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Short Communication

Bacterial degradation of spermine and expression of spermidine/spermine acetyltransferase in *Bacillus subtilis* (natto) under liquid cultivation

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The polyamines spermine (Spm), spermidine (Spd), and their precursor, putrescine (Put), are aliphatic polycations that have important roles in a wide variety of basic cellular functions (Pegg, 2016). Although all organisms, from bacteria to animals and plants, have biosynthetic pathways for the production of polyamines (Kusano et al., 2008), polyamine levels decrease as organisms age due to a reduction in the activities of biosynthetic enzymes (Nishimura et al., 2006). Previous studies have shown that this reduction in polyamine levels is a trigger for several age-associated diseases that can be treated through the administration of exogenous polyamines (Soda et al., 2013).

Soybean fermented using *Bacillus subtilis* (natto) is one of the most polyamine-rich foods available due to the high amounts of these chemicals in soybean (Kalač, 2014; Nishibori et al., 2007; Okamoto et al., 1997). In Japan, fermented soybean is called "natto". It is reported that the long-term intake of natto increases levels of Spm in the blood of human volunteers (Soda et al., 2009). Our previous studies have shown that polyamines persist throughout the natto production processes (Kobayashi et al., 2016, 2017) and additional Spd was produced by *B. subtilis* (natto). However, bacterial fermentation decreases levels of Spm.

Since the levels of polyamine in cells are regulated by biosynthesis, degradation, and transport, *B. subtilis* (natto) also possesses a polyamine degradation mechanism. However, it is widely accepted that bacteria do not produce Spm (Michael, 2016) and direct degradation of Spm by *B. subtilis* (natto) is unproven. Two proteins, BltD (Woolridge et al., 1997, 1999) and PaiA (Forouhar et al., 2005), have been reported from *B. subtilis* 168 to be components of a putative spermidine/spermine acetyltransferase (SSAT) that could initiate polyamine degradation via acetylation. The genes encoding SSAT (*bltD* and *paiA*) are not essential for *B. subtilis* 168 according to BSORF *Bacillus subtilis* genome database (http://bacillus.genome.jp/). Additionally, it is unclear if these proteins play an important role in polyamine degradation in *B. subtilis* (natto). Our study therefore investigated polyamine degradation by *B. subtilis* (natto) to determine if bacterial fermentation affects exogenous polyamine levels.

In this study, polyamine degradation by *B. subtilis* (natto) BEST 195 was evaluated during liquid cultivation. We used E9 medium (Birrer et al., 1994), a synthetic minimal culture, and Luria-Bertani broth, a natural medium, to observe the effects of nutrient conditions on polyamine degradation. *B. subtilis* (natto) BEST 195 was inoculated into 50 mL of LB broth and cultivated overnight with continuous shaking. After reaching OD_{660} 1.0 in a 1% NaCl solution, a 0.1-mL aliquot of pre-culture was added to 10 mL of E9 medium supplemented with 10^{-4} g/L of biotin or LB broth in glass tubes and cultivated at 37°C with continuous shaking at 120 rpm.

Polyamines were extracted as previously described (Kobayashi et al., 2016). Before extraction, 1 μmol of diaminohexane dihydrochloride was added to the sample as an internal standard. Polyamines were analyzed by post-column chromatography using *o*-phthalaldehyde, as previously described (Kobayashi et al., 2017).

Firstly, polyamine degradation was determined using two

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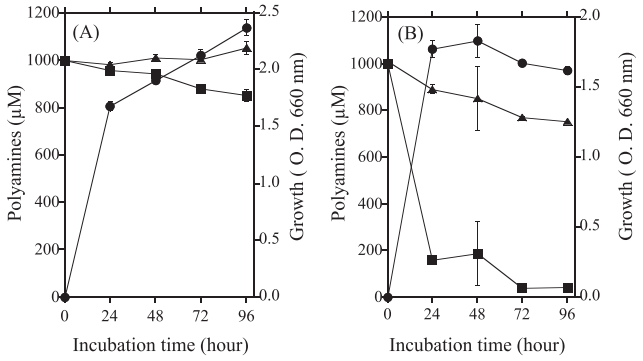

Fig. 1. Polyamine degradation by *Bacillus subtilis* (natto) BEST 195.

Degradation of polyamine in (A) E9 medium, and (B) LB broth. Circles, triangles, and squares indicate growth, spermidine, and spermine, respectively. Data are means ± standard error, obtained from triplicate assays.

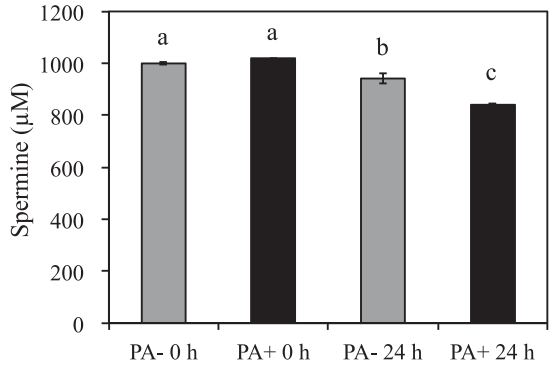

Fig. 2. Spermine degradation by crude enzyme extracted from cells.

Gray and black columns indicate assays with and without polyamine supplementation, respectively. Data are means ± standard error, obtained from triplicate assays. Statistical analysis was performed using the Tukey-Kramer method. Each letter indicates a significant difference ($p < 0.05$).

liquid cultures supplemented with 1 mM Spd and 1 mM Spm. While only small reductions in Spm was observed using the E9 minimal medium (Fig. 1A), *B. subtilis* (natto) BEST 195 degraded both Spd and Spm in LB broth (Fig. 1B). We observed that Spm degradation occurred faster than Spd degradation, supporting previous studies suggesting a reduction in Spm during the natto fermentation process (Kobayashi et al., 2016, 2017). Spm has been reported to be the preferred substrate for SSAT (Forouhar et al., 2005; Woolridge et al., 1999). From these data, nutrient conditions appear to influence polyamine degradation by *B. subtilis* (natto).

To confirm that polyamine degradation was related to the SSAT reaction, we performed an acetylation assay following the method developed by Woolridge et al. (1999), with slight modification. Briefly, crude enzyme was extracted from cells after incubation using LB broth supplemented with or without polyamines (1 mM Spd and 1 mM Spm). Cells in the exponential growth phase were pelleted by centrifugation and re-suspended in 50 mM Tris buffer (pH 7.5) supplemented with ProteoGuard TM EDTA-Free Protease Inhibitor cocktail (Clontech Laboratories, Inc., CA). Cells were lysed by vortexing with glass beads and

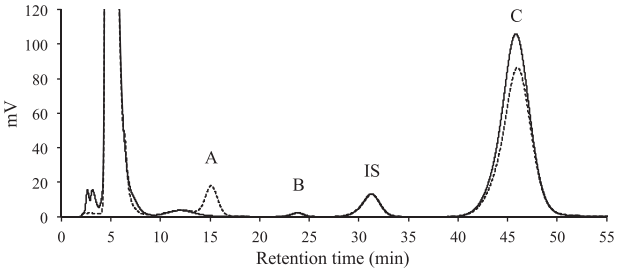

Fig. 3. Comparison of polyamine separations by HPLC between before and after acetylation assay.

Polyamine was labeled with *o*-phthalaldehyde and detected using a florescence detector. A: N^1-acetylspermine, B: spermidine, C: spermine, IS: diaminohexane. Solid and broken lines show PA+ 0 hours and PA+ 24 hours, respectively.

Table 1. Primer sequences.

Gene	Primer	Sequences	mer	Amplicon size (bp)
bltD	Forward	acctgtcgggctttactatga	21	99
	Reverse	agccagactcgtccgttttt	20	
paiA	Forward	tgggcttggcaaacatctgt	20	138
	Reverse	ccggtctgaacaaaccccat	20	
16S rRNA	Forward	actcctacgggaggcagcagt	21	200
	Reverse	gtattaccgcggctgctggcacg	23	

centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was mixed with equal parts 2 mM Spm, 500 μM Acetyl-CoA, 50 mM Tris-HCl (pH 7.5), 1 mM DTT, supplemented with the protease inhibitor cocktail and then incubated at 37°C for 24 hours. Finally, the solution was supplemented with equal parts 0.4 M $HClO_4$ supplemented with 100 nmol of diaminohexane dihydrochloride to halt the reaction and the Spm level was determined with HPLC. After the reaction, the Spm levels decreased slightly to 94.2% in the extracts from cells incubated without supplementation, while polyamine supplementation led to a significant decrease in Spm levels to 84.3% (Fig. 2). In addition, N^1-acetylspermine was detected in the chromatogram after incubation (Fig. 3). These results demonstrate that polyamine degradation involves SSAT and that polyamine acetylation is enhanced by polyamine supplementation.

SSAT levels are strongly regulated by transcription, translation, and post-translation (Perez-Leal and Merali, 2012). Therefore, to investigate whether SSAT was involved in polyamine degradation, we evaluated the mRNA levels of genes that encode SSAT (*bltD* and *paiA*) with quantitative RT-PCR. *B. subtilis* (natto) BEST 195 was cultivated to the exponential growth phase in two liquid cultures, either with or without polyamine supplementation. Total bacterial RNA was extracted using ISOGEN (Nippon gene, Toyama, Japan) according to the manufacturer's protocol. Total RNA (200 ng) was reverse-transcribed with a PrimeScript 1st strand cDNA synthesis kit (TAKARA BIO INC., Shiga, Japan) using random primers at 30°C for 10 minutes and then at 42°C for 30 min-

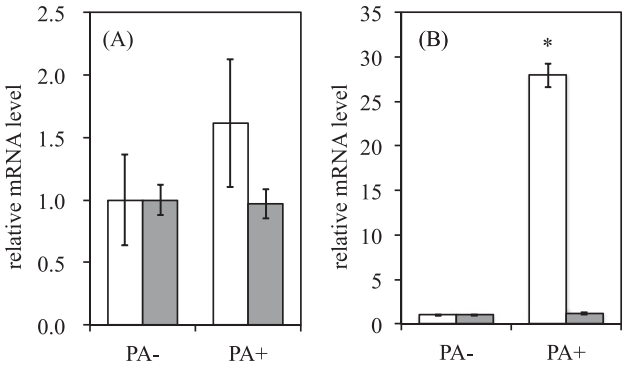

Fig. 4. Induction of SSAT gene expression by polyamine supplementation.

Expression in (A) E9 medium, and (B) LB broth. White and gray columns indicate the expression of *bltD* and *paiA*, respectively. Statistical analysis was performed using a *t*-test. * indicates a significant difference at $p < 0.0001$.

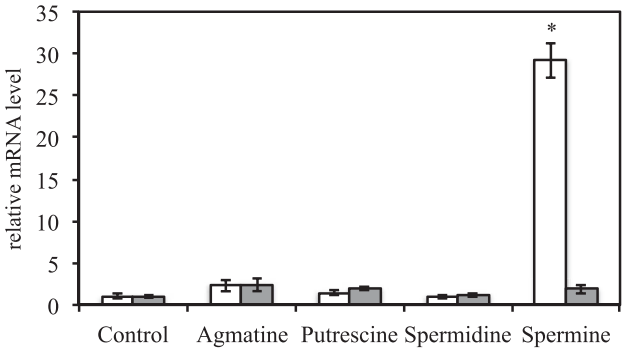

Fig. 5. Comparison of induction of SSAT gene expression among four different polyamines.

White and gray columns indicate the expression of *bltD* and *paiA*, respectively. Statistical analysis was carried out using a Dunnett's test. * indicates a significant difference between the test and a control at $p < 0.0001$.

utes. Nucleotide sequences of primer used in PCR for the gene expression analysis are listed in Table 1. Primers were designed as follows; the sequences of the *B. subtilis* 168 *bltD* and *paiA* genes were downloaded from the BSORF website (BSORF gene entry: *bltD*, BG10906; *paiA*, BG10695) and queried against the *B. subtilis* (natto) BEST 195 sequence on the NCBI database with BLASTn to identify high homology genes (Accession number: *bltD*, AP011541 region 2481040–2481498; *paiA*, AP011541 region 3066302–3066820). Subsequently, primer sets to amplify these genes were designed with Primer3plus (http://www.primer3plus.com/). The 16S rRNA gene was used as housekeeping control gene employing the primer set and conditions described in Wang and Qian (2009), with slight modification. cDNA amplification was performed using SYBR premix Ex Taq II (TAKARA). Each reaction contained 12.5 μL of SYBR premix; 0.4 μM forward and reverse primers; 2.0 μL of 1:10 dilution of the cDNA; and water to 25 μL.

When using LB broth for incubation, polyamine supplementation strongly induced *bltD* gene expression, while *paiA* mRNA levels did not change (Fig. 4B). The expression of both genes was not significantly altered when bacteria were cultivated using minimal culture (Fig. 4A). These observations coincided with the polyamine degradation experiment (Fig. 1) and suggested that *bltD* gene expression depends on exogenous polyamine and nutrient conditions.

To compare the induction of SSAT expression by different polyamines, we determined the mRNA levels of *bltD* and *paiA* in cells cultivated in LB broth supplemented with four different polyamines. Each medium was supplemented with 1 mM Agmatine (Agm), Put, Spd, or Spm. We determined that the addition of Agm, Put, and Spd did not increase mRNA levels of either SSAT gene, whereas *bltD* gene expression was strongly increased by Spm addition (Fig. 5). This demonstrates that exogenous Spm induces *bltD* gene expression and suggested that polyamine acetylation influenced polyamine degradation.

In summary, this study has demonstrated that *B. subtilis* (natto) degrades Spm in the nutrient-rich liquid medium. Additionally, we propose that BltD is involved in the bacterial Spm degradation. However, further studies are needed to elucidate the regulation of *bltD* gene by exogenous Spm and medium conditions. This study has provided a better understanding of the changes that occur in polyamine content during the production of fermented soybean natto.

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