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Improvement of Fibrinolytic Activity of *Bacillus subtilis* 168 by Integration of a Fibrinolytic Gene into the Chromosome

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Fibrinolytic enzyme genes (*aprE2*, *aprE176*, and *aprE179*) were introduced into the *Bacillus subtilis* 168 chromosome without any antibiotic resistance gene. An integration vector, pDG1662, was used to deliver the genes into the *amyE* site of *B. subtilis* 168. Integrants, SJ3-5nc, SJ176nc, and SJ179nc, were obtained after two successive homologous recombinations. The integration of each fibrinolytic gene into the middle of the *amyE* site was confirmed by phenotypes (Amy^-, $Spec^S$) and colony PCR results for these strains. The fibrinolytic activities of the integrants were higher than that of *B. subtilis* 168 by at least 3.2-fold when grown in LB broth. *Cheonggukjang* was prepared by inoculating each of *B. subtilis* 168, SJ3-5nc, SJ176nc, and SJ179nc, and the fibrinolytic activity of *cheonggukjang* was 4.6 ± 0.7, 10.8 ± 0.9, 7.0 ± 0.6, and 8.0 ± 0.2 (U/g of *cheonggukjang*), respectively at 72 h. These results showed that construction of *B. subtilis* strains with enhanced fibrinolytic activities is possible by integration of a strong fibrinolytic gene *via* a marker-free manner.

Keywords: *Bacillus subtilis*, fibrinolytic genes, chromosome integration, integration vector

Introduction

Bacillus subtilis and closely related species play important roles for the fermentation of various Asian soy foods, including Korean *doenjang*, *cheonggukjang*, and *ganjang*, Japanese *natto*, Chinese *douchi*, and Indonesian *gembus* [14]. Bacilli secrete amylases and proteases, which are responsible for the degradation of nutrients in soybeans, production of peptides and amino acids, and flavoring compounds [13, 16]. Some secreted proteases possess fibrinolytic activities and degrade fibrin directly. Nattokinase secreted by some *B. subtilis* strains is the most well-known example [18]. In recent years, fibrinolytic enzymes from bacilli have been the subject of extensive studies because of their ability to degrade fibrin, the major cause of thrombolytic diseases such as acute myocardial infarction and cerebral infarction [12]. In addition to serving as a source for therapeutic agents, bacilli strains with fibrinolytic activities are useful as starters for fermented soy foods. *Cheonggukjang*, a Korean fermented soy food, is prepared by inoculation of cooked soybeans with bacilli and following fermentation for 2 days at 37–42°C. *Cheonggukjang* is a rich source for bacilli and bioactive metabolites, including fibrinolytic enzymes. *Cheonggukjang* is consumed after being boiled with added condiments. If *cheonggukjang* is consumed without heat treatment after fermentation, the fibrinolytic activity of *cheonggukjang* could be enjoyed in addition to the probiotic effect of bacilli. If the fibrinolytic activities of fermented soybean foods are to be increased, *Bacillus* strains with strong fibrinolytic activities should be used as starters. An efficient method to increase the fibrinolytic activity of a strain is the introduction of a fibrinolytic gene into the strain. However, the introduction of a gene should be conducted by a food-grade way. In most genetic

Table 1. Bacterial strains and plasmids used in this study.

Strains, plasmids, or primers	Description	Source or reference
E. coli DH5α	φ80d*lacZ*ΔM15, Δ*(lacZYA-argF)*U169, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hdR17* (r_K^-, m_K^+), *supE44*, *relA1*, *deoR*, *phoA*	Lab strain
B. subtilis 168	*trpC2*	Lab strain
B. subtilis CH3-5	Wild-type strain, high fibrinolytic activity	[9]
B. subtilis HK176	Wild-type strain, high fibrinolytic activity	[11]
B. subtilis PD3-5nc	*B. subtilis* 168 derivative, pDG3-5nc inserted into the *amyE* locus of 168	This study
B. subtilis PD176nc	*B. subtilis* 168 derivative, pDG176nc inserted into the *amyE* locus of 168	This study
B. subtilis PD179nc	*B. subtilis* 168 derivative, pDG179nc inserted into the *amyE* locus of 168	This study
B. subtilis SJ3-5nc	*B. subtilis* 168 derivative, *amyE::aprE2*	This study
B. subtilis SJ176nc	*B. subtilis* 168 derivative, *amyE::aprE176*	This study
B. subtilis SJ179nc	*B. subtilis* 168 derivative, *amyE::aprE179*	This study
Plasmids		
pDG1662	6.98 kb, Am^r, Spc^r, Cm^r, *amyE* segments	BGSC
pDG3-5nc	pDG1662 (ΔCm^r) with 1.7 kb *Bam*HI-*Sal*I fragment containing *aprE2*	This study
pDG176nc	pDG1662 (ΔCm^r) with 1.5 kb *Bam*HI-*Sal*I fragment containing *aprE176*	This study
pDG179nc	pDG1662 (ΔCm^r) with 1.5 kb *Bam*HI-*Sal*I fragment containing *aprE179*	This study

Am^r, ampicillin resistance gene; Spc^r, spectinomycin resistance gene; Cm^r, chloramphenicol resistance gene; *amyE*, α-amylase gene.
BGSC, *Bacillus* Genetic Stock Center (Columbus, OH, USA)

engineering studies, a target gene is introduced together with an antibiotic resistance gene, which is used as a selection marker [4]. However, an antibiotic resistance gene is not allowed if the host organism is used for food fermentation.

In previous studies, we characterized strong fibrinolytic enzymes secreted by *B. subtilis* strains; AprE2 from *B. subtilis* CH3-5 [7, 8] and AprE176 from *B. subtilis* HK176 [9]. We also improved AprE176 by error-prone PCR [9]. In this work, we introduced *aprE2*, *aprE176*, and *aprE179* (a mutant from *aprE176*) into the chromosome of *B. subtilis* 176 without an antibiotic resistance gene. We measured the fibrinolytic activities of the recombinant strains and prepared *cheonggukjang* using the integrants. Introduction of a fibrinolytic gene into a nonessential gene on the chromosome of a *Bacillus* strain *via* a food-grade manner seems an effective method to improve the fibrinolytic capacity of a *Bacillus* strain.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. All *B. subtilis* recombinants were derived from *B. subtilis* 168. *B. subtilis* and *E. coli* DH5α were grown in LB broth (Luria Bertani broth; Acumedia, Lansing, MI, USA) at 37°C with aeration. *B. subtilis* PD3-5nc, PD176nc, and PD179nc were grown in LB containing spectinomycin (100 μg/ml; Sigma, St. Louis, MO, USA). For *E. coli* cells harboring pDG1662, pDG3-5nc, pDG176nc, or pDG179nc, ampicillin (100 μg/ml, Sigma) was included in the LB medium.

Construction of Integration Plasmids

AprE2 was amplified from *B. subtilis* CH3-5 by using a primer pair, aprEFB and aprERS (Table 2). *aprE176* was amplified from *B. subtilis* HK176 by using a primer pair, 51F and 51R-S (Table 2). The PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles consisting of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min. The *aprE179* gene was amplified by splicing overlap extension PCR (SOE-PCR) as described previously [11]. The SOE-PCR was completed through two rounds of PCR. The 179up (1–1,206 bp) and 179down (1,185–1,528 bp) fragments of *aprE179* were amplified using the 51F and 179siteR, and 179siteF and 51R-S primer pairs, respectively. One microliter of the first-stage PCR product was used as the template for the second-stage PCR, and the primers 51F and 51R-S were used to amplify the full-length *aprE179*. The PCR conditions were as follows: 94°C for 5 min, followed by the first 10 cycles consisting of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min and the next 20 cycles consisting of 94°C for 30 sec, 63°C for 30 sec, and 72°C for 1 min.

The amplified *aprE2*, *aprE176*, and *aprE179* genes were inserted into plasmid pDG1662 after being digested with *Bam*HI and *Sal*I (Fig. 1). *E. coli* DH5α competent cells were prepared and transformed by electroporation as described previously [9]. *B. subtilis* 168 competent cells were prepared and transformed by the two-step transformation method of Cutting and Vander Horn [3].

Table 2. Primers used in this study.

Primers	Sequences (5′ to 3′)	References
aprEFB	GCGGATCCGCCGCATCTGTGTCTTTG (*Bam*HI site underlined)	This study
aprERS	GCGTCGACGAGAACAGAGAAGCCGCT (*Sal*I site underlined)	This study
51F	AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC (*Bam*HI site underlined)	[12]
51R-S	AGTCGACTTCAGAGGGAGCCACCCGTCGATCA (*Sal*I site underlined)	This study
179siteF	TTCTGTCATTACGGTAGGCGCT (substitution site underlined)	This study
179siteR	AGCGCCTACCGTAATGACAGAA (substitution site underlined)	This study
amylF	TGGCAACCGTTACTTAGG	This study
amylR	ATACCTGCCATCAGGCAA	This study
spcF	GGTTCAGCAGTAAATGGTGG	This study
spcR	TCCTTCCCACTTATCATCAC	This study

Two-Step Replacement Recombinations

The two-step replacement recombination procedures are described in Fig. 2 [17]. In the first step, *B. subtilis* 168 cells harboring each integration plasmid were cultivated in LB broth containing spectinomycin (100 μg/ml) at 37°C and integrants were screened on LB plates with spectinomycin. In the second step, an integrant obtained from the first step was cultivated in LB broth without an antibiotic for 18 h at 37°C. Then the temperature was increased to 45°C and the plates were incubated for the next 24 h. Then spectinomycin-sensitive (Spc^S) colonies were screened on LB plates. Colony PCR was used to confirm the structure of Spc^r clones from the first integration stage and Spc^S clones from the second crossover events. A small portion of cells was scraped from a colony and introduced into a 0.2 ml Eppendorf tube containing 10 μl of 2× PCR mixture (GoTag Long PCR Master; Promega, Madison, WI, USA). PCRs were done using various primer pairs and the amplification program consisted of 93°C for 3 min, 35 cycles of 93°C for 15 sec, 62°C for 30 sec, and 68°C for

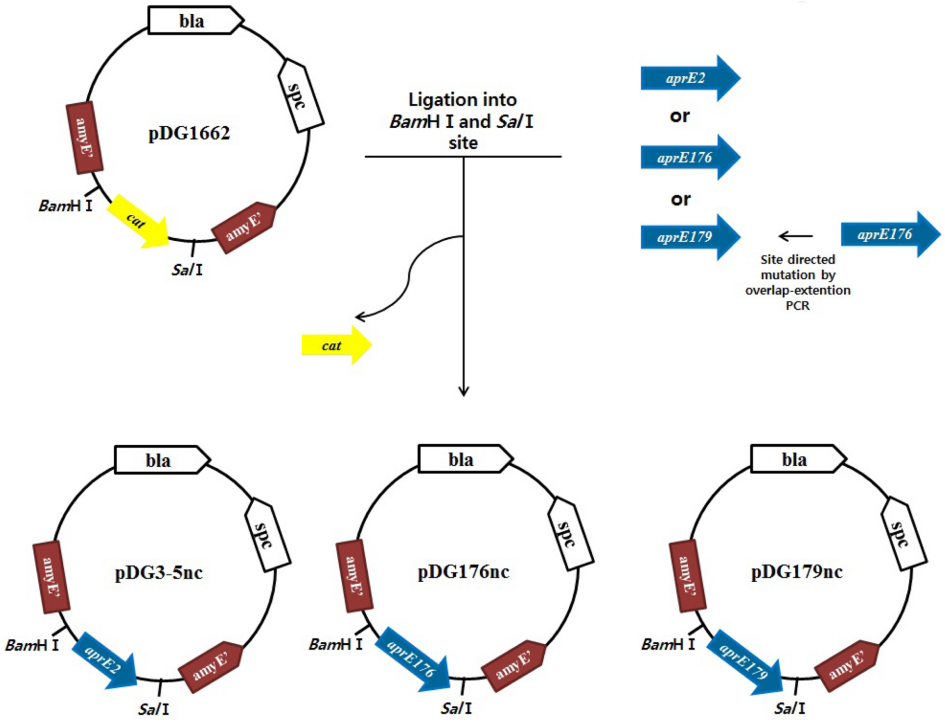

Fig. 1. Construction of integration plasmids.

The fibrinolytic enzyme gene fragments were ligated into the *Bam*HI and *Sal*I site of the pDG1662 integration vector. bla, ampicillin resistance gene; spc, spectinomycin resistance gene; cat, chloramphenicol resistance gene; *amyE*, α-amylase gene.

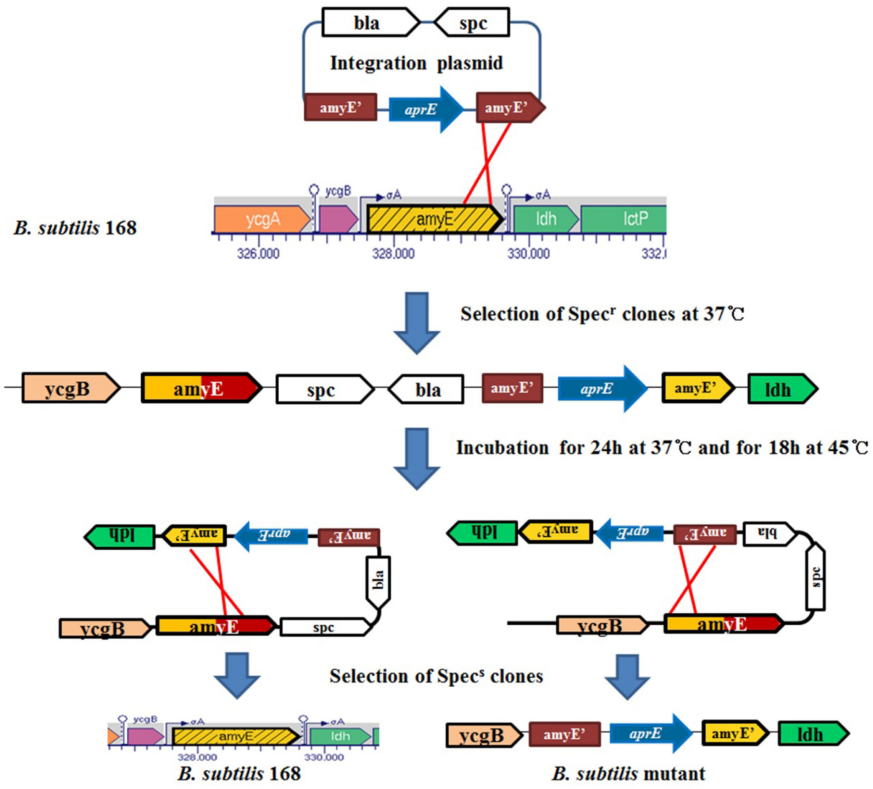

Fig. 2. Schematic of the markerless gene insertion procedures conducted in *B. subtilis* 168.
The first crossover event is shown in the upper panel, and the second crossover event is in the lower panel. The first crossover between the target gene (*amyE*). bla, ampicillin resistance gene; spc, spectinomycin resistance gene; cat, chloramphenicol resistance gene; *amyE*, α-amylase gene; *aprE*, fibrinolytic enzyme gene.

4 min. After the PCR, 5 μl of each amplified product was analyzed by agarose gel (1% (w/v)) electrophoresis.

Preparation of *Cheonggukjang*

Cheonggukjang was prepared from soybeans (2013 crop year, Hamyang, Gyeongnam, Korea). Soybeans (300 g) were washed and soaked in water for 15 h at room temperature. After the water was decanted, the whole soybeans were autoclaved for 45 min at 121°C. Soybeans were inoculated with *B. subtilis* strains (2% inoculum size, dry soybean weight (v/w)): 168 (control *cheonggukjang*), SJ3-5nc, SJ176nc, and SJ179nc. Fermentation was proceeded for 3 days at 37°C and *cheonggukjang* samples were taken at time points (6, 12, 24, 36, 48, 60, and 72 h) for measuring the cell numbers and fibrinolytic activities. The fibrinolytic activities of culture supernatant and *cheonggukjang* were assayed by using the fibrin plate method as described previously [7, 10]. Plasmin (Sigma) was spotted on a fibrin plate at different concentrations (3–40 mU) and the plate was incubated for 18 h at 37°C. The size of the lysis zone was measured using a Vernier caliper and a standard curve was obtained. The protein concentration of a sample was determined by the Bradford method [2] using bovine serum albumin as the standard. All measurements were done in triplicates and the means were represented with standard deviations.

Results and Discussion

Construction of Integration Plasmids

A 1.7 kb fragment containing gene *aprE2* was amplified from the *B. subtilis* CH3-5 genome. The fragment included the putative promoter sequences and the possible transcription terminator. By the same way, a 1.5 kb *aprE176* gene was amplified from *B. subtilis* HK176. *aprE179*, a mutant derived from *aprE176*, was obtained by the splicing overlap extension PCR technique. *aprE179* differs from *aprE176* in a single nucleotide. The 526th nucleotide from the start codon, GTG, is G in *aprE176* but A in *aprE179*, causing the amino acid change Ala to Thr [9]. Each amplified fragment was cloned into pDG1662 at the *Bam*HI and *Sal*I sites, resulting in pDG3-5nc, pDG176nc, and pDG179nc, respectively (Fig. 1). The three integration plasmids had the same structure and a fibrinolytic gene was located in the middle of *amyE*.

Integration of a Fibrinolytic Gene into the Chromosome of *B. subtilis* 168

B. subtilis 168 was selected as the host for the integration of the fibrinolytic genes because this strain has a basal level of fibrinolytic activity and is easily transformed. Transformation of *B. subtilis* CH3-5 and *B. subtilis* HK176, wild-type strains isolated from *cheonggukjang*, was not successful.

Selection of the integrants, where the whole plasmid was integrated into the *amyE* site of the *B. subtilis* 168 chromosome, was performed using LB plates containing spectinomycin. Colonies on LB plates with spectinomycin were examined for the plasmid integration by colony PCR (Fig. 3). Amplification of the *amyE* and spectinomycin resistance genes was carried out. When primers for *amyE* were used, two bands were amplified from the integrants but a single band was amplified from *B. subtilis* 168 (Fig. 3A, lanes a1−a4). There were two *amyE* genes in the integrants; one copy was an intact *amyE* and the other contained *aprE* in the middle of the gene (Fig. 2). When primers for the spectinomycin resistance gene were used, a 600 bp fragment was amplified from *B. subtilis* PD3-5nc, PD176nc, and PD179nc but not from *B. subtilis* 168 (Fig. 3A, lanes b1−b4). These results indicated that pDG1662 plasmids containing different fibrinolytic genes were individually integrated into the chromosome of *B. subtilis* 168 by single crossover.

B. subtilis PD3-5nc, PD176nc, and PD179nc, integrants obtained from the first round of single crossover, were forced to undergo the second round of crossover as mentioned in the Methods section. Colonies on LB plates were examined. Recombinants were expected, which were generated by the second homologous recombination between two *amyE* sequences [6]. Depending upon the location where homologous recombination occurred, two different recombinants were expected (Fig. 3). One was reverted to wild type, *B. subtilis* 168, and the other was the strain where a fibrinolytic gene remained in the middle of the *amyE* site but the Spc^r and Amp^r genes were deleted (Fig. 2). Colonies on LB agar plates were spotted onto LB plates with 1% soluble starch or spectinomycin (100 μg/ml). After several trials, colonies showing Spe^S and Amy^- were obtained. When PCR was done using the amylF/amylR primer pair, a 1.5−1.7 kb fragment was amplified, whereas a smaller band was amplified from *B. subtilis* 168 (Fig. 3B, lanes a1, a5, and a7). When the amylF/51R-S primer pair was used, 1.6−1.7 kb bands were amplified from the three recombinants but no band was amplified from *B. subtilis* 168 (Fig. 3B, lanes c1, c5, c6, and c7). These colony PCR

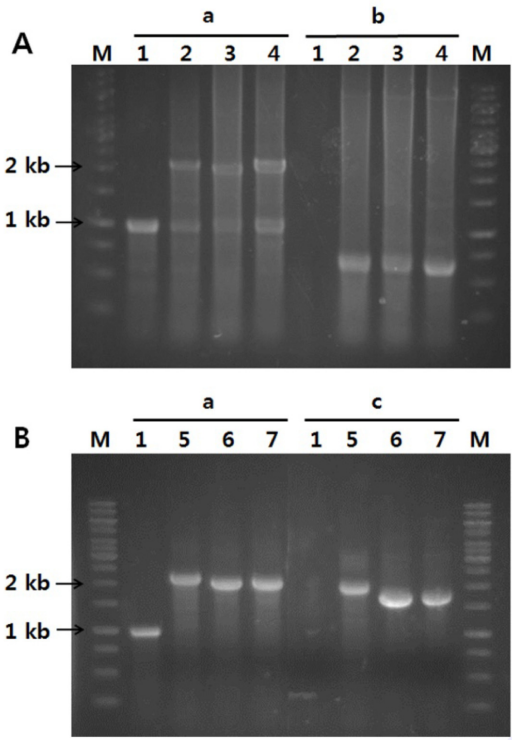

Fig. 3. Colony PCR analyses of recombinants.
(**A**) Colonies after the first crossover. (**B**) Colonies after the second crossover. Fragments were amplified using the amylF/amylR primer pair (a), the spcF/spcR primer pair (b), and the amylF/51R-S primer pair (c). Lanes M, size marker (Gene Ruler 1 kb DNA ladder; Fermentas Vilnius, Lithuania); 1, *B. subtilis* 168; 2, *B. subtilis* PD3-5nc; 3, *B. subtilis* PD176nc; 4, *B. subtilis* PD179nc; 5, *B. subtilis* SJ3-5nc; 6, *B. subtilis* SJ176nc; and 7, *B. subtilis* SJ179nc.

results together with the observed phenotypes confirmed that the strains were obtained through the 2nd homologous recombination. They were named *B. subtilis* SJ3-5nc, SJ176nc, and SJ179nc, respectively.

Incubation temperature is an important factor to increase the frequency of the second crossover event. The desired recombinants were obtained only after cells were first incubated for 24 h at 37°C and another 18 h at 45°C. Several researchers used plasmids with temperature-sensitive replication for the integration into the host chromosome because the transformation efficiency is much higher than non-replicating DNA. Inside the host, the plasmid replicates at the permissive temperature but cannot replicate when the temperature increases. Colonies grown on plates with an antibiotic are those cells where the entire plasmid was integrated into the host chromosome. pMAD, a replication-thermosensitive mutant of pE194, was used for two-step gene replacement in some gram-positive

bacteria [1]. pNZT1, a plasmid with thermosensitive replication, was used for the replacement of the native *glcU-gdh* operon promoter with the *pur* operon promoter in *Bacillus amyloliquefaciens* [19]. Unlike these vectors, pDG1662 does not replicate in *Bacillus* species, and it is in fact an *E. coli* plasmid [5]. Thus, the efficiency for the integration of pDG1662 into *B. subtilis* 168 chromosome was not high. Still, colonies were obtained that grew on LB with spectinomycin. Integrants where the whole plasmids were inserted into the *amyE* site of *B. subtilis* 168 were incubated at different temperatures (37°C, 40°C, and 45°C) and times for the second crossover event. We found a condition under which the frequency of the second single crossover event was increased. By incubating the integrants for 18 h at 37°C and another 24 h at 45°C, the desired strains were obtained.

Fibrinolytic Activity of Recombinant Strains

B. subtilis SJ3-5nc, SJ176nc, and SJ179nc were cultured in LB broth for 144 h and their growth and fibrinolytic activities were measured (Fig. 4). No differences were observed in growth between *B. subtilis* 168 and its derivatives. The OD_{600} values reached 1.5–1.6 in 96 h, and then decreased rapidly for all four cultures (Fig. 4). The fibrinolytic activities of the recombinants increased gradually and reached the maximum values at 120 h. The highest fibrinolytic activities of *B. subtilis* 168, SJ3-5nc, SJ176nc, and SJ179nc were 5.0 ± 0.3, 23.3 ± 1.3, 15.8 ± 1.5, and 17.4 ± 1.0 U/ml, respectively (Fig. 4B). *B. subtilis* SJ3-5nc showed 4.7-fold higher activity than *B. subtilis* 168. *B. subtilis* SJ179nc showed 3.5-fold and *B. subtilis* SJ176nc showed 3.2-fold higher activity than *B. subtilis* 168. *B. subtilis* SJ3-5nc, where gene *aprE2* was integrated into the chromosome, showed higher activity than those with the *aprE176* or *aprE179* gene was integrated at the same locus. The fibrinolytic activities of *B. subtilis* 168 derivatives constructed through this work are less than those of *B. subtilis* CH3-5 and *B. subtilis* HK176 (data not shown). One of the reasons is that only a single gene was introduced into *B. subtilis* 168, but actually many gene products contribute to the fibrinolytic activity of *Bacillus* strains. *B. subtilis* CH3-5 and *B. subtilis* HK176 possess high levels of fibrinolytic activities, but all the responsible enzymes are not well understood, necessitating further research.

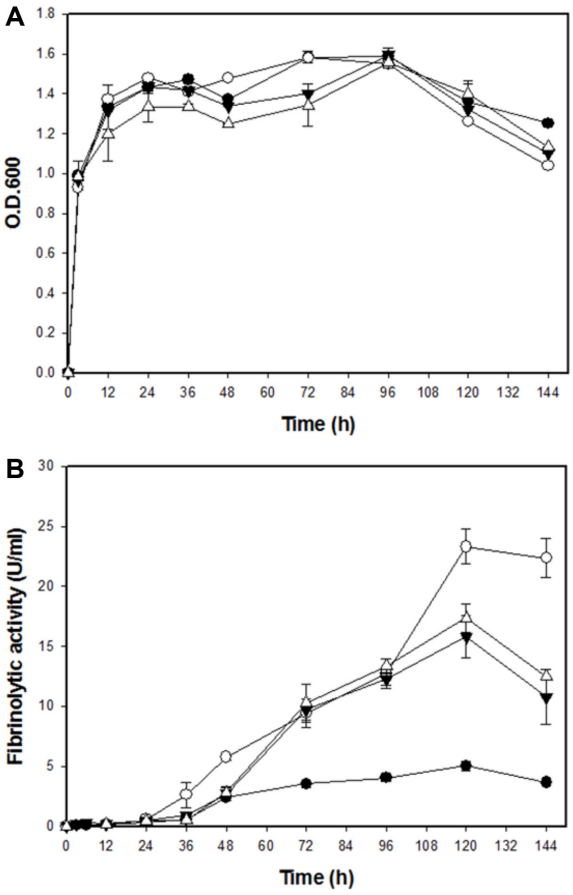

Fig. 4. Growth and fibrinolytic activities of *B. subtilis* mutants.
B. subtilis 168 (●), SJ3-5nc (○), SJ176nc (▼), and SJ179nc (△) were grown in LB for 144 h.

Cheonggukjang Fermentation

B. subtilis 168, SJ3-5nc, SJ176nc, and SJ179nc were individually inoculated into cooked soybeans and *cheonggukjang* fermentation was performed at 37°C. All

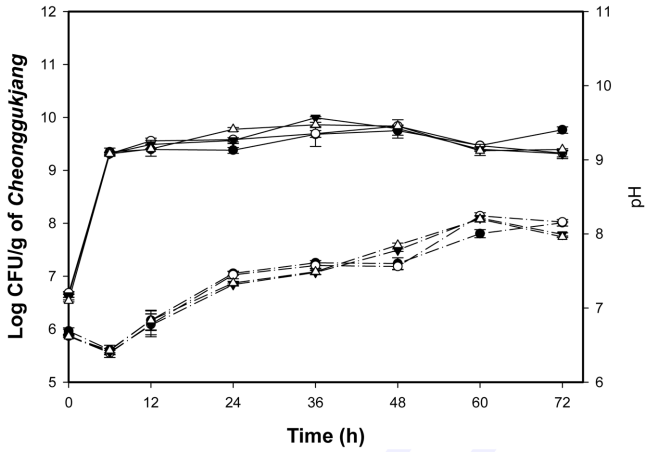

Fig. 5. Viable cell counts and pH change during *cheonggukjang* fermentation at 37°C for 72 h.
B. subtilis 168 (●), SJ3-5nc (○), SJ176nc (▼), and SJ179nc (△) were used as starters. Solid line, viable cell counts; dashed line, pH.

four bacilli strains showed good growth, and the viable counts increased rapidly from 10^6 to 10^9 CFU/g of *cheonggukjang* within the first 6 h (Fig. 5). The pH of *cheonggukjang* increased from 7.0 to 8.0 after 60 h (Fig. 5). The increase in pH was probably the result of proteolysis and the release of ammonia following the utilization of amino acids by bacilli. Sarkar *et al.* [15] reported that the increase in pH of soybean fermented with *Bacillus* sp. DK-WI was coincident with the increase in the proteolytic activity of *Bacillus* and ammonia concentration during fermentation [15].

The fibrinolytic activities of *cheonggukjang* remained at basal levels during the first 6 h, and then increased, except for *cheonggukjang* fermented with *B. subtilis* 168 (Fig. 6). The fibrinolytic activities increased gradually and reached the highest values at 72 h. The fibrinolytic activity of *cheonggukjang* fermented with *B. subtilis* 168, SJ3-5nc, SJ176nc, and SJ179nc was 4.6 ± 0.7, 10.8 ± 0.9, 7.0 ± 0.6, and 8.0 ± 0.2 U/g of *cheonggukjang*, respectively. *Cheonggukjang* fermented with *B. subtilis* SJ3-5nc showed the highest fibrinolytic activity. *B. subtilis* SJ3-5nc also showed the highest activity among the recombinants when grown in LB broth. *Cheonggukjang* prepared with *B. subtilis* CH3-5 showed the fibrinolytic activity of 72 U/g at 60 h and *cheonggukjang* prepared with *B. subtilis* HK176 showed 55 U/g at 48 h (data not shown). The results were not surprising because *B. subtilis* 168 has a low level of fibrinolytic activity and the introduction of a gene was not enough to increase the fibrinolytic activity drasticially. Because the purpose of this work was to examine the possibility of improving the fibrinolytic activity of *Bacillus* strains through the introduction of a gene without an antibiotic marker, properties of *cheonggukjang* such as flavor, texture, and production of metabolites were not examined in detail at this time. Unlike *cheonggukjang* prepared with *B. subtilis* CH3-5 or *B. subtilis* HK176, *cheonggukjang* prepared with *B. subtilis* 168 and its integrants did not produce slime materials. As the next step for the production of high-quality *cheonggukjang*, *Bacillus* strains conferring good organoleptic properties to *cheonggukjang* will be selected and their fibrinolytic activities will be improved by the methods shown in this work.

The results show a possibility that *Bacillus* strains can be improved and become more suitable starters for soyfood fermentations. Fibrinolytic enzymes from *Bacillus* strains are important bioactive compounds that can increase the functionality of fermented foods. Introduction of fibrinolytic genes into *Bacillus* hosts is an effective way for improving host strains. However, it should be carried out *via* a food-grade way because an antibiotic resistance gene is not allowed for starters used for food production. In addition to antibiotic resistance markers, food-grade vectors and hosts should not contain any sequences derived from harmful or potentially harmful organisms such as *E. coli*. In this respect, the strains constructed through this work can be regarded as food-grade hosts because the fibrinolytic genes are derived from *B. subtilis* strains, which have been used for food fermentations and are considered generally recognized as safe organisms. In the future, construction of strains with higher fibrinolytic activities than strains constructed through this work should be tried. The usefulness of such strains is also checked through soyfood fermentations.

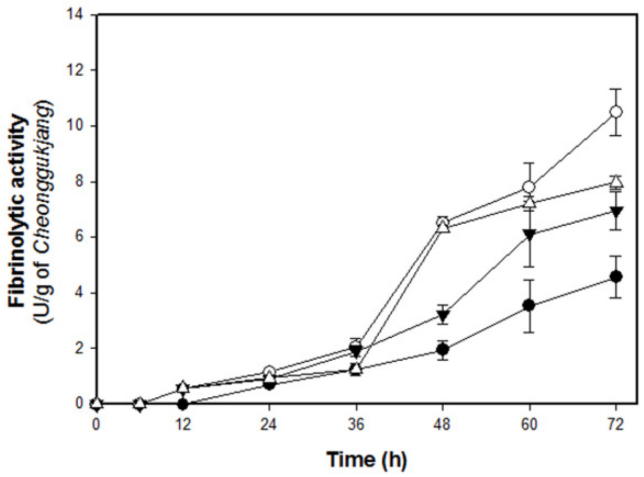

Fig. 6. Fibrinolytic activities of *cheonggukjang* during fermentation at 37°C for 72 h.
B. subtilis 168 (●), SJ3-5nc (○), SJ176nc (▼), and SJ179nc (△) were used as starters.

Acknowledgments

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