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# Regulation and Characterization of a Newly Deduced Cell Wall Hydrolase Gene (*cwlJ*) Which Affects Germination of *Bacillus subtilis* Spores

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**The predicted amino acid sequence of *Bacillus subtilis ycbQ* (renamed *cwlJ*) exhibits high similarity to those of the deduced C-terminal catalytic domain of SleBs, the specific cortex-hydrolyzing enzyme of *B. cereus* and the deduced one of *B. subtilis*. We constructed a *cwlJ::lacZ* fusion in the *B. subtilis* chromosome. The β-galactosidase activity and results of Northern hybridization and primer extension analyses of the *cwlJ* gene indicated that it is transcribed by E$\sigma^E$ RNA polymerase. *cwlJ*-deficient spores responded to both L-alanine and AGFK, the $A_{580}$ values of spore suspensions decreased more slowly than in the case of the wild-type strain, and the mutant spores released less dipicolinic acid than did those of the wild-type strain during germination. However, the mutant spores released only slightly less hexosamine than did the wild-type spores. In contrast, *B. subtilis sleB* spores did not release hexosamine at a significant level. While *cwlJ* and *sleB* spores were able to germinate, CJSB (*cwlJ sleB*) spores could not germinate but exhibited initial germination reactions, e.g., partial decrease in $A_{580}$ and slow release of dipicolinic acid. CJSB spores became slightly gray after 6 h in the germinant, but their refractility was much greater than that of *sleB* mutant spores. The roles of the *sleB* and *cwlJ* mutations in germination and spore maturation are also discussed.**

During sporulation and germination of *Bacillus subtilis*, the action of autolysins is assumed to be required for asymmetric septum peptidoglycan hydrolysis, engulfment, cortex maturation, mother cell lysis, and cortex hydrolysis during germination (28, 33). Mother cell lysis depends on the compensatory effect of cell wall hydrolases CwlB (LytC) and CwlC (11, 13, 34). For cortex maturation, a defect in the *cwlD* gene leads to a lack of germination and blocking of the formation of muramic acid lactam structure in the cortex (2, 26, 31). Recently, Makino and colleagues reported that the *B. cereus sleB* gene encodes a 24-kDa mature germination-specific *N*-acetylmuramoyl-L-alanine amidase which degrades decoated spores from various organisms (18, 22). *B. subtilis sleB* is homologous to *B. cereus sleB*, and *B. subtilis sleB* mutant spores are able to germinate and form colonies. However, *B. subtilis* SleB showed no activity against degraded decoated spores or other substrates (21).

Our work on the *B. subtilis* genome sequencing project has revealed the *ycbQ* gene, which is homologous with the cortex-hydrolyzing *sleB* genes (22, 25). In this study, we describe the regulation and function of the *cwlJ* (*ycbQ*) gene and the compensatory effect of the CwlJ and *B. subtilis* SleB proteins on germination.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The strains of *B. subtilis* and *Escherichia coli* and the plasmids used in this study are described in Table 1. *B. subtilis* was grown on nutrient agar medium (Difco) at 30°C for about 10 h, then inoculated into DSM (Schaeffer medium) (30), and shaken at 37°C. If necessary, erythromycin and spectinomycin were added to the medium to final concentrations of 0.3 and 50 μg/ml, respectively. *E. coli* was grown in LB medium (29) at 37°C. If necessary, ampicillin was added to a final concentration of 50 or 100 μg/ml.

**Plasmid construction.** To construct a *B. subtilis cwlJ* mutant, an internal fragment of the *cwlJ* gene was amplified by PCR using two primers, forward primer cbQHF (5′-GCCGAAGCTTG$_{10}$*TGAGAGCAACGAGTGC*$_{26}$; the internal sequence of the *cwlJ* region is italicized, numbering is with respect to the first A of the translational start codon of *cwlJ*, and the *Hin*dIII site is underlined) and reverse primer cbQBR (5′-GCGCGGATCCT$_{212}$*ATCCATGAGTCACAGCC*$_{195}$; the sequence complementary to the internal region of *cwlJ* is italicized, and the *Bam*HI site is underlined), with *B. subtilis* 168 DNA as a template. The PCR fragment was digested with *Hin*dIII and *Bam*HI. To remove an extra cloning site region, pGEM-3zf(+) was digested with *Eco*RI and *Sma*I, blunt ended with mung bean nuclease, and then self-ligated. The resulting plasmid, pGEMΔES, and pMUTin2 were digested with *Hin*dIII and *Bam*HI and then ligated to the digested PCR fragment, followed by transformation of *E. coli* JM109. The resulting plasmid, pGEMcJ, was used to synthesize an RNA probe, and pMUTin2cJ was used for the transformation of *E. coli* C600 to produce concatemeric DNAs (3).

To construct a *B. subtilis sleB* mutant, the entire *sleB* region was amplified by PCR using two primers, forward primer soPF (5′-GCCGCTGCAGC$_{-165}$ *GTTCCGTTAATATGATGC*$_{-147}$; the upstream sequence of the *sleB* gene is italicized, numbering is with respect to the first A of the translational start codon of *sleB*, and the *Pst*I site is underlined) and reverse primer soER (5′-GCGCGAATTCC$_{2383}$*GCATTCAATATACTCACG*$_{2365}$; the sequence complementary to the internal region of *sleB* is italicized, and the *Eco*RI site is underlined), with *B. subtilis* 168 DNA as a template. The PCR fragment was digested with *Pst*I and *Eco*RI, followed by ligation into the corresponding sites of pUC118. The resultant plasmid, pUCSO, was digested with *Hin*cII and *Sma*I and then ligated into the *Hin*cII-*Stu*I fragment of the spectinomycin resistance (Sp$^r$) cassette plasmid pDG1727 (7). The resultant plasmid, pUCSOSP, was used to construct a *B. subtilis sleB* mutant.

**Mutant construction.** A *cwlJ*-deficient mutant, cbQ, was constructed by transformation of *B. subtilis* 168 with pMUTin2cJ. Disruption of the *cwlJ* gene by means of Campbell-type recombination was confirmed by PCR. Thus, the cbQ mutant was a *cwlJ-lacZ* transcriptional fusion strain. *sleB* and *sleB cwlJ* mutants 168SB and CJSB, respectively, were constructed by transformation of *B. subtilis* with *Sca*I-digested pUCSOSP DNA. The double-crossover recombination event was confirmed by PCR using primers soPF and soER.

**Transformation of *E. coli* and *B. subtilis*.** *E. coli* transformation was performed as described by Sambrook et al. (29), and *B. subtilis* transformation was performed by the competent cell method (1).

**Spore germination.** *B. subtilis* 168, cbQ, 168SB, and CJSB were cultured in DSM for 2 days at 37°C. Spores were suspended in deionized water and then washed by centrifugation as described by Nicholson and Setlow (23) until all cell

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype | Source or reference |
|---|---|---|
| *B. subtilis* | | |
| 168 | *trpC2* | D. Ehrlich |
| 1S38 | *trpC2 spoIIIC94* | BGSC[a] |
| 1S60 | *leuA8 tal-1 spoIIG41* | BGSC |
| 1S86 | *trpC2 spoIIA1* | BGSC |
| SpoIIIGΔ1 | *trpC2 spoIIIGΔ1* | 10 |
| cbQ | *cwlJ*::pMUTin2cJ | This study |
| 168SB | *sleB*::Sp$^r$ | This study |
| CJSB | *cwlJ*::pMUTin2cJ *sleB*::Sp$^r$ | This study |
| *E. coli* | | |
| JM109 | *recA1* Δ(*lac-proAB*) *endA1 gyrA96 thi-1 hsdR17 relA1 supE44* [F′ *traD36 proA$^+$B$^+$ lacI$^q$ lacZ*ΔM15] | Takara |
| C600 | *supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21* | Laboratory stock |
| Plasmids | | |
| pMUTin2 | *lacZ lacI bla erm* | D. Ehrlich |
| pGEM-3Zf(+) | *lacZ bla* | Promega |
| pMUTin2cJ | *lacZ lacI ΔcwlJ bla erm* | This study |
| pGEMcJ | *lacZ bla ΔcwlJ* | This study |
| pUCSO | *lacZ* Ap$^r$ *sleB* | This study |
| pDG1727 | Sp$^r$ | BGSC |
| pUCSOSP | *sleB*::Sp$^r$ | This study |

[a] BGSC, *Bacillus* Genetic Stock Center, Ohio State University.

debris and vegetative cells had been removed. The spores were heat activated at 80°C for 20 min, unless otherwise noted, and then diluted with a 10 mM Tris-HCl buffer (pH 8.4). Germination was initiated by the addition of L-alanine to 10 mM or AGFK (L-asparagine, D-glucose, D-fructose, KCl) to 10 mM each ingredient. At appropriate times, the $A_{580}$ of the mixture was measured and an 11-ml sample was taken and centrifuged in a microcentrifuge. The supernatant (1 ml) was used for the measurement of released dipicolinic acid as described by Nicholson and Setlow (23). The rest of the supernatant was dried with a concentrator (model CC-180; TOMY), followed by measurement of the released reducing groups by a modification (35) of the method of Park and Johnson, with *N*-acetylglucosamine as a standard. Dipicolinic acid in sporulating cells was determined by the method of Jannsen et al. as described by Nicholson and Setlow (23).

**β-Galactosidase assay.** The β-galactosidase assay was performed basically as described by Shimotsu and Henner (32). One unit of β-galactosidase activity was defined as the amount of enzyme necessary to release 1 nmol of 2-nitrophenol from 2-nitrophenyl-β-D-galactopyranoside in 1 min.

**Northern blot and primer extension analyses.** Cells (15 units of optical density at 600 nm) cultured in DSM were harvested and then suspended in 1 ml of chilled killing buffer (36). After centrifugation at 12,000 × *g* for 1 min at 4°C, the pellet was suspended in 1 ml of SET buffer containing lysozyme (final concentration, 6 mg/ml). After incubation for 10 min at 0°C, the suspension was centrifuged at 12,000 × *g* for 1 min at 4°C. The pellet was used for RNA preparation with Isogen (Nippon Gene) according to the manufacturer's instructions. Agarose-formaldehyde gel electrophoresis was performed as described by Sambrook et al. (29). The transfer of RNAs onto a nylon membrane (Magnagraph, Micron Separations) was performed with a vacuum blotter (model BE-600; BIO-CRAFT). The DNA fragment used for preparing an RNA probe was amplified by PCR with M13(−21) and M13RV (Takara) as primers and with pGEMcJ DNA, containing the internal region of *cwlJ*, as a template. The amplified fragment was digested with *Hin*dIII, and then fragments were purified by phenol and chloroform treatment, followed by precipitation with ethanol. The RNA probe was prepared with a DIG RNA labeling kit (Boehringer Mannheim), and Northern (RNA) hybridization was performed according to the manufacturer's instructions. Primer extension analysis was performed as described previously (31), using primer PEXcbQ (5′-$G_{44}$CCATCAAATCGACATCC$_{27}$; 5′ and 3′ ends correspond to the complementary nucleotides at positions 44 and 27 with respect to the 5′ end of the *cwlJ* gene).

## RESULTS

The *B. subtilis* genome project has revealed the existence of many cell wall hydrolase homologs, one of which is the product of the *ycbQ* gene located at 24° on the *B. subtilis* chromosome map. The amino acid sequence of YcbQ (renamed CwlJ) exhibits 28 and 30% identity with those of the deduced catalytic domains of *B. cereus* SleB and *B. subtilis* SleB (Fig. 1). The SleB proteins contain signal sequences and direct repeated sequences in their N-terminal regions, but CwlJ lacks both signal and repeated sequences.

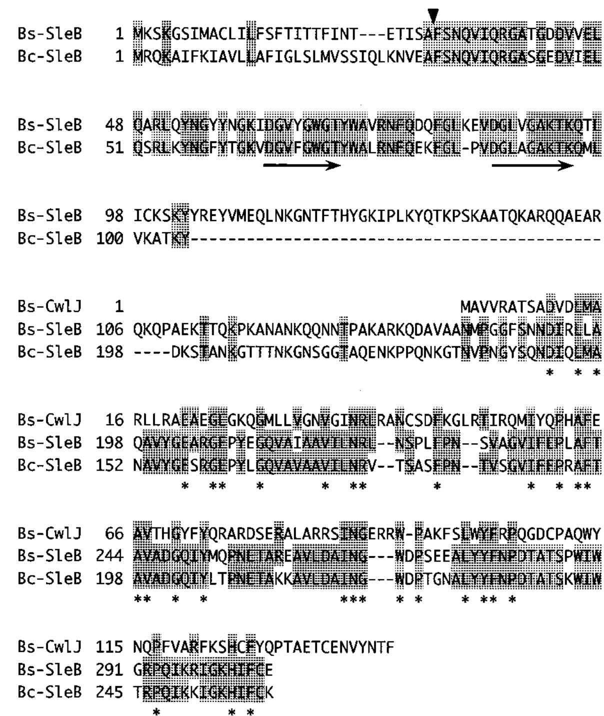

FIG. 1. Alignment of the deduced amino acid sequences of *B. subtilis* CwlJ (Bs-CwlJ; YcbQ) (25), *B. subtilis* (Bs-SleB; deduced *B. subtilis* cortex-hydrolyzing amidase) (22), and *B. cereus* SleB (Bc-SleB; *B. cereus* cortex-hydrolyzing amidase) (21). Amino acid identities are indicated by shading, and amino acids identical in the three proteins are indicated by asterisks. Amino acids are numbered from the N termini of the proteins, and dashes indicate the introduction of gaps in the alignment. The arrowhead and arrows indicate a signal sequence cleavage site and repeated sequences, respectively. The nucleotide sequence $G_{256}GC_{258}$ (numbering with respect to the first A of the translational start codon of *cwlJ*) under GSDB, EMBL, DDBJ, and NCBI accession no. D30808 should be corrected to CGG; thus, the corresponding amino acid, $G_{86}$ (numbered with respect to the N-terminal amino acid) should be corrected to R.

**Expression of the *cwlJ* gene.** The *cwlJ-lacZ* fusion strain, cbQ, was cultured in DSM, and β-galactosidase activity was determined. The activity was first detected after $t_2$ (2 h after the onset of sporulation) and was maximal at around $t_7$ (Fig. 2A), which indicates that the *cwlJ* expression is regulated by a sporulation-specific sigma factor. RNAs from four sigma factor-deficient strains, 1S86 (SigF$^-$), 1S60 (SigE$^-$), SpoIIIGΔ1 (SigG$^-$), and 1S38 (SigK$^-$), were analyzed by Northern blotting using an RNA probe containing the internal region of the *cwlJ* gene. Hybridizing bands for the 168, SigG$^-$, and SigK$^-$ RNAs were detected at 0.47 kb, but no band was observed for the SigF$^-$ and SigE$^-$ RNAs (Fig. 2B). The *cwlJ* gene consists of 426 nucleotide residues followed by a deduced rho-independent terminator ($\Delta G = -21.6$ kcal/mol) (Fig. 3) (25). Therefore, these results indicate that the *cwlJ* gene is transcribed by E$\sigma^E$ RNA polymerase as a monocistronic mRNA. To determine the promoter region, primer extension analysis was performed with 168, SigF$^-$, SigE$^-$, SigG$^-$, and SigK$^-$ RNAs along with primer PEXcbQ. Extended products were found at

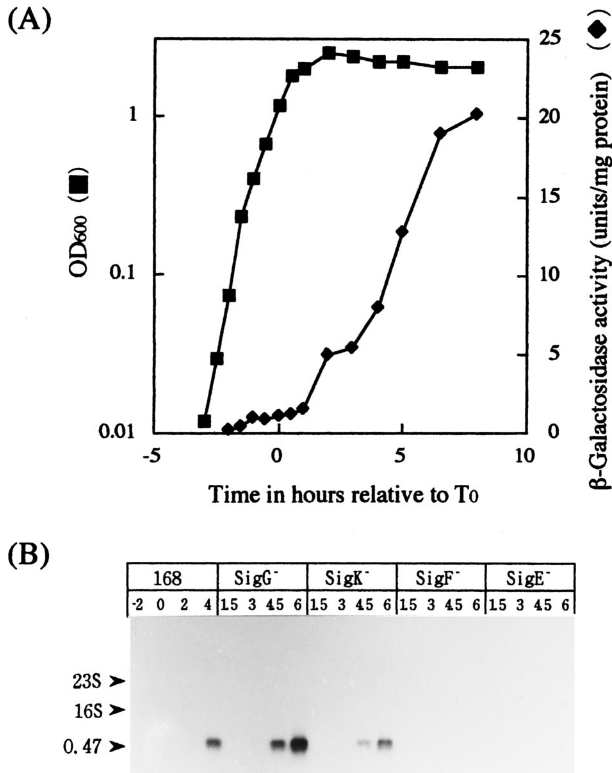


FIG. 2. Time course of the production of the *cwlJ-lacZ* fusion protein (A) and Northern blot analysis of the *cwlJ* region (B). (A) *cwlJ*-directed β-galactosidase activity of strain cbQ was determined at the indicated times after the onset of sporulation. Squares, cell growth at an optical density of 600 nm ($OD_{600}$); diamonds, β-galactosidase activity. (B) Northern hybridization performed with the *cwlJ*-specific RNA probe as described in Materials and Methods. The lanes contained 5 μg of RNA from *B. subtilis* 168 at $t_{-2}$ (−2), $t_0$ (0), $t_2$ (2), or $t_4$ (4), and *B. subtilis* 1S86 (SigF⁻), 1S60 (SigE⁻), SpoIIIGΔ1 (SigG⁻), and 1S38 (SigK⁻) at $t_{1.5}$ (1.5), $t_3$ (3), $t_{4.5}$ (4.5), or $t_6$ (6). 0.47 indicates the size (in kilobases) of the hybridizing RNA in comparison with the migration of 23S and 16S RNAs.

$t_{4.5}$ and/or $t_{7.5}$ for 168, SigG⁻, and SigK⁻ RNAs, but no products were observed at $t_{4.5}$ for the SigF⁻ and SigE⁻ RNAs (Fig. 3A). The products started at A, G, and A (residues 23, 22, and 21 bp upstream, respectively, of the translational start codon) (Fig. 3). The upstream sequences TCATcAc and aATAtgAT (capital letters denote consensus sequence), with their spacing of 14 bp, were very similar to the −35 and −10 consensus sequences of $\sigma^E$-dependent promoters ([T/G][A/C]ATA[A/T][A/T] and CATACA(A/C)T, respectively, with a spacing of 14 bp) (8).

**Construction and characterization of *cwlJ*, *sleB*, and *cwlJ sleB* disruptants.** cbQ (*cwlJ*), 168SB (*sleB*), and CJSB (*cwlJ sleB*) disruptants were constructed as described in Materials and Methods. The cbQ strain showed normal cell growth, cell separation, and motility and produced highly refractile spores. A previous report indicated that *B. subtilis sleB*-deficient spores did not complete darkening and released less dipicolinic acid during germination but still produced viable colonies after germination (21). Since CwlJ exhibits high amino acid sequence similarity with the C-terminal region of *B. subtilis* SleB (Fig. 1), germination was compared among 168, cbQ, 168SB, and CJSB spores. During germination in L-alanine-containing buffer, the 168SB spores showed a smaller decrease in $A_{580}$ than the wild-type 168 spores. The decrease in absorbance of cbQ spores exhibited a different pattern: there were large differences between cbQ spores and 168 and 168SB spores 30 min after the onset of germination. Levels of release of dipicolinic acid by these three strains during spore germination were also different, as 168SB and cbQ spores released dipicolinic acid in slightly lower and much lower amounts, respectively, than did 168 spores. CJSB spores exhibited only a 25% reduction in $A_{580}$ at 6 h after germination and a slower decrease in absorbance at 30 min after the onset of germination. CJSB spores released dipicolinic acid more slowly than the cbQ spores (Fig. 4). The cbQ spores released only slightly less hexosamine than those of the wild type, but the 168SB and CJSB spores did not release significant amounts of hexosamine. These results suggest that SleB is a major germination-specific cortex hydrolase and CwlJ is a minor one. The absorbance profiles of the spores in AGFK as a germinant were very similar to those in L-alanine

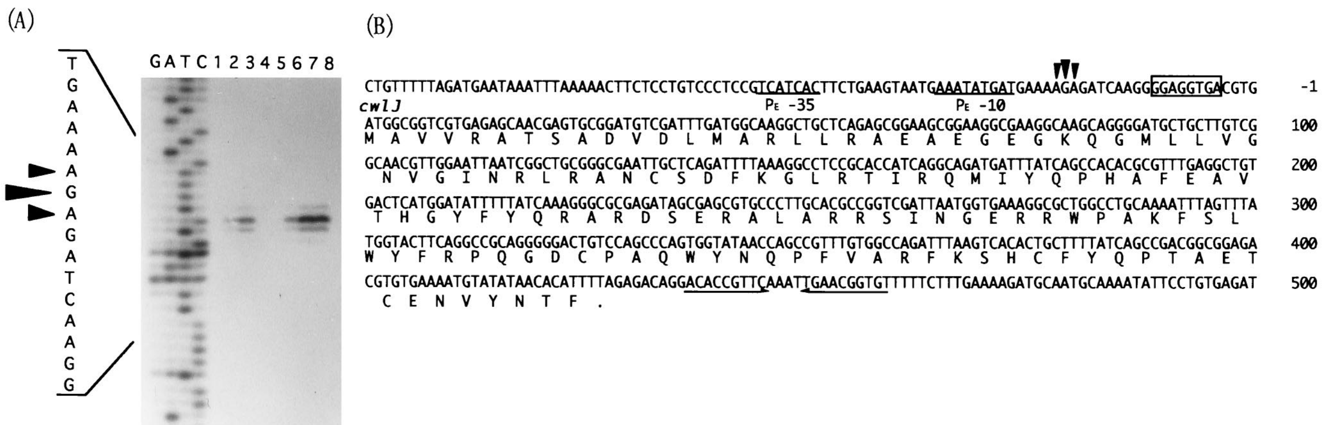


FIG. 3. Determination of the transcriptional start sites by primer extension analysis (A) and nucleotide sequence of the upstream region of *cwlJ* (B). (A) RNA (5 μg) from *B. subtilis* 168 at $t_{1.5}$ (lane 1), $t_{4.5}$ (lane 2), or $t_{7.5}$ (lane 3), 1S86 (SigF⁻) at $t_{4.5}$ (lane 4), 1S60 (SigE⁻) at $t_{4.5}$ (lane 5), SpoIIIGΔ1 (SigG⁻) at $t_{4.5}$ (lane 6), or 1S38 (SigK⁻) at $t_{4.5}$ (lane 7) or $t_{7.5}$ (lane 8) was hybridized with a labeled cbQPEX1 primer, which is complementary to nucleotides 27 to 44 in the sequence in panel B. The primer-extended products obtained with reverse transcriptase were subjected to electrophoresis in 12% (wt/vol) polyacrylamide sequencing gels, followed by autoradiography with an imaging plate (BAS-MP; Fuji). The dideoxy-DNA sequencing reaction mixtures with the cbQPEX1 primer were electrophoresed in parallel (lanes G, A, T, and C). The positions of the products are indicated by arrowheads. The boxed area indicates the −10 region of the $\sigma^E$ promoter. In panel B, the promoter regions (−35 and −10) of $\sigma^E$ and the transcriptional start position are indicated by underlines and arrowheads, respectively. The deduced rho-independent terminator is indicated by opposing arrows.

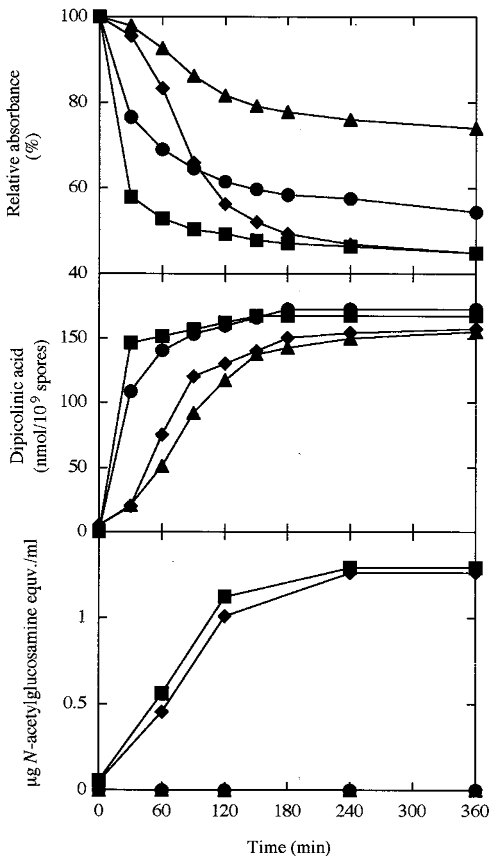


FIG. 4. Spore germination of *B. subtilis* 168, cbQ (*cwlJ*), 168SB (*sleB*), and CJSB (*cwlJ sleB*). The germination of spores of the *B. subtilis* strains was monitored at $A_{580}$ at the indicated times after the addition of L-alanine and is expressed as relative absorbance. The released dipicolinic acid and reducing groups in the supernatants of the spore suspensions were also measured. Squares, diamonds, circles, and triangles indicate *B. subtilis* 168, cbQ, 168SB, and CJSB, respectively.

as a germinant (9). Therefore, these effects were common to these germination pathways.

**Germination frequencies and microscopic observation of the mutant spores.** Table 2 shows the germination frequencies of the mutant spores with or without heat activation at 80°C for 20 min. These results indicate that the cbQ and 168SB spores are similar with respect to heat resistance and viability to spores of 168 under the conditions used. Spores of strain CJSB did not produce colonies on LB agar even without heat activation. Thus, this defect was not caused by heat sensitivity of the CJSB spores. Phase-contrast microscopy of the wild-type and mutant spores is shown in Fig. 5. Dormant spores of the mutants and the wild type were bright, and it was difficult to observe a difference in refractility. The refractility of cbQ spores changed from bright to dark after 6 h under the germination conditions, whereas 168SB spores became dark gray and CJSB spores became weakly bright under the same conditions. These phenomena show good agreement with the germination frequencies (Table 2). Therefore, these results indicate that both CwlJ and SleB are necessary for normal germination behavior of *B. subtilis* spores.

## DISCUSSION

*B. cereus* SleB was extracted from dormant spores, and the protein, which had been processed by signal peptidase, was active against the decoated spores of various bacilli (18, 22). However, the gene product of *B. subtilis sleB*, which had been cloned by colony hybridization with the *B. cereus sleB* fragment as a probe, was not detected in the germination exudate of the *B. subtilis* spores (21). *B. subtilis* CwlJ did not contain a signal sequence or direct repeated sequences in its N-terminal region (Fig. 1). Since the *cwlJ* gene was transcribed by $E\sigma^E$ RNA polymerase (Fig. 3), expression of *cwlJ* is mother cell specific, and a signal sequence may not be necessary for CwlJ to localize in the spore cortex. Direct repeated sequences have been found in the noncatalytic domains of many cell wall hydrolases (e.g., CwlA, CwlB [LytC], CwlC, LytD [CwlG], and XlyA from *B. subtilis* [11–13, 15, 17, 19, 27] and CwlL, CwlM, and CwlX from *B. licheniformis* and a cell wall hydrolase from *Bacillus* species [14, 16, 24]). In the case of CwlM, the noncatalytic domain containing direct repeats was related to the substrate specificity (14). In the case of the major autolysin, CwlB, the truncated protein comprising only the noncatalytic domain, bound tightly to cell walls (12). Therefore, CwlJ, lacking the noncatalytic domain, may have a wide substrate specificity and/or bind weakly to the *B. subtilis* cortex.

In *Clostridium perfringens*, two cortex-hydrolyzing enzymes (spore cortex lytic amidase SleC and cortical fragment-lytic muramidase SleM) play roles in cortex hydrolysis during spore germination (4, 20). Foster and Johnstone proposed a germination mechanism for *B. megaterium* (6), and Foster recently proposed a germination mechanism in *B. subtilis* in which three types of enzymes with different substrate specificities (amidase, endopeptidase, and transglycosylase) were required for cortex hydrolysis during germination in that order (5). Since the *sleB*-deficient mutant 168SB did not show significant hexosamine release during germination, SleB plays a major role in cortex hydrolysis during germination. Therefore, SleB probably corresponds to the amidase proposed by Foster. However, the 168SB spores did form colonies on LB agar plates. Therefore, there should be another lytic enzyme (probably an amidase) that plays a role in cortex hydrolysis at the early stage of germination. CwlJ is such a candidate, because spores of the *cwlJ*-deficient mutant cbQ exhibited a slow decrease in the $A_{580}$

TABLE 2. Germination frequencies of the *cwlJ* and *cwlJ sleB* mutants

| Strain | No. of spores | No. of colonies | | Germination frequency[b] (%) | |
|---|---|---|---|---|---|
| | | No heat | Heat[a] | No heat | Heat |
| 168 (wild type) | $1.3 \times 10^8$ | $3.2 \times 10^7$ | $2.8 \times 10^7$ | 24.6 | 21.5 |
| cbQ (*cwlJ*) | $9.8 \times 10^7$ | $2.7 \times 10^7$ | $1.7 \times 10^7$ | 27.6 | 17.3 |
| 168SB (*sleB*) | $9.3 \times 10^7$ | $5.0 \times 10^7$ | $4.0 \times 10^7$ | 53.8 | 43.0 |
| CJSB (*cwlJ sleB*) | $1.2 \times 10^8$ | $<1.0 \times 10^3$ | $<1.0 \times 10^3$ | $<8.5 \times 10^{-4}$ | $<8.5 \times 10^{-4}$ |

[a] Spore heat treatment was performed at 80°C for 20 min in 10 mM Tris-HCl (pH 8.4).
[b] Defined as the ratio of the number of colonies on LB agar after heat treatment or no heat treatment to the number of spores determined under a microscope.

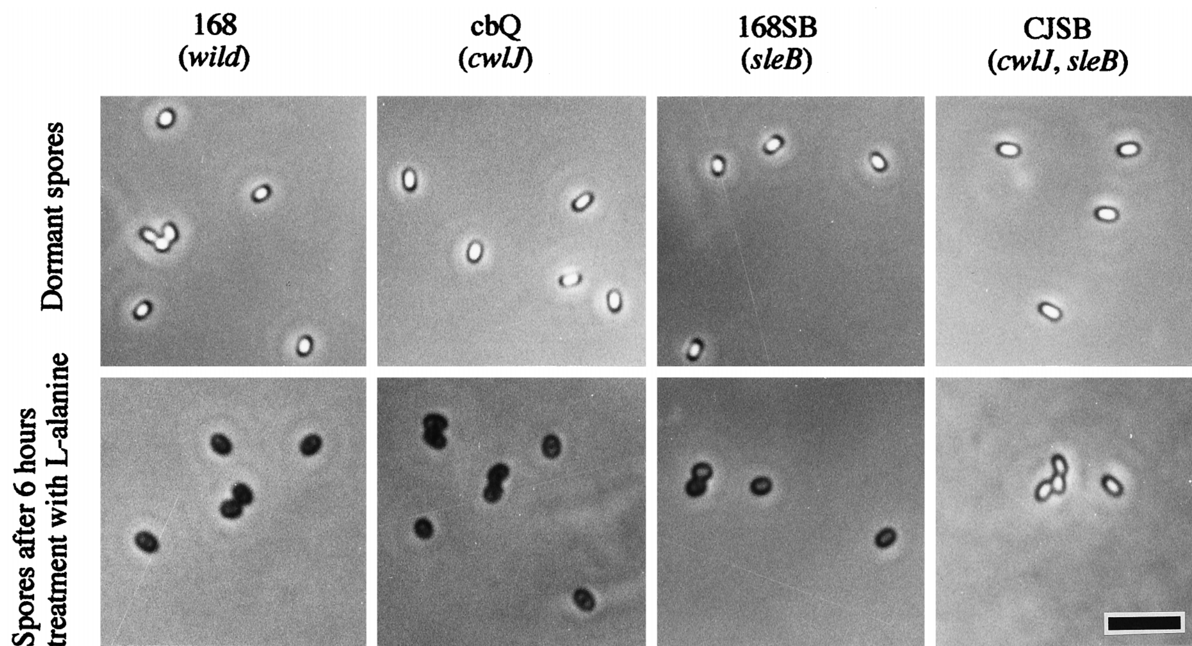


FIG. 5. Phase-contrast microscopy of *B. subtilis* 168, cbQ, 168SB, and CJSB spores treated with L-alanine. Spores were germinated at 37°C for 6 h as described for Fig. 4, with the addition of germination buffer (10 mM L-alanine, 10 mM Tris-HCl [pH 8.4]), and then observed by phase-contrast microscopy. Bar, 5 μm.

by 30 min after the onset of germination, and its dipicolinic acid release rate was lower than that of 168SB; cbQ also released slightly less hexosamine than did the wild-type 168 (Fig. 4). Moreover, the combination of these two proteins was essential for normal spore germination, because the *cwlJ sleB* double-mutant (CJSB) spores did not form colonies on LB agar plates (Table 2) and showed a change in refractility from only bright to faint gray (Fig. 5).

Obviously CwlJ plays a different role from SleB in spore germination. In addition to the foregoing results, the initial response of germination was severely affected by the *cwlJ sleB* double mutation, there being a smaller decrease in the $A_{580}$ value. The absorbance curve seems to be the combined pattern of the single mutations (Fig. 4). Therefore, CwlJ may be a minor cortex-lytic enzyme during germination. But another possibility, that CwlJ is a cortex maturation enzyme, cannot be eliminated at this stage. Previously, spores of the *cwlD*-deficient mutant ADD1, which lacks muramic acid lactam-forming activity, were found not to germinate (2, 26, 31). However, the cbQ spores germinated at the normal level (Table 2). Therefore, the latter possibility presupposes the presence of a new cortex hydrolase that acts at the early stage of germination and is unable to digest the cortex of cbQ spores.

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