

Development of natto with germination-defective mutants of *Bacillus subtilis* (natto)

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Abstract The effects of cortex-lysis related genes with the *pdaA*, *sleB*, and *cwlD* mutations of *Bacillus subtilis* (natto) NAFM5 on sporulation and germination were investigated. Single or double mutations did not prevent normal sporulation, but did affect germination. Germination was severely inhibited by the double mutation of *sleB* and *cwlD*. The quality of natto made with the *sleB cwlD* double mutant was tested, and the amounts of glutamic acid and ammonia were very similar to those in the wild type. The possibility of industrial development of natto containing a reduced number of viable spores is presented.

Keywords Germination · *sleB* · *cwlD* · *pdaA* · *Bacillus subtilis* · Natto

Introduction

Natto is a traditional and popular food in Japan (Murooka and Yamashita 2008). It has unique features regarding stickiness and taste, which are related to the digestion of soy proteins. It is made from steamed soybeans using

Bacillus subtilis (natto) as a starter. *B. subtilis* (natto) forms spores that are found in the natto. *Bacillus* spores are extremely stable as to chemical and/or physical treatments. Therefore, the killing of spores in natto is not easy without loss of food quality, i.e. taste, flavor, color, and/or stickiness. For these reasons, the utilization of natto for processed foods has not increased and natto containing a decreased amount of spores has been desired. For the industrial development of natto, sporulation and/or germination mutants are needed as starters. Since enzymes produced during the sporulation phase usually affect the quality of natto, germination mutants are expected to be the strongest candidates. In *B. subtilis*, various germination-deficient mutants have been investigated. The major group of germination-deficient mutants is related to the germination receptors for germinants [L-alanine, AGFK (a mixture of L-asparagine, D-glucose, D-fructose and KCl), and Ca²⁺-dipicolinic acid]. This group contains *gerA*, *gerB*, *gerK*, and *gerD* (Paidhungat and Setlow 2002; Ragkousi et al. 2003). The mutants of this group are inadequate for our purpose, because germinants comprise not only L-alanine, AGFK, and Ca²⁺-dipicolinic acid but also possible unknown nutrients (inosine is known as a good germinant for other bacilli; Moir 1992). The second group is related to spore coat deficiency. The *gerT* and *gerQ* mutants belong to this group but their spores germinate at high frequency on rich media (Ragkousi et al. 2003; Ferguson et al. 2007). The third group affects vegetative growth and/or sporulation. The *gerC*, *gerF*, *gerG*, and *gerE* mutants belong to this group (Paidhungat and Setlow 2002; Piggot and Losick 2002; Moir 1981). These mutants are also inadequate because the mutants are not clear germination-defective mutants and may affect the quality of natto. The fourth group is related to cortex-lysis or modification and it contains *cwlD*, *sleB*, *cwlJ*, and *pdaA*

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(Sekiguchi et al. 1995; Moriyama et al. 1996; Ishikawa et al. 1998). CwlD, SleB, and CwlJ were considered to be cortex-lytic enzymes. SleB recognizes muramic- δ -lactam, which is a unique peptidoglycan component in the cortex (Warth and Strominger 1969). Muramic- δ -lactam is produced by PdaA (polysaccharide deacetylase) and CwlD (*N*-acetylmuramoyl-L-alanine amidase homologue) (Sekiguchi et al. 1995; Fukushima et al. 2002; Gilmore et al. 2004; Fukushima et al. 2005). The mutations in the fourth group affect neither vegetative growth nor sporulation, but severely affect germination. Therefore, in this study we investigated the effect of disruption of *pdaA*, *cwlD*, and/or *sleB* on the germination of *B. subtilis* (*natto*) and applied the mutants to the production of natto.

Materials and methods

Bacterial strains and plasmids

The strains of *B. subtilis* 168, *B. subtilis* (*natto*) NAFM5 (Kimura et al. 2004), and *Escherichia coli* and the plasmids used in this study are listed in Table 1. NAFM5 was a derivative of the major natto starter, Miyagino strain, and NAFM5 had the competence ability. *B. subtilis* was grown on LB medium (Sambrook et al. 1989) at 37°C overnight, inoculated into DSM (Schaeffer sporulation medium) (Schaeffer et al. 1965), and then shaken at 37°C. If necessary, kanamycin and tetracycline were added to the medium to final concentrations of 20 and 10 $\mu\text{g ml}^{-1}$, respectively. *E. coli* was grown in LB medium at 37°C. If necessary, kanamycin, tetracycline, and ampicillin were added to the medium to final concentrations of 20, 20, and 50 $\mu\text{g ml}^{-1}$, respectively.

Plasmids construction

To construct *pdaA* mutants, the upstream region of *pdaA* was amplified by PCR using two primers, pdaAUP1 and pdaAUP2 (Table 2), with *B. subtilis* (*natto*) NAFM5 DNA as a template. The PCR fragment was digested with *Kpn*I and *Bam*HI and ligated to pUC118 at the corresponding sites, followed by transformation of *E. coli* JM109, plasmid pDA1 being generated. pDG780 was digested with *Bam*HI and *Sal*I, and the 1.5-kb fragment (kanamycin cassette) was inserted into pDA1 at the corresponding sites to generate plasmid pDA2. Similarly, the downstream region of *pdaA* was cloned into pUC118 with two primers, pdaADW1 and pdaADW2, to generate plasmid pDA3. pDA2 was digested with *Kpn*I and *Sal*I and the 2.5-kbp fragment was inserted into pDA3 at the corresponding sites. The resulting plasmid, pDA4, was used to construct a *pdaA*-deficient mutant. pDA4-Tc containing the tetracycline cassette from

pDG1515 instead of the kanamycin cassette of pDA4 was used to construct a *pdaA sleB*-double mutant.

Likewise, for *sleB* mutants, pSB4 was constructed using the upstream and downstream regions of *sleB* and the kanamycin cassette from pDG780. The fragment was amplified by PCR with *B. subtilis* (*natto*) NAFM5 DNA as a template and primers sleBUP1 and sleBUP2 or primers sleBDW1 and sleBDW2. For *cwlD* mutants, pCD4 was constructed using the upstream and downstream regions of *cwlD* and the tetracycline cassette from pDG1515. The fragment was amplified by PCR with *B. subtilis* (*natto*) NAFM5 DNA as a template and primers cwlDUP1 and cwlDUP2 or primers cwlDDW1 and cwlDDW2.

Mutant construction

pdaA-deficient mutants, 16A and NAA, were constructed by transformation of *B. subtilis* 168 and *B. subtilis* (*natto*) NAFM5, respectively, with pDA4. Disruption of the *pdaA* gene was confirmed by PCR using primers pdaAUP1 and pdaADW2. *sleB*-deficient mutants, 16B and NAB, were constructed by transformation of 168 and NAFM5, respectively, with pSB4. *cwlD*-deficient mutants, 16D and NAD, were constructed by transformation of 168 and NAFM5, respectively, with pCD4. *pdaA* and *sleB* double-deficient mutants, 16AB and NAAB, were constructed by transformation of 16B and NAB, respectively, with pDA4-Tc. *pdaA cwlD*-double mutants, 16AD and NAAD, were constructed by transformation of 16A and NAA, respectively, with pCD4. *sleB cwlD*-double mutants, 16BD and NABD, were constructed by transformation of 16B and NAB, respectively, with pCD4.

Transformation of *E. coli* and *B. subtilis* strains

E. coli transformation was performed as described by Sambrook et al. (1989) and *B. subtilis* and *B. subtilis* (*natto*) transformation was performed by the procedure of Anagnostopoulos and Spizizen (1961).

Viable spore counts

B. subtilis 168, 16A, 16B, and 16D and *B. subtilis* (*natto*) NAFM5, NAA, NAB, and NAD were shake-cultured in LB medium overnight at 37°C. These precultures were inoculated into DSM followed by shake-culturing for 3 days at 37°C. The numbers of spores were determined with a bacterial counting chamber on a microscope. Heat resistance spores (80°C, 10 min) were counted by a standard agar method (Nissui Pharm) as viable colonies after 3 days at 37°C.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
<i>Bacillus subtilis</i>		
168	<i>trpC2</i>	D. Ehrlich
16A	<i>trpC2</i> Δ <i>pdaA</i> ::Km ^r	168←pDA4
16B	<i>trpC2</i> Δ <i>sleB</i> ::Km ^r	168←pSB4
16D	<i>trpC2</i> Δ <i>cwlD</i> ::Tc ^r	168←pCD4
16AD	<i>trpC2</i> Δ <i>pdaA</i> ::Km ^r Δ <i>cwlD</i> ::Tc ^r	16A←pCD4
16BD	<i>trpC2</i> Δ <i>sleB</i> ::Km ^r Δ <i>cwlD</i> ::Tc ^r	16B←pCD4
16AB	<i>trpC2</i> Δ <i>pdaA</i> ::Tc ^r Δ <i>sleB</i> ::Km ^r	16B←pDA4-Tc
<i>B. subtilis</i> (natto)		
NAFM5	Rif ^r <i>bio</i>	K. Kimura
NAA	Rif ^r <i>bio</i> Δ <i>pdaA</i> ::Km ^r	NAFM5←pDA4
NAB	Rif ^r <i>bio</i> Δ <i>sleB</i> ::Km ^r	NAFM5←pSB4
NAD	Rif ^r <i>bio</i> Δ <i>cwlD</i> ::Tc ^r	NAFM5←pCD4
NAAD	Rif ^r <i>bio</i> Δ <i>pdaA</i> ::Km ^r Δ <i>cwlD</i> ::Tc ^r	NAA←pCD4
NABD	Rif ^r <i>bio</i> Δ <i>sleB</i> ::Km ^r Δ <i>cwlD</i> ::Tc ^r	NAB←pCD4
NAAB	Rif ^r <i>bio</i> Δ <i>pdaA</i> ::Tc ^r Δ <i>sleB</i> ::Km ^r	NAB←pAD4-Tc
<i>Escherichia coli</i>		
JM109	<i>recA1</i> Δ (<i>lac-proAB</i>) <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> <i>relA1</i> <i>supE44</i> [F' <i>traD36</i> <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ³ <i>lacZ</i> Δ M15]	
Plasmid		
pUC118	Ap ^r <i>lacZ</i>	Takara
pDG780	Ap ^r Km ^r	BGSC
pDG1515	Ap ^r Tc ^r	BGSC
pDA1	Ap ^r <i>pdaA</i> upstream	This study
pDA2	Ap ^r <i>pdaA</i> upstream -Km ^r	This study
pDA3	Ap ^r <i>pdaA</i> downstream	This study
pDA4	Ap ^r Δ <i>pdaA</i> ::Km ^r	This study
pDA4-Tc	Ap ^r Δ <i>pdaA</i> ::Tc ^r	This study
pSB4	Ap ^r Δ <i>sleB</i> ::Km ^r	This study
pCD4	Ap ^r Δ <i>cwlD</i> ::Tc ^r	This study

Sources shown before and after the arrows indicate donor plasmid DNA and recipient cells on transformation, respectively

BGSC Bacillus Genetic Stock Center, Ohio State University

Spore germination

B. subtilis 168, 16AB, 16AD, and 16BD and *B. subtilis* (natto) NAFM5, NAAB, NAAD, and NABD were shake-cultured in DSM for 3 days at 37°C. The spores were purified by lysozyme treatment followed by salt and detergent washes as described by Nicholson and Setlow (1990). The purified spores were adjusted to $A_{600}=1$ and their viability was assayed as described above. To measure spore germination as the loss of optical density and release of dipicolinic acid, spores were diluted with a 10 mM Tris-HCl buffer (pH 8.4). Germination was initiated by the addition of L-Ala (10 mM). At appropriate times, A_{600} of the mixture was measured and a 3-ml sample was taken and centrifuged with a microcentrifuge. The supernatant was used for the measurement of released dipicolinic acid as described by Nicholson and Setlow (1990).

Analysis of natto

B. subtilis (natto) NAFM5 and NABD were shake-cultured in LB medium at 37°C overnight and then the cultures were diluted to 10^5 cells ml⁻¹ with saline. To make natto, 1 ml of a diluent was inoculated into 50 g of autoclaved soybeans (121°C, 50 min), followed by fermentation at 40°C for 17 h. One hundred milliliters of saline were added to the natto, followed by agitating for 1 h at 4°C. Then the supernatant was used to count the viable spores and to measure the glutamate and ammonia contents for evaluation of the natto. The amounts of glutamate and ammonia were determined with an F-kit L-glutamic acid (Roche) and an F-kit ammonia, respectively, according to the manufacturer's instructions. The organoleptic evaluation of stickiness and flavor were performed by lifting the natto after mixing with a spatula.

Table 2 Primers used in this study

Primer	Sequence	Restriction site
<i>pdaA</i> (789 bp)		
pdaAUP1	5'-CGGGGTACC ₋₉₁₅ GAAGGTCGGTTTTCTGTCCA ₋₈₉₆	<i>KpnI</i>
pdaAUP2	5'-CGCGGATCC ₂₉ GCGCAGCATATTGAACACAT ₁₀	<i>BamHI</i>
pdaADW1	5'-ACGCGTCGAC ₇₇₂ ATGAGGCTGCCGTCTTTGTA ₇₉₁	<i>SalI</i>
pdaADW2	5'-CCCAAGCTT ₁₆₇₁ GACTCGGCAATATGGGACAG ₁₆₅₂	<i>HindIII</i>
<i>sleB</i> (915 bp)		
sleBUP1	5'-GCCGAGCTC ₋₉₇₇ GAAAAACGGCAGGTTTCAC ₋₉₆₀	<i>SacI</i>
sleBUP2	5'-CGCGGATCC ₋₁ CTTTTCAAGCCTCCTACTGC ₋₂₀	<i>BamHI</i>
sleBDW1	5'-ACGCGTCGAC ₈₇₃ GCGTCCGCAGATTAAG ₈₉₀	<i>SalI</i>
sleBDW2	5'-CCCAAGCTT ₁₈₇₀ CTTTAAAAATGCAAGCGCTC ₁₈₅₁	<i>HindIII</i>
<i>cwID</i> (711 bp)		
cwIDUP1	5'-GCCGAGCTC ₋₉₈₁ CGGGTTTTGTCAATCCTGTT ₋₉₆₂	<i>SacI</i>
cwIDUP2	5'-CGCGGATCC ₁₃ GCTTTTTCCTCATCCCTTCC ₋₇	<i>BamHI</i>
cwIDDW1	5'-ACGCGTCGAC ₆₉₅ AAGGAGACCCTCCGGAGTAA ₋₇₁₄	<i>SalI</i>
cwIDDW2	5'-CCCAAGCTT ₁₆₆₃ TCCCCTGTCTTTGCACTTTC ₁₆₄₄	<i>HindIII</i>

Subscript numbers in the sequence are with respect to the 5'-terminal nucleic acid residues of PdaA, SleB, and CwID, respectively. Restriction site is italicized

Results

Effects of single mutants as to cortex-lysis-related genes

SleB is a cortex-lytic enzyme (Moriyama et al. 1996) and is assumed to digest the cortex-containing muramic delta-lactam, a unique peptidoglycan component (Warth and Strominger 1969). The delta-lactam structure is produced by CwID and PdaA (Sekiguchi et al. 1995; Fukushima et al. 2002; Gilmore et al. 2004; Fukushima et al. 2005). Since the genome organizations of *B. subtilis* 168 and *B. subtilis* (*natto*) are very similar (Qiu et al. 2004), we attempted amplification of the upstream and downstream regions of *sleB*, *cwID*, and *pdaA* of *B. subtilis* (*natto*) with *B. subtilis* (*natto*) DNA as a template and primers based on the sequences of *B. subtilis* 168. We were able to amplify these three gene fragments and then three disruption plasmids, pDA4 (for *pdaA*), pSB4 (*sleB*), and pCD4 (*cwID*), were constructed. The corresponding genes in *B. subtilis* 168 and *B. subtilis* (*natto*) were disrupted by conventional transformation with these plasmids. Then we examined the spore germination frequencies of the *sleB*, *cwID*, and *pdaA* mutants of *B. subtilis* 168 and *B. subtilis* (*natto*) NAFM5 (Fig. 1). Microscopic observation revealed that all the mutants formed spores and the numbers of spores were similar to those in the wild type (10^8 to 10^9 spores ml^{-1}). In the case of *B. subtilis* 168, the viable numbers of mutant spores measured as heat resistant ones were different from those in the wild type. In particular, the *pdaA* and *cwID* mutants showed greatly decreased numbers of viable spores (2.4×10^5 and 5.1×10^4 spores ml^{-1} , respectively), but the *sleB* mutation did not affect viability significantly (1.7×10^8 spores ml^{-1}). In the case of *B. subtilis* (*natto*) NAFM5, the *pdaA* and *cwID* mutants showed decreased numbers of

viable spores (1.5×10^6 and 1.6×10^6 spores ml^{-1} , respectively), but the effects were much less than those in *B. subtilis* 168. The *sleB* mutation in *B. subtilis* (*natto*) decreased the number of viable spores (1.1×10^7 spores ml^{-1}). The degrees of the effects of these mutations in *B. subtilis* 168 were $\Delta cwID$, $\Delta pdaA$, and $\Delta sleB$ in decreasing order and those in *B. subtilis* (*natto*) were ($\Delta pdaA$, $\Delta cwID$) and $\Delta sleB$, in decreasing order.

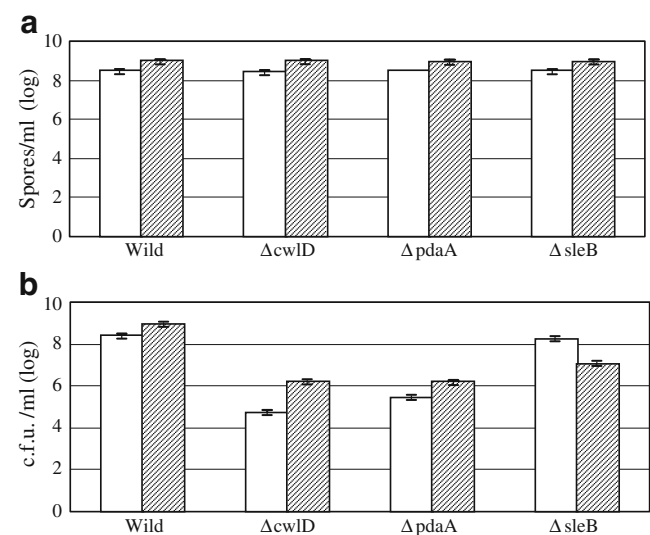


Fig. 1 The spore numbers of the single mutants and the wild types. The single mutants and the wild types of *B. subtilis* 168 and *B. subtilis* (*natto*) NAFM5 were cultured in DSM for 3 days at 37°C. The numbers of spores were determined under a microscope (a). Heat-resistant spores (80°C, 10 min), as viable spores, were plated, followed by counting after 3 days at 37°C (b). Open and slashed boxes indicate *B. subtilis* 168 and *B. subtilis* (*natto*) NAFM5, respectively. c.f.u. colony forming units

Effects of double mutants as to cortex-lysis-related genes

Although the single mutants as to the cortex-lysis-related genes showed decreased numbers of viable spores, the effects were not complete. Therefore, we examined the effect of double mutants as to cortex-lysis-related genes, i.e. the *pdaA* and *sleB*, *pdaA* and *cwID*, and *sleB* and *cwID* mutations. All the double mutants formed spores at frequencies similar to in the single mutants. To analyze the germination of mutants, the spores were purified and then they ($A_{600}=1$) were heated for 10 min at 80°C, followed by plating on agar medium to determine viable spore numbers (Fig. 2). Colonies were counted after 3 days, because the colonies of mutants were very small at 24 h.

The most dramatic effect was found for the double mutant as to *sleB* and *cwID*. *B. subtilis* 168 spores with these mutations gave 8.6×10^2 viable cells ml^{-1} and *B. subtilis* (*natto*) spores gave 1.0×10^2 viable cells ml^{-1} (Fig. 2b). Since the *sleB* gene product was considered to only degrade the cortex with muramic- δ -lactam (Masayama et al. 2006), the effects of the mutations were different from each other (*sleB* is not associated with muramic- δ -lactam formation). Therefore, the synergetic effect of the *sleB* and *cwID* mutations was found for both *B. subtilis* 168 and *B. subtilis* (*natto*). From a practical aspect, the *sleB cwID*-double mutant is a good candidate for producing natto with spores deficient in significant germination.

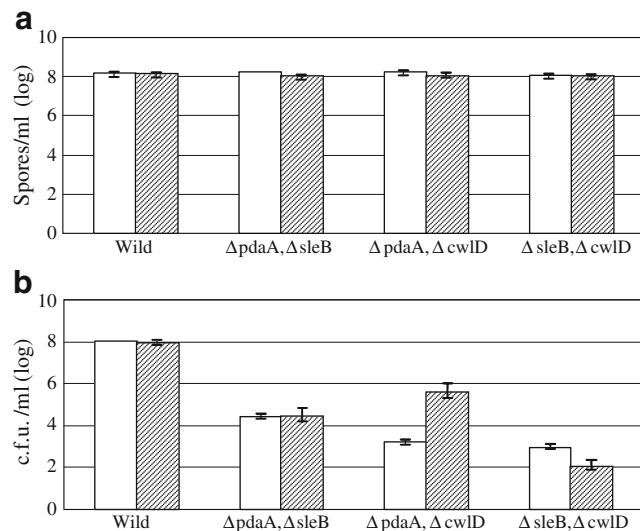


Fig. 2 The spore numbers of the double mutants and the wild types. The double mutants and the wild types of *B. subtilis* 168 and *B. subtilis* (*natto*) NAFM5 were cultured in DSM for 3 days at 37°C. The numbers of spores were determined under a microscope (a). Heat-resistant spores (80°C, 10 min) were counted after cultivation for 3 days at 37°C (b). Open and slashed boxes indicate 168 and NAFM5, respectively

Analysis of spore germination

Since the *sleB cwID*-double mutants exhibited the lowest germination, germination was compared among 168, 16BD ($\Delta sleB \Delta cwID$), NAFM5, and NABD ($\Delta sleB \Delta cwID$) spores (Fig. 3). During germination in L-Ala buffer, the 16BD and NABD spores showed a slower decrease in A_{600} than the wild-type spores of 168 and NAFM5, respectively. Therefore, the germination of both strains was severely affected by the double mutations. Dipicolinic acid release from spores is also a marker of the early germination event. But the levels of the release of dipicolinic acid from these spores were almost the same as that in the wild type. These results suggest that an early germination event (i.e., dipicolinic acid release) was not affected by the double mutations in either strain. These data were supported by the results for each single mutation in *B. subtilis* (Sekiguchi et al. 1995; Moriyama et al. 1996). Phase-contrast microscopy of the wild type and mutants is shown in Fig. 4. The refractility of spores of the wild types, 168 and NAFM5,

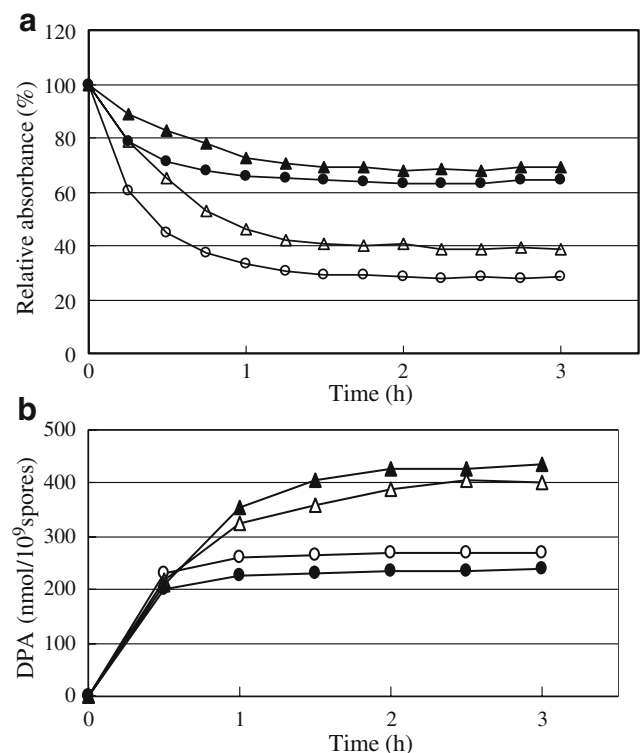
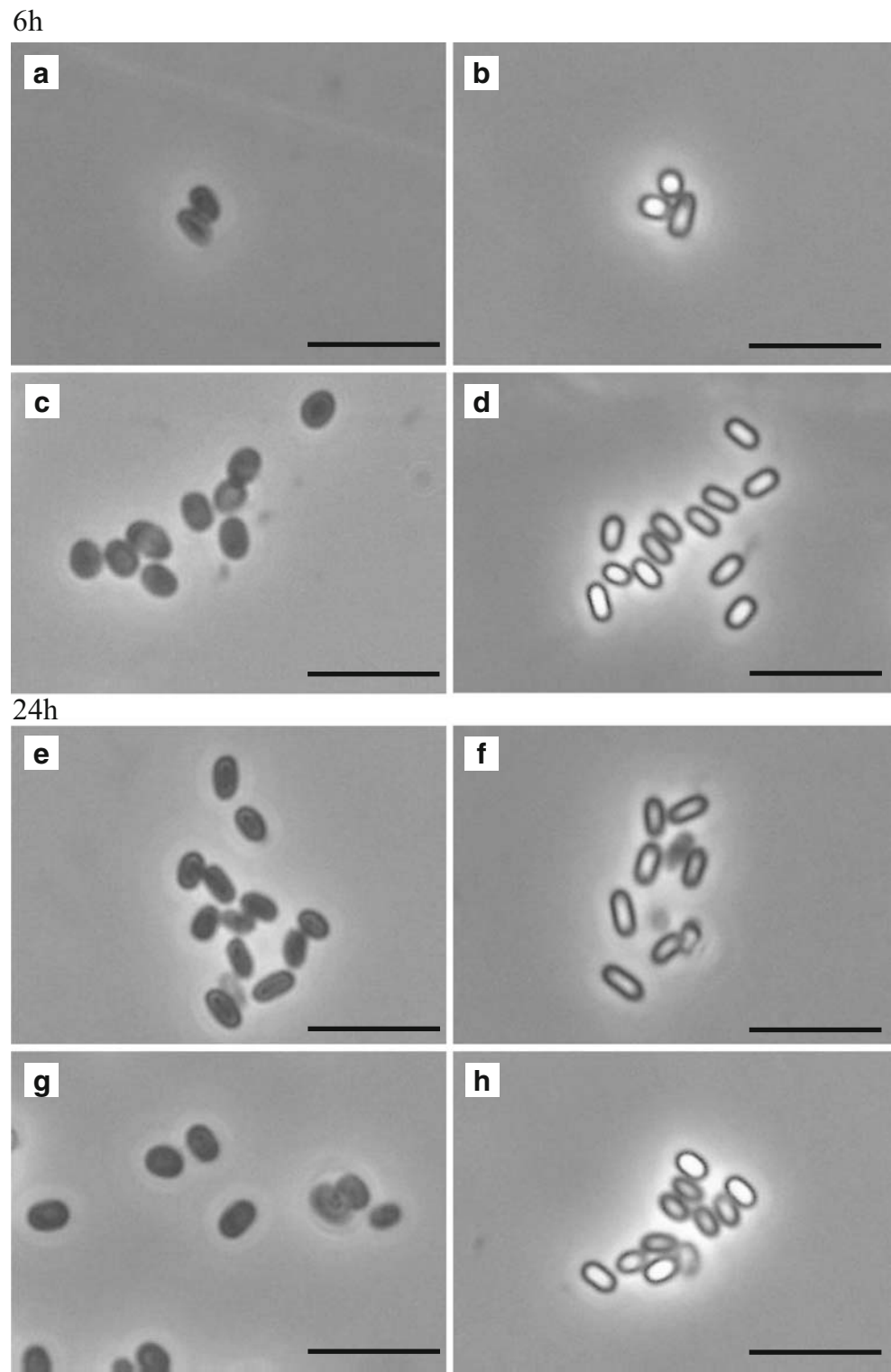


Fig. 3 The germination properties of the *sleB cwID*-double mutants and the wild types. The germination of spores of the *sleB cwID*-double mutants and the wild types was monitored at A_{600} at the indicated times after addition to a germination buffer (10 mM L-alanine, 10 mM Tris-HCl (pH 8.4)). Relative absorbance is shown in (a). The dipicolinic acid (DPA) released into the supernatants of spore suspensions during germination is shown in (b). Open and closed circles indicate 168 and its *sleB cwID*-double mutant (16BD), respectively. Open and closed triangles indicate NAFM5 and its *sleB cwID*-double mutant (NABD), respectively

Fig. 4 Phase-contrast microscopy of spores of the *sleB cwID*-double mutants derived from 168 and NAFM5. Spores were germinated at 37°C for 6 h (**a** to **d**) or 24 h (**e** to **h**) in the germination buffer. **a** and **e** Show 168 and **b** and **f** show the *sleB cwID*-double mutant of 168 (16BD). **c** and **g** Show NAFM5 and **d** and **h** show the *sleB cwID*-double mutant of NAFM5 (NABD). *Bar*=5 μ m



changed from bright to dark after 6 h, whereas 16BD and NABD spores became phase gray (or blight) under the same conditions. After 24 h, the 16BD and NABD spores were still phase gray (Fig. 4f, h). These results correspond

to the spore viability and the loss of optical density. On the other hand, it is interesting that the dipicolinic acid content of *B. subtilis* (*natto*) spores was 1.5 times larger than that of *B. subtilis* ones (Fig. 3b).

Analysis of natto

Since the *sleB cwID*-double mutants formed spores but viable spores were very few, we made natto with NAFM5 and NABD, and then determined the numbers of spores and quality of natto (Fig. 5). Microscopy of extracts of natto indicated that the spore formation of NABD was normal, but the viable spore number decreased similarly in a liquid culture (Fig. 5). The organoleptic test of stickiness and flavor and both the glutamic acid and ammonia contents of NABD were similar to those of the wild type (Fig. 5 and data not shown). These results indicated that the natto made with NABD exhibited normal qualities.

Discussion

Spores of *B. subtilis* were extremely stable as to chemical or physical attack. Natto is a soybean-fermented food and *B. subtilis* (natto) is used as a starter, therefore natto contains many spores. On the other hand, low bacterial numbers in processed foods are desirable from the

viewpoint of shelf life. For this reason, natto is not used as a processed food. Spores from natto contaminate machinery used for manufacturing foods and thus the foods have high initial viable cell counts. Therefore, natto with no or few spores is required.

There have been many reports about germination-defective mutants of *B. subtilis*. Fukushima et al. (2002) reported that the germination rate of *pdaA* mutants was 0.0008%. In this study, the germination rates of the *pdaA* mutants were 0.075% and 0.17% for 168 and NAFM5, respectively. The germination rate of the NAFM5 mutant was apparently higher than that of the 168 mutant. The germination rate of the 168 mutant shown in this study was higher than that previously reported. Ishikawa et al. (1998) reported the germination rate of the *sleB* mutant in *B. subtilis* was 43% and our results were 61% and 1.2% for 168 and NAFM5, respectively. The mutation of *sleB* was more effective in NAFM5 than in 168. Sekiguchi et al. (1995) reported that the germination rate of the *cwID* mutant in *B. subtilis* was less than 3.7×10^{-8} , but our results were 1.9×10^{-4} and 1.7×10^{-3} for 168 and NAFM5, respectively. Popham et al. (1996) showed that the germination rate of the *cwID* mutant in *B. subtilis* was 5×10^{-4} (with the respect to the wild type).

We investigated the effects of double mutations of these three genes. The *cwID* and *sleB* double mutation strongly affected the germination frequency. The loss of optical density of the spores of the *cwID sleB*-double mutant was low compared with in the wild type and the refractility of the spores stopped at the phase gray stage. But the DPA release level was almost the same as that in the wild type. These results indicate that the germination of the *cwID sleB*-double mutant was blocked at a late stage of germination. This phenomenon resembled the case of the *sleB cwIJ*-double mutant (Ishikawa et al. 1998). The release of dipicolinic acid of NAFM5 was higher than that of 168. The reason was not clear but the spore size was larger than that of 168 (Fig. 4). The difference of DPA release may be attributed to the different spore size.

Stickiness and the amounts of glutamic acids and ammonia are important for natto qualities. The natto made with the *cwID sleB*-double mutant has the same qualities as the wild type. This indicates that the *cwID sleB*-double mutant has no effect on natto qualities. But natto contains vegetative cells in addition to spores. The vegetative cells in natto also affect the shelf life of processed foods. Therefore, process to decrease the number of vegetative cells is required. Such processes are heating, freezing, and addition of preservatives such as glycine or lysozyme and they are milder than killing spores. Consequently, the effect on food quality seems to be very little. Similarly, decolonization of vegetative cells contaminated on machine is easier than that of spores. The introduction of a cold-

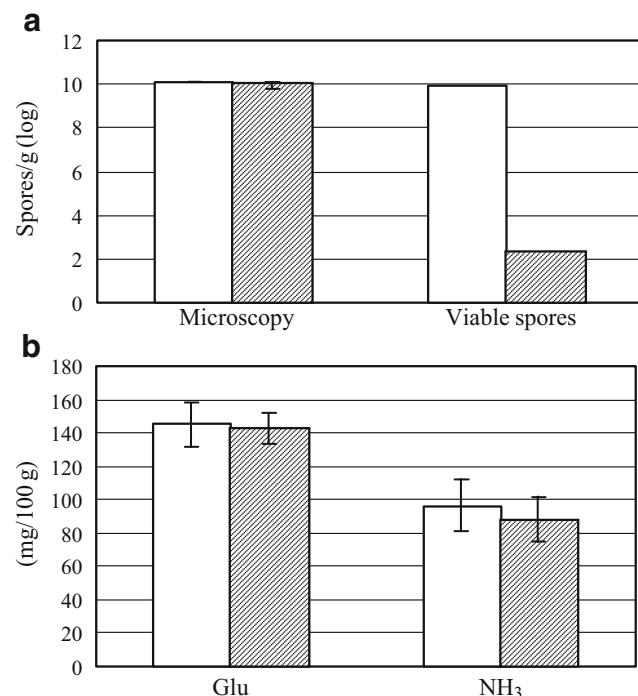


Fig. 5 The spore numbers and the amounts of glutamic acid and ammonia in natto. Natto made with the *sleB cwID*-double mutant and the wild type was added to saline, followed by agitation for 1 h at 4°C. **a** The numbers of spores in the supernatants were determined under a microscope or by plating after heat treatment (80°C, 10 min) followed by cultivation for 3 days at 37°C. **b** The amounts of L-glutamic acid and ammonia in the supernatants were measured with F-kits to evaluate the quality of the natto. Open and slashed boxes indicate NAFM5 and its *sleB cwID*-double mutant (NABD), respectively

sensitive mutation to the germination mutant may become an improvement process. Moreover, natto contains abundant vitamin K that is expected to prevent osteoporosis. However, because vitamin K weakens the effect of an anti-blood coagulant pharmaceutical agent, it is not preferable that patients take agent with natto (Homma et al. 2006). It is known that *B. subtilis* spores germinate in intestines (Casula and Cutting 2002). It means *B. subtilis* produces vitamin K in intestines (Kaneki et al. 2001). In contrast to spores, vegetative cells do not have the tolerance to peptic juice (Duc et al. 2003). Therefore, the control of the germination of spores in intestines is preferable and thus the germination mutant may be able to be used for its application. In this manuscript, we present the possibility of the improved natto production with a *sleB cwLD* germination-defective mutant.

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