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

1 **Introduction**
2
3 Natto is a traditional and popular food in Japan (Murooka and Yamashita 2008). It has
4 unique features regarding stickiness and taste, which are related to the digestion of soy
5 proteins. It is made from steamed soybeans using *Bacillus subtilis* (*natto*) as a starter. *B.*
6 *subtilis* (*natto*) forms spores that are found in the natto. *Bacillus* spores are extremely
7 stable as to chemical and/or physical treatments. Therefore, the killing of spores in natto
8 is not easy without loss of food quality, i.e. taste, flavor, color and/or stickiness. For
9 these reasons, the utilization of natto for processed foods has not increased, and natto
10 containing a decreased amount of spores has been desired. For the industrial
11 development of natto, sporulation and/or germination mutants are needed as starters.
12 Since enzymes produced during the sporulation phase usually affect the quality of natto,
13 germination mutants are expected to be the strongest candidates. In *B. subtilis*, various
14 germination-deficient mutants have been investigated. The major group of
15 germination-deficient mutants is related to the germination receptors for germinants
16 [L-alanine, AGFK (a mixture of L-asparagine, D-glucose, D-fructose and KCl), and
17 Ca^{2+}-dipicolinic acid]. This group contains *gerA*, *gerB*, *gerK*, and *gerD* (Paidhungat and
18 Setlow 2002; Ragkousi et al. 2003). The mutants of this group are inadequate for our
19 purpose, because germinants comprise not only L-alanine, AGFK, and Ca^{2+}-dipicolinic
20 acid but also possible unknown nutrients (inosine is known as a good germinant for
21 other bacilli; Moir 1992). The second group is related to spore coat deficiency. The *gerT*
22 and *gerQ* mutants belong to this group but their spores germinate at high frequency on
23 rich media (Ragkousi et al. 2003; Ferguson et al. 2007). The third group affects
24 vegetative growth and/or sporulation. The *gerC*, *gerF*, *gerG,* and *gerE* mutants belong
25 to this group (Paidhungat and Setlow 2002; Piggot and Losick 2002; Moir 1981). These
26 mutants are also inadequate, because the mutants are not clear germination-defective
27 mutants and may affect the quality of natto. The fourth group is related to cortex-lysis or
28 modification, and it contains *cwlD*, *sleB*, *cwlJ,* and *pdaA* (Sekiguchi et al. 1995;
29 Moriyama et al. 1996; Ishikawa et al. 1998). CwlD, SleB, and CwlJ were considered to
30 be cortex-lytic enzymes. SleB recognizes muramic-delta-lactam, which is a unique
31 peptidoglycan component in the cortex (Warth and Strominger 1969).
32 Muramic-delta-lactam is produced by PdaA (polysaccharide deacetylase) and CwlD
33 (*N*-acetylmuramoyl-L-alanine amidase homologue) (Sekiguchi et al. 1995; Fukushima
34 et al. 2002; Gilmore et al. 2004; Fukushima et al. 2005). The mutations in the fourth
35 group affect neither vegetative growth nor sporulation, but severely affect germination.
36 Therefore, in this study we investigated the effect of disruption of *pdaA*, *cwlD* and/or

1 *sleB* on the germination of *B. subtilis* (*natto*), and applied the mutants to the production
2 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 μg 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 μg 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. Co.) as viable colonies after 3 days at 37 °C.

1 Spore germination
2
3 *B. subtilis* 168, 16AB, 16AD, and 16BD, and *B. subtilis* (*natto*) NAFM5, NAAB,
4 NAAD, and NABD were shake-cultured in DSM for 3 days at 37 °C. The spores were
5 purified by lysozyme treatment followed by salt and detergent washes as described by
6 Nicholson and Setlow (1990). The purified spores were adjusted to A_{600}=1 and their
7 viability was assayed described above. To measure spore germination as the loss of
8 optical density and release of dipicolinic acid, spores were diluted with a 10 mM
9 Tris-HCl buffer (pH 8.4). Germination was initiated by the addition of L-Ala (10 mM).
10 At appropriate times, A_{600} of the mixture was measured, and a 3 ml sample was taken
11 and centrifuged with a microcentrifuge. The supernatant was used for the measurement
12 of released dipicolinic acid as described by Nicholson and Setlow (1990).
13
14 Analysis of natto
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16 *B. subtilis* (*natto*) NAFM5 and NABD were shake-cultured in LB medium at 37 °C
17 overnight, and then the cultures were diluted to 10^5 cells ml^{-1} with saline. To make natto,
18 1 ml of a diluent was inoculated into 50 g of autoclaved soybeans (121 °C, 50 min),
19 followed by fermentation at 40 °C for 17 h. One hundred ml of saline were added to the
20 *natto*, followed by agitating for 1 h at 4 °C. Then the supernatant was used to count the
21 viable spores, and to measure the glutamate and ammonia contents for evaluation of the
22 *natto*. The amounts of glutamate and ammonia were determined with an F-kit
23 L-glutamic acid (Roche) and an F-kit ammonia, respectively, according to the
24 manufacturer's instructions. The organoleptic evaluation of stickiness and flavor were
25 performed by lifting the natto after mixing with a spatula.
26

1 **Results**

2

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

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5 SleB is a cortex-lytic enzyme (Moriyama et al. 1996) and is assumed to digest the
6 cortex containing muramic delta-lactam, a unique peptidoglycan component (Warth and
7 Strominger 1969). The delta-lactam structure is produced by CwlD and PdaA
8 (Sekiguchi et al. 1995; Fukushima et al. 2002; Gilmore et al. 2004; Fukushima et al.
9 2005). Since the genome organizations of *B. subtilis* 168 and *B. subtilis* (*natto*) are very
10 similar (Qiu et al. 2004), we attempted amplification of the upstream and downstream
11 regions of *sleB, cwlD,* and *pdaA* of *B. subtilis* (*natto*) with *B. subtilis* (*natto*) DNA as a
12 template and primers based on the sequences of *B. subtilis* 168. We were able to amplify
13 these three gene fragments and then three disruption plasmids, pDA4 (for *pdaA*), pSB4
14 (*sleB*), and pCD4 (*cwlD*), were constructed. The corresponding genes in *B. subtilis* 168
15 and *B. subtilis* (*natto*) were disrupted by conventional transformation with these
16 plasmids. Then we examined the spore germination frequencies of the *sleB*, *cwlD* and
17 *pdaA* mutants of *B. subtilis* 168 and *B. subtilis* (*natto*) NAFM5 (Fig. 1). Microscopic
18 observation revealed that all the mutants formed spores and the numbers of spores were
19 similar to those in the wild type (10^8 to 10^9 spores ml^{-1}). In the case of *B. subtilis* 168,
20 the viable numbers of mutant spores measured as heat resistant ones were different from
21 those in the wild type. In particular, the *pdaA* and *cwlD* mutants showed greatly
22 decreased numbers of viable spores (2.4×10^5 and 5.1×10^4 spores ml^{-1}, respectively),
23 but the *sleB* mutation did not affect viability significantly (1.7×10^8 spores ml^{-1}). In the
24 case of *B. subtilis* (*natto*) NAFM5, the *pdaA* and *cwlD* mutants showed decreased
25 numbers of viable spores (1.5×10^6 and 1.6×10^6 spores ml^{-1}, respectively), but the
26 effects were much less than those in *B. subtilis* 168. The *sleB* mutation in *B. subtilis*
27 (*natto*) decreased the number of viable spores (1.1×10^7 spores ml^{-1}). The degrees of the
28 effects of these mutations in *B. subtilis* 168 were Δ*cwlD*, Δ*pdaA* and Δ*sleB* in
29 decreasing order, and those in *B. subtilis* (*natto*) were (Δ*pdaA,* Δ*cwlD*) and Δ*sleB*, in
30 decreasing order.

31

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

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34 Although the single mutants as to the cortex-lysis related genes showed decreased
35 numbers of viable spores, the effects were not complete. Therefore, we examined the
36 effect of double mutants as to cortex-lysis related genes, i.e. the *pdaA* and *sleB*, *pdaA*

1 and *cwlD*, and *sleB* and *cwlD* mutations. All the double mutants formed spores at
2 frequencies similar to in the single mutants. To analyze the germination of mutants, the
3 spores were purified and then they (A_{600}=1) were heated for 10 min at 80°C, followed
4 by plating on agar medium to determine viable spore numbers (Fig. 2). Colonies were
5 counted after 3 days, because the colonies of mutants were very small at 24 h.
6 The most dramatic effect was found for the double mutant as to *sleB* and *cwlD*. *B.*
7 *subtilis* 168 spores with these mutations gave 8.6 x 10^2 viable cells ml^{-1}, and *B. subtilis*
8 (*natto*) spores gave 1.0 x 10^2 viable cells ml^{-1} (Fig. 2B). Since the *sleB* gene product
9 was considered to only degrade the cortex with muramic-delta-lactam (Masayama et al.
10 2006), the effects of the mutations were different from each other (*sleB* is not associated
11 with muramic-delta lactam formation). Therefore, the synergetic effect of the *sleB* and
12 *cwlD* mutations was found for both *B. subtilis* 168 and *B. subtilis* (*natto*). From a
13 practical aspect, the *sleB cwlD*-double mutant is a good candidate for producing natto
14 with spores deficient in significant germination.
15
16
17 Analysis of spore germination
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19 Since the *sleB cwlD*-double mutants exhibited the lowest germination, germination was
20 compared among 168, 16BD (Δ*sleB* Δ*cwlD*), NAFM5, and NABD (Δ*sleB* Δ*cwlD*)
21 spores (Fig. 3). During germination in L-Ala buffer, the 16BD and NABD spores
22 showed a slower decrease in A_{600} than the wild type spores of 168 and NAFM5,
23 respectively. Therefore, the germination of both strains was severely affected by the
24 double mutations. Dipicolinic acid release from spores is also a marker of the early
25 germination event. But the levels of the release of dipicolinic acid from these spores
26 were almost the same as that in the wild type. These results suggest that an early
27 germination event (i.e., dipicolinic acid release) was not affected by the double
28 mutations in either strain. These data were supported by the results for each single
29 mutation in *B. subtilis* (Sekiguchi et al. 1995; Moriyama et al. 1996). Phase-contrast
30 microscopy of the wild type and mutants is shown in Fig. 4. The refractility of spores of
31 the wild types, 168 and NAFM5, changed from bright to dark after 6 h, whereas 16BD
32 and NABD spores became phase gray (or blight) under the same conditions. After 24 h,
33 the 16BD and NABD spores were still phase gray (Fig. 4F and H). These results
34 correspond to the spore viability and the loss of optical density. On the other hand, it is
35 interesting that the dipicolinic acid content of *B. subtilis* (*natto*) spores was 1.5 times
36 larger that that of *B. subtilis* ones (Fig. 3B).

1
2 Analysis of natto
3
4 Since the *sleB cwlD*-double mutants formed spores but viable spores were very few, we
5 made natto with NAFM5 and NABD, and then determined the numbers of spores and
6 quality of natto (Fig. 5). Microscopy of extracts of natto indicated that the spore
7 formation of NABD was normal, but the viable spore number decreased similarly in a
8 liquid culture (Fig. 5). The organoleptic test of stickiness and flavor, and both the
9 glutamic acid and ammonia contents of NABD were similar to those of the wild type
10 (Fig. 5 and data not shown). These results indicated that the natto made with NABD
11 exhibited normal qualities.
12

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 *cwlD* mutant in *B. subtilis* was less than 3.7 x 10^{-8}, but our results were 1.9x 10^{-4} and 1.7 x 10^{-3} for 168 and NAFM5, respectively. Popham et al. (1996) showed that the germination rate of the *cwlD* mutant in *B. subtilis* was 5 x 10^{-4} (with the respect to the wild type).

We investigated the effects of double mutations of these three genes. The *cwlD* and *sleB* double mutation strongly affected the germination frequency. The loss of optical density of the spores of the *cwlD 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 *cwlD sleB*-double mutant was blocked at a late stage of germination. This phenomenon resembled the case of the *sleB cwlJ*-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 *cwlD sleB*-double mutant has the same qualities as the wild type. This indicates that the *cwlD 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-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 taking the agent have natto (Homma et al. 2006). It is known that *B. subtilis* spores germinate in intestines (Casula G and Cutting SM 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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5

Figure legends

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 resistance 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.

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 resistance 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.

Fig. 3 The germination properties of the *sleB cwlD*-double mutants and the wild types. The germination of spores of the *sleB cwlD*-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, 10mM Tris-HCl (pH 8.4)). Relative absorbance is shown in panel A. The dipicolinic acid (DPA) released into the supernatants of spore suspensions during germination is shown in panel B. *Open* and *closed circles* indicate 168 and its *sleB cwlD*-double mutant (16BD), respectively. *Open* and *closed triangles* indicate NAFM5 and its *sleB cwlD*-double mutant (NABD), respectively.

Fig. 4 Phase-contrast microscopy of spores of the *sleB cwlD*-double mutants derived from 168 and NAFM5. Spores were germinated at 37 °C for 6 h (panels A to D) or 24 h (panels E to H) in the germination buffer. Panels A and E show 168, and panels B and F show the *sleB cwlD*-double mutant of 168 (16BD). Panels C and G show NAFM5, and panels D and H show the *sleB cwlD*-double mutant of NAFM5 (NABD). *Bar,* 5 µm.

1 **Fig. 5** The spore numbers and the amounts of glutamic acid and ammonia in natto.
2 Natto made with the *sleB cwlD*-double mutant and the wild type was added to saline,
3 followed by agitation for 1 h at 4°C. (A) The numbers of spores in the supernatants
4 were determined under a microscope or by plating after heat treatment (80°C, 10 min)
5 followed by cultivation for 3 days at 37 °C. (B) The amounts of L-glutamic acid and
6 ammonia in the supernatants were measured with F-kits to evaluate the quality of the
7 natto. *Open* and *slashed boxes* indicate NAFM5 and its *sleB cwlD*-double mutant
8 (NABD), respectively.
9

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Bacillus subtilis		
168	*trpC2*	D. Ehrlich
16A	*trpC2* Δ*pdaA*::Km^r	168←pDA4
16B	*trpC2* Δ*sleB*::Km^r	168←pSB4
16D	*trpC2* Δ*cwlD*::Tc^r	168←pCD4
16AD	*trpC2* Δ*pdaA*::Km^r Δ*cwlD*::Tc^r	16A←pCD4
16BD	*trpC2* Δ*sleB*::Km^r Δ*cwlD*::Tc^r	16B←pCD4
16AB	*trpC2* Δ*pdaA*::Tc^r Δ*sleB*::Km^r	16B←pDA4-Tc
B. subtilis (*natto*)		
NAFM5	Rif^r *bio*	K. Kimura
NAA	Rif^r *bio* Δ*pdaA*::Km^r	NA←pDA4
NAB	Rif^r *bio* Δ*sleB*::Km^r	NA←pSB4
NAD	Rif^r *bio* Δ*cwlD*::Tc^r	NA←pCD4
NAAD	Rif^r *bio* Δ*pdaA*::Km^r Δ*cwlD*::Tc^r	NAA←pCD4
NABD	Rif^r *bio* Δ*sleB*::Km^r Δ*cwlD*::Tc^r	NAB←pCD4
NAAB	Rif^r *bio* Δ*pdaA*::Tc^r Δ*sleB*::Km^r	NAB←pAD4-Tc
Escherichia coli		
JM109	*recA1* Δ (*lac-proAB*) *endA1* *gyrA96 thi-1 hsdR17 relA1* *supE44* [F' *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15]	
Plasmid		
pUC118	Ap^r *lacZ*	Takara
pDG780	Ap^r Km^r	BGSC
pDG1515	Ap^r Tc^r	BGSC
pDA1	Ap^r *pdaA* upstream	This study
pDA2	Ap^r *pdaA* upstream -Km^r	This study
pDA3	Ap^r *pdaA* downstream	This study
pDA4	Ap^r Δ*pdaA*::Km^r	This study
pDA4-Tc	Ap^r Δ*pdaA*::Tc^r	This study
pSB4	Ap^r Δ*sleB*::Km^r	This study

pCD4	Ap^r Δ*cwlD*::Tc^r	This study

BGSC, Bacillus Genetic Stock Center, Ohio State University

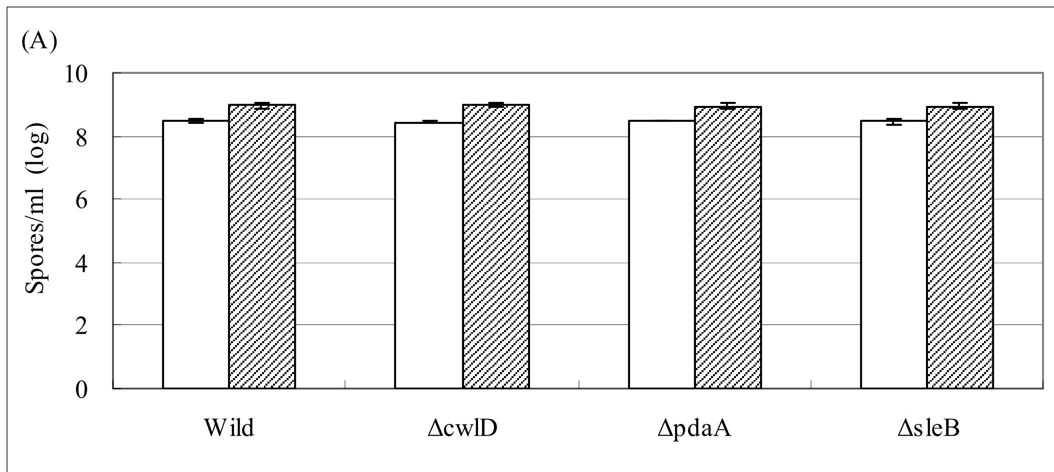
Sources shown before and after the arrows indicate donor plasmid DNA and recipient cells on transformation, respectively

Table 2 Primers used in this study

Primer	Sequence	Restriction site
pdaA (789bp)		
pdaAUP1	5'- CGGGGTACC $_{-915}$GAAGGTCGGTTTTCTGTCCA$_{-896}$	*Kpn*I
pdaAUP2	5'- CGCGGATCC $_{29}$GCGCAGCATATTGAACACAT$_{10}$	*Bam*HI
pdaADW1	5'- ACGCGTCGAC $_{772}$ATGAGGCTGCCGTCTTTGTA$_{791}$	*Sal*I
pdaADW2	5'- CCCAAGCTT $_{1671}$GACTCGGCAATATGGGACAG$_{1652}$	*Hin*dIII
sleB (915bp)		
sleBUP1	5'- GCCGAGCTC $_{-977}$GAAAACGGCAGGTTTCAC$_{-960}$	*Sac*I
sleBUP2	5'- CGCGGATCC $_{-1}$CTTTTCAAGCCTCCTACTGC$_{-20}$	*Bam*HI
sleBDW1	5'- ACGCGTCGAC $_{873}$GCGTCCGCAGATTAAAAG$_{890}$	*Sal*I
sleBDW2	5'- CCCAAGCTT $_{1870}$CTTTAAAAATGCAAGCGCTC$_{1851}$	*Hin*dIII
cwlD (711bp)		
cwlDUP1	5'- GCCGAGCTC $_{-981}$CGGGTTTTGTCAATCCTGTT$_{-962}$	*Sac*I
cwlDUP2	5'- CGCGGATCC $_{13}$GCTTTTTCCTCATCCCTTCC$_{-7}$	*Bam*HI
cwlDDW1	5'- ACGCGTCGAC $_{695}$AAGGAGACCCTCCGGAGTAA$_{714}$	*Sal*I
cwlDDW2	5'- CCCAAGCTT $_{1663}$TCCCCTGTCTTTGCACTTTC$_{1644}$	*Hin*dIII

Subscript numbers in the sequence are with respect to the 5'-terminal nucleic acid residues of PdaA, SleB and CwlD, respectively

Restriction site is underlined



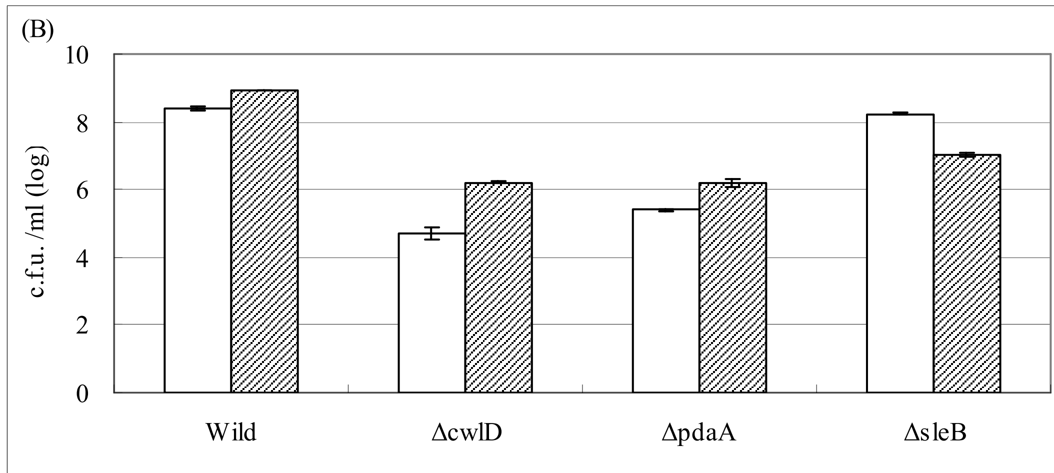
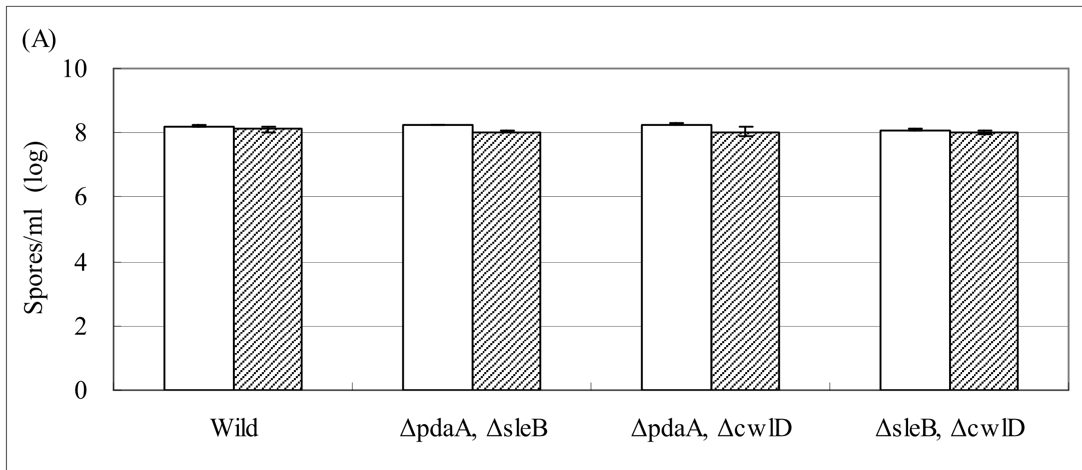


Fig. 1



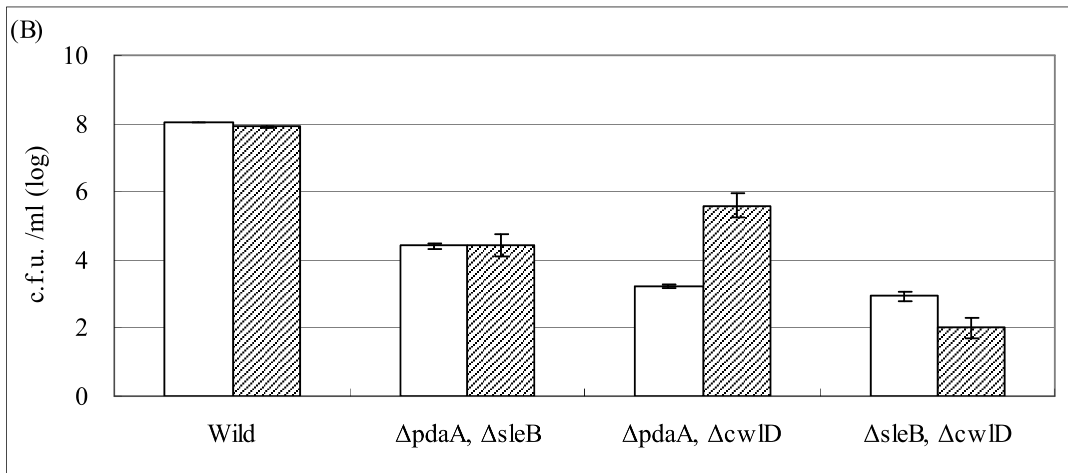
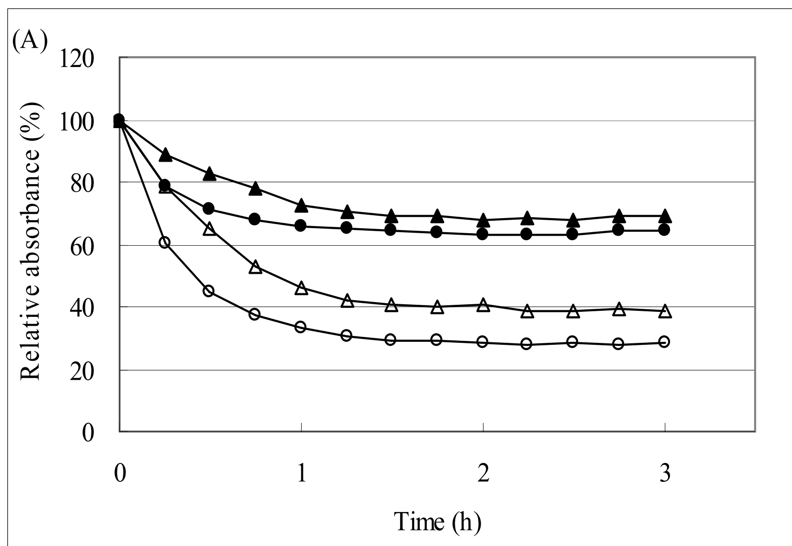


Fig. 2



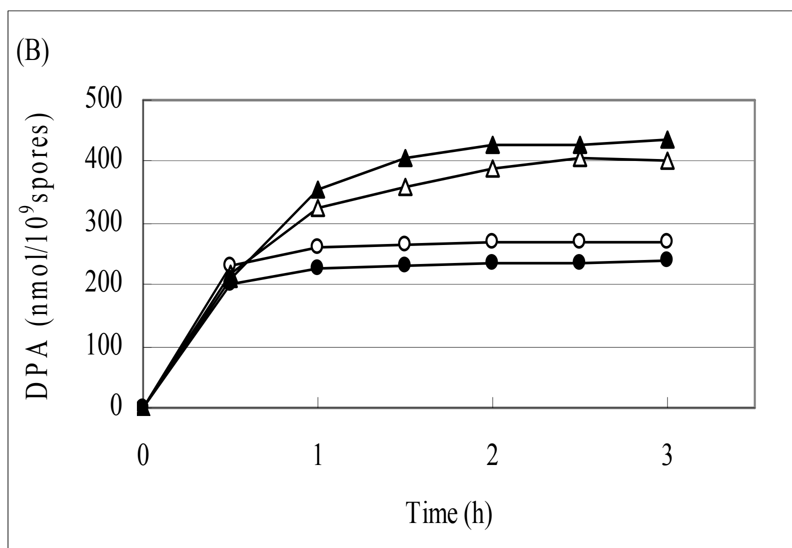
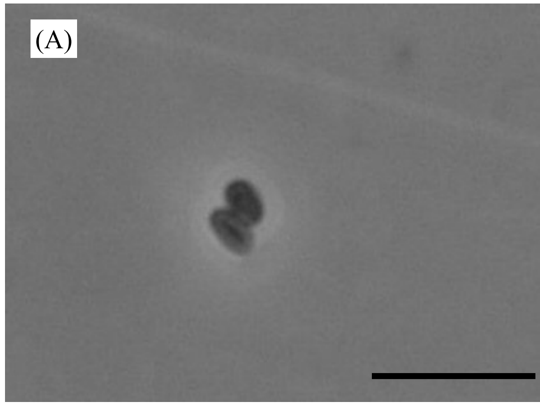
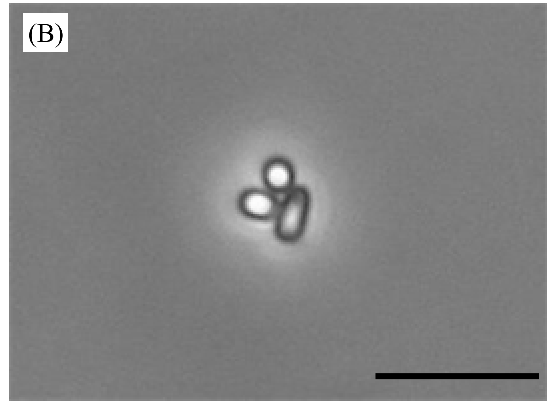
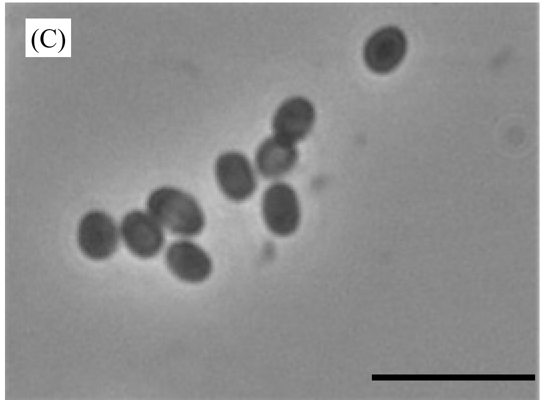


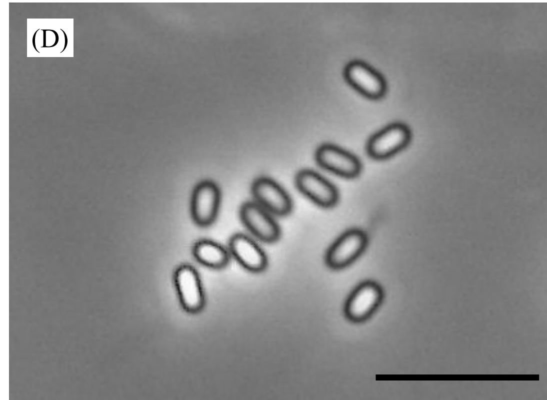
Fig. 3

6h









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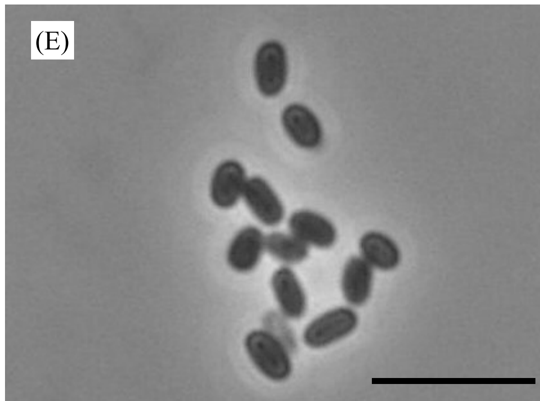
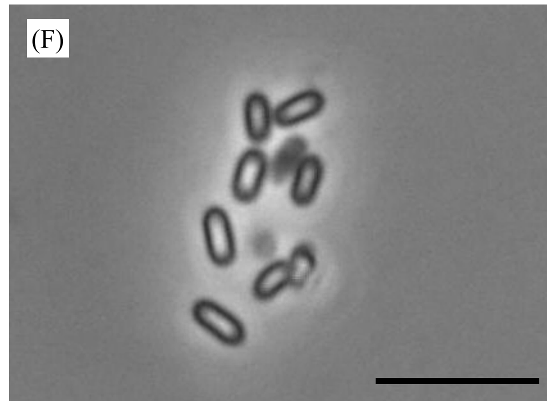
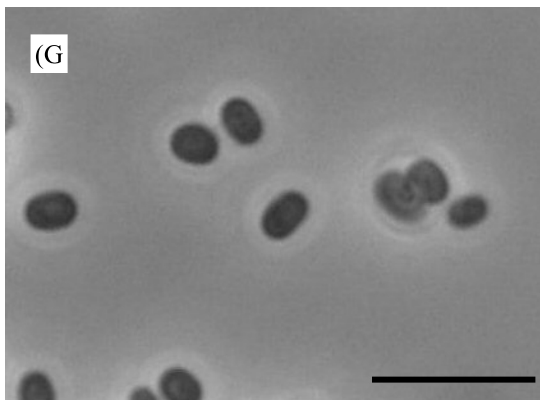






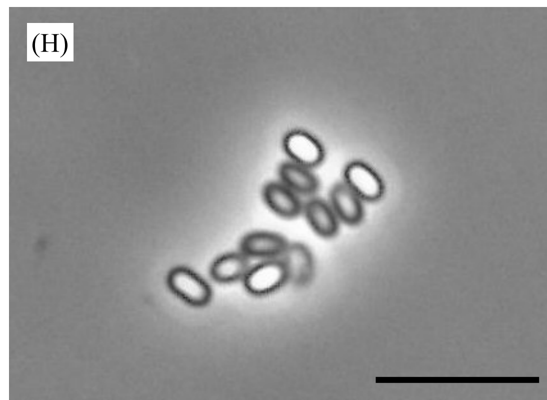
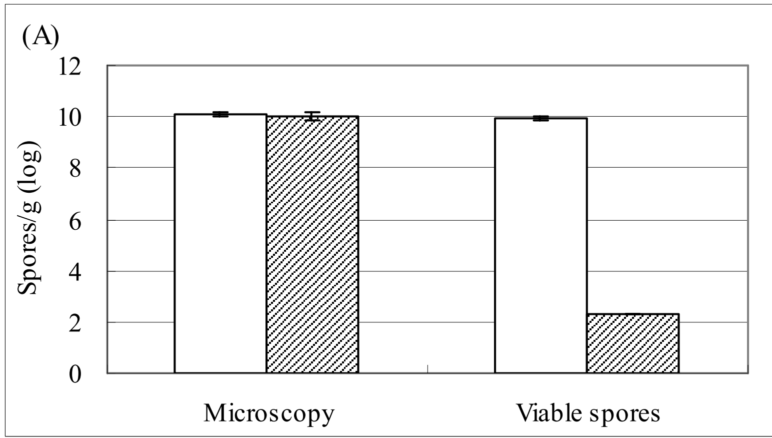


Fig. 4



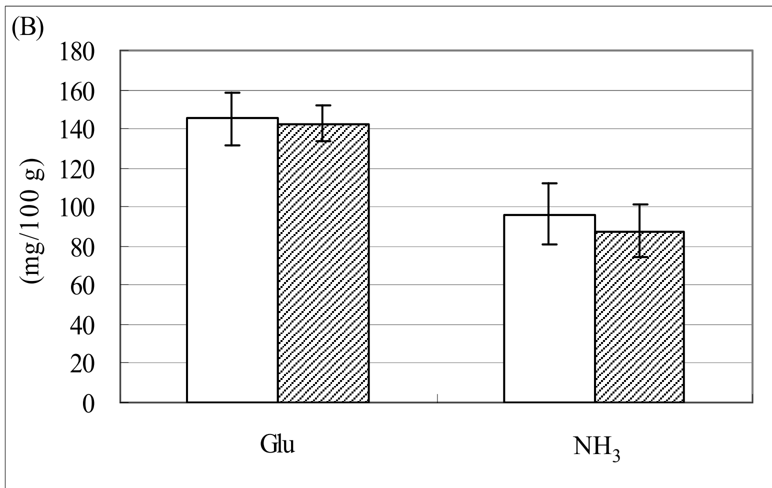


Fig. 5