genes from *B. amyloliquefaciens* EMD17 The antimicrobial substance secreted by *B. amyloliquefaciens* EMD17 was not a bacteriocin, but was one of many different antimicrobials produced by bacilli. Lipopeptides are the major compounds responsible for antibacterial and antifungal activities. Surfactin, iturin, and fengycin are the most common lipopeptides with antifungal activities (21). *B. amyloliquefaciens* strains secrete lipopeptides including surfactin, iturin, and fengycin into culture supernatants. When culture supernatants from *B. amyloliquefaciens* S13-3 were analyzed using ESI-MS/MS analysis, 11 lipopeptides were identified (20). Surfactin, the most common lipopeptide and the most powerful biosurfactant, is a lipoheptapeptide interlinked with a hydroxyl fatty acid of length C12-C17 (22). Surfactin shows antibacterial, antifungal, antiviral, and antitumoral activities and also inhibits fibrin clot formation. Considering antibacterial and antifungal activities, and high substance stability, surfactin was suspected to be the major agent, if not the sole agent, for the antimicrobial activity of *B. amyloliquefaciens* EMD17.

The presence of a surfactin synthetase gene in the genome of *B.*

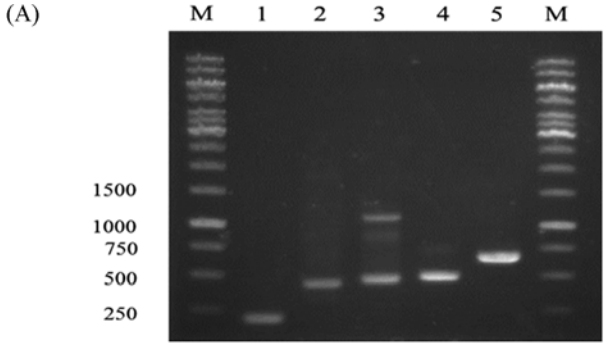

Fig. 2. PCR amplification of selected lipopeptide genes. (A) agarose gel showing amplified fragments. Lane M, 1 kb DNA ladder; 1, *srfAA*; 2, *srf/lch*; 3, *fen* (fengycin); 4, *itu D* (iturine D); 5, *itu A* (iturin A). (B) partial sequence of the PCR product (lane 1 in A). Translated amino acids are shown below nucleotide sequences.

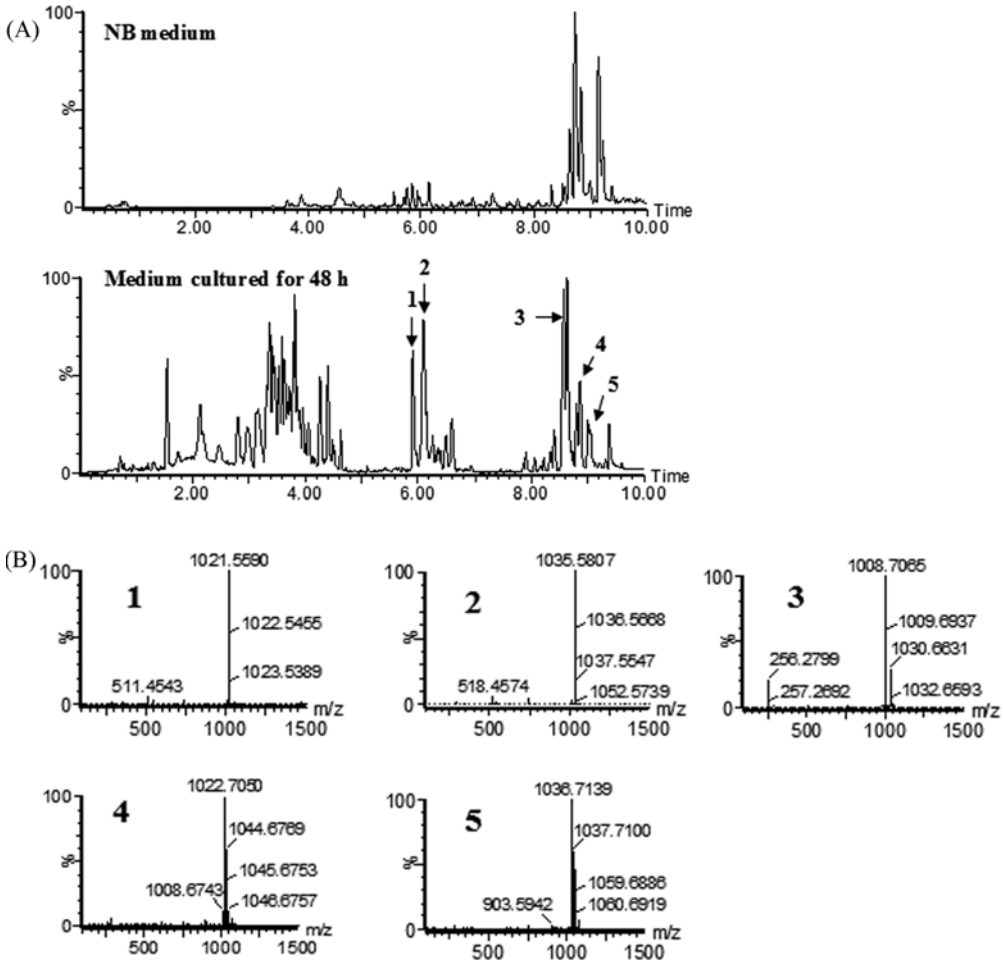

Fig. 3. LC-MS chromatograms of the supernatant from a *B. amyloliquefaciens* EMD17 culture and blank NB medium (A), and MS spectra of cyclic lipopeptides (B).

amyloliquefaciens EMD17 was examined using PCR (Table 2). A 210 bp fragment was amplified and sequenced (Fig. 2A lane 1, and 2B). The sequence was 99% homologous to the surfactin synthetase A gene *srfAA* from *B. amyloliquefaciens* IT-45, to *B. amyloliquefaciens* subsp. *plantarum* CAU B946, and to *B. amyloliquefaciens* SQR9 (results not shown). The fragment was an internal region of a large gene encoding surfactin synthetase A of 3,584 amino acids, and the region was part of the condensation domain (WP_015416786.1). In addition to genes encoding surfactin, genes encoding fengycin and iturin were also present. DNA fragments were amplified using PCR and apparent fragment sizes matched expected sizes (Fig. 2A). Fragment nucleotide sequences were also identical with sequences of genes encoding fengycin and iturin (results not shown). Thus, lipopeptides, including surfactin, fengycin, and iturin, were probably produced and were important for the antimicrobial activity of *B. amyloliquefaciens* EMD17. The *srfA* and *srf/lch* genes were also identified in *B. subtilis* H27, *B. subtilis* W42, and *B. amyloliquefaciens* MJ1-4 when the same primers were used (results not shown). However, the main antibacterial substances inhibiting *B. cereus* are bacteriocins for *B. subtilis* H27 and *B. subtilis* W42 because inhibitory activities were destroyed by protease treatment (8,17), which was not observed for *B. amyloliquefaciens* EMD17.

Mass spectra of culture supernatants *B. amyloliquefaciens* EMD17 was grown in NB for 48 h and the resulting supernatant was analyzed using MS. Many metabolites in the size range of 1,000-1,200 Da were detected (Fig. 3A). Main peak sizes were 1,008.7065, 1,021.5590, 1,022.7050, 1,035.5807, and 1,036.7189 (Fig. 3B). Sizes of peaks 4 and 5 (Fig. 3B) were identical to reported values of surfactin E of 1,022.7 and surfactin of 1,036.7 (20). Sizes of peaks 1 and 2 (Fig. 3B) were similar to sizes of lichenysin A (1,021.7, 1,035.7) produced by *Bacillus licheniformis* (26). Lichenysin A is a cyclic lipoheptapeptide that is structurally similar to surfactin. Surfactins, fengycins, and iturins have similar sizes although the exact size is variable depending upon the specific structure of each lipopeptide (20). PCR and mass spectrometry results indicated that *B. amyloliquefaciens* EMD17 produced lipopeptides, including surfactin, as the main inhibitory substances. Lipopeptides are generally believed to serve as weapons for hosts against other competing organisms in the same environment, but not enough studies have been done to understand the exact role

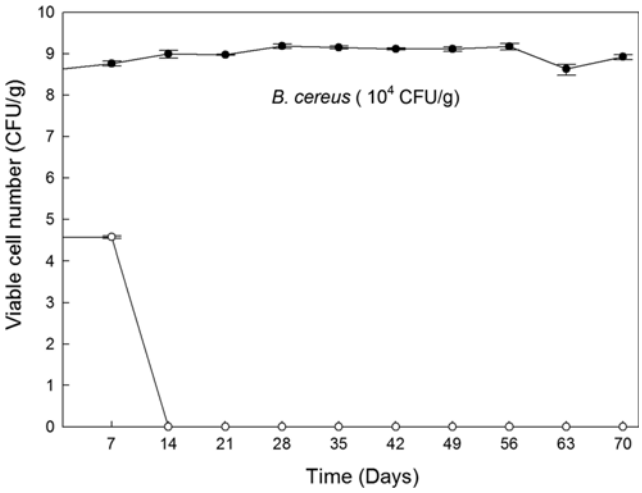

Fig. 4. Changes in total bacilli and *B. cereus* counts during *Doenjang* fermentation. Total bacillus cells (-●-) and *B. cereus* cells (-○-) were counted using LB and Pemba plates, respectively. *Doenjang* fermentation was started via inoculation with *B. amyloliquefaciens* EMD17 at 10^5 cells/g of soybeans, *Pichia farinosa* SY80 at 10^5 cells/g of soybeans, and *Rhizopus oryzae* at 10^5 spores/g of soybeans into cooked soybeans. *Doenjang* was spiked with *B. cereus* ATCC14579 at 10^4 cells/g of soybeans and fermented for 70 days at 25°C.

and benefit of each compound (22). Each *Bacillus* strain is differentially equipped with antimicrobials and, thus, characterization of antimicrobial substances should be done for each strain. Further studies of antimicrobial compounds of *B. amyloliquefaciens* EMD17 are necessary to understand exact inhibitory mechanisms, which will facilitate use of *B. amyloliquefaciens* EMD17 and other bacilli producing antimicrobial compounds as starters for fermented foods.

Inhibition of *B. cereus* by *B. amyloliquefaciens* EMD17 during soyfood fermentation During *Cheonggukjang* fermentation in which *B. amyloliquefaciens* EMD17 and *B. cereus* were co-inoculated, *B. cereus* cells were not detected after 12 h of fermentation and after 10 days of storage at 4°C (results not shown). Thus, *B. cereus* cells were killed rapidly by *B. amyloliquefaciens* EMD17. *B. amyloliquefaciens* EMD17 showed higher inhibition activities against *B. cereus* than against other bacilli. *B. subtilis* W42, a bacteriocin producer, was used as a starter for *Cheonggukjang* and inhibited spiked *B. cereus* ATCC 11778 cells at $1x10^5$ cells/g of dry soybean weight during *Cheonggukjjang* fermentation. However, inhibition was not complete and $1.43x10^3$ cells still remained after 72 h (27).

A high *B. amyloliquefaciens* level of anti-*B. cereus* activity was also observed during *Doenjang* fermentation. *Doenjang* was prepared using whole *Meju* soybeans. After 2 weeks of fermentation at 25°C, *B. cereus* cells were no longer detected (Fig. 4). Total bacilli counts were stable during a 10-week fermentation period, and no *B. cereus* cells were detected on Pemba plates after 2 weeks. Thus, the strong inhibition activity of *B. amyloliquefaciens* EMD17 against *B. cereus* cells in soybean fermentation environments was confirmed. Since the number of intentionally spiked *B. cereus* cells was high at 10^4 cells/g of soybeans, *B. amyloliquefaciens* EMD17 probably prevented environmental *B. cereus* contamination of fermented soyfoods. Although many bacilli inhibit growth of *B. cereus* cells based on agar spot and well diffusion method results, real efficacies have not been confirmed in food systems. In this respect, *B. amyloliquefaceines* EMD17 is different from many other bacilli as the strain efficiently inhibited growth of *B. cereus* in *Cheonggukjang* and *Doenjang* fermentations. Potential use of *B. amyloliquefaciens* EMD17 as a starter for production of microbially safe fermented soyfoods is indicated. Detailed properties of *Doenjang* will be described in the future.

In addition to an antimicrobial activity, *B. amyloliquefaciens* EMD17 also exerted a fibrinolytic activity. Supernatants from 30 h cultures in TSB showed a highest fibrinolytic activity of 61.02 U among LB, BHI, TSB, and NB media (results not shown). Supernatants from 24 h cultures in LB showed the second highest activity of 46.83 U. *B. amyloliquefaciens* EMD17 exerts the 2 desirable properties of pathogenic microorganism inhibition and dissolution of fibrin and, thus, can be useful as a starter for fermented soy foods. The microbial safety of fermented soy foods can be improved by use of starters capable of inhibiting the growth of toxinogenic organisms, such as *B. cereus* and toxin producing fungi. Multiple starters consisting of 2-4 bacilli with different properties will be an effective method for increasing the microbial safety and the functionality of fermented soy foods. In this respect, *B. amyloliquefaciens* EMD17 can be used as a starter singly or in association with multiple starters. The real effectiveness of *B. amyloliquefaciens* as a starter needs to be further investigated.

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