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Nucleotide Sequence and Regulation of a New Putative Cell Wall Hydrolase Gene, *cwlD*, Which Affects Germination in *Bacillus subtilis*

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DNA sequencing of a region upstream of the *mms223* gene of *Bacillus subtilis* showed the presence of two open reading frames, *orf1* and *orf2*, which may encode 18- and 27-kDa polypeptides, respectively. The predicted amino acid sequence of the latter shows high similarity to a major autolysin of *B. subtilis*, CwlB, with 35% identity over 191 residues, as well as to other autolysins (CwlC, CwlM, and AmiB). The gene was tentatively named *cwlD*. Bright spores produced by a *B. subtilis* mutant with an insertionally inactivated *cwlD* gene were committed to germination by the addition of L-alanine, and spore darkening, a slow and partial decrease in A_{580}, and 72% dipicolinic acid release compared with that of the wild-type strain were observed. However, degradation of the cortex was completely blocked. Spore germination of the *cwlD* mutant measured by colony formation after heat treatment was less than 3.7×10^{-8}. The germination deficiency of the *cwlD* mutant was only partially removed when the spores were treated with lysozyme. Analysis of the chromosomal transcription of *cwlD* demonstrated that a transcript (RNA2) appearing 3 h after initiation of sporulation may have originated from an internal σ^E-dependent promoter of the *cwlD* operon, and a longer transcript (RNA1) appearing 4.5 h after sporulation may have originated from a σ^G-dependent promoter upstream of the *orf1* gene. The *cwlD* mutant harboring a *B. subtilis* vector plasmid containing the intact *cwlD* gene recovered germination at a frequency 26% of the wild-type level.

Bacillus subtilis produces several autolysins (9), including two major autolysins (CwlB [LytC] and CwlG [LytD]) (23, 28, 31, 40). CwlB is a 50-kDa *N*-acetylmuramoyl-L-alanine amidase (amidase) which cleaves the amide bond between the lactyl group of muramic acid and the α-amino group of L-alanine (23, 28), and CwlG is a 90-kDa endo-β-*N*-acetylglucosaminidase which cleaves the glycosyl bond between glucosamine and muramic acid (31, 40).

During sporulation and germination, the action of autolysins is assumed to be required for asymmetric septum peptidoglycan hydrolysis, which is a morphogenic transition between sporulation stages II and III, cortex maturation, mother cell lysis, and cortex hydrolysis during germination (5, 6, 13, 42, 45). The spore cortex, with a chemical structure slightly distinct from that of vegetative cell wall peptidoglycan, is apparently responsible for the maintenance of spore dormancy (6, 9). At the onset of germination, the cortex is selectively hydrolyzed, leaving a thin layer of vegetative cell peptidoglycan which forms the basis of the new vegetative cell wall (11). Germination-specific cortex-lytic enzymes which are apparently responsible for hydrolysis of the spore cortex during the germination response have been purified from spores of *Bacillus megaterium* KM (11, 12) and *Bacillus cereus* (30). It has proved difficult to solubilize autolysins from spores of *B. subtilis* (5, 9), although several sporulation-specific lytic activities have been identified by means of synthetic substrates (16) or by using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with substrate-containing gels (9). We recently cloned a sporulation-specific cell wall hydrolase gene (*cwlC*) from *B. subtilis* (20). CwlC degraded spore cortex peptidoglycan, but its function is still obscure.

We report here that a new gene exhibiting sporulation phase-specific gene expression, *cwlD*, encodes a putative cell wall hydrolase and that spores from a mutant having an insertionally inactivated *cwlD* gene are deficient in germination.

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MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The strains of *B. subtilis* used in this study are described in Table 1. *Escherichia coli* JM109 {[*recA1* Δ(*lac-proAB*) *endA1 gyrA96 thi-1 hsdR17 supE44 relA1* [F′ (*traD36 proAB lacI*^{q}ZΔM15)]} and plasmids pUC118 and pHY300PLK were purchased from Takara Shuzo Co. (Kyoto, Japan). Plasmid pKP1500 and strain spoIIIGΔ1 were kindly gifted by T. Miki (32) and P. Setlow (19), respectively. For nucleotide sequencing, M13mp18 and M13mp19 (Takara) were used. pGB223 is a pUC19 derivative containing a 2.5-kb *Eco*RI fragment from *B. subtilis* chromosomal DNA (14, 15). *B. subtilis* and *E. coli* were grown in LB medium (5 g of yeast extract, 10 g of polypeptone, and 10 g of NaCl per liter [pH 7.2]) at 37°C. If necessary, ampicillin, tetracycline, and chloramphenicol were added to final concentrations of 50, 20 and 10 μg/ml, respectively. For *B. subtilis* sporulation, Schaeffer medium (44) was used.

DNA sequencing. The *B. subtilis* 2.5-kb *Eco*RI chromosomal DNA fragment containing *orf1*, *orf2* (*cwlD*), *mms223* (methyl methanesulfonate phenotype), and part of the *gerD* genes present on plasmid pGB223 (Fig. 1) was subcloned into phages M13mp18 and M13mp19. The templates for nucleotide sequence determination were produced by cloning the 1.5-kb *Eco*RI-*Pst*I (containing *orf1* and *cwlD*), the 1.0-kb *Pst*I-*Eco*RI (containing the central to 3′-terminal region of *mms223* and the 3′-terminal region of *gerD*) and the 0.5-kb *Eco*RI-*Sph*I (con-

TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype	Reference or source
AC327	*purB his-1 smo-1*	1
1S38	*trpC2 spoIIIC94*	BGSC[a]
1S60	*leuA8 tal-1 spoIIG41*	BGSC
1S86	*trpC2 spoIIA1*	BGSC
spoIIIGΔ1	*trpC2 spoIIIGΔ1*	P. Setlow (19)
ADD1	*purB his-1 smo-1 cwlD::cat*	This study
YB886	*attSPβ amyE metB5 trpC2 xin-1*	3
BG305	*attSPβ amyE metB5 trpC2 xin-1 cwlD::cat*	This study
BG307	*attSPβ amyE metB5 trpC2 xin-1 mms223::cat*	This study

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

taining the 5′ terminus to the end to the 3′-terminal region of *orf1*) DNA subfragments into M13mp18 and M13mp19. Several specific primers were used when no suitable restriction sites were available. The complete nucleotide sequence of each strand was determined by using overlapping clones.

The upstream region of *orf1* was amplified by PCR, using an in vitro cDNA cloning kit (Takara) according to the manufacturer's instructions. *Bgl*II-digested fragments of *B. subtilis* AC327 chromosomal DNA were ligated into the dephosphorylated *Bam*HI site of pUC118. DNA was PCR amplified from the ligation mixture with the M13 primer RV (Takara) and primer Dscal, which is complementary to nucleotides 380 to 397 (Fig. 2). An amplified DNA (350 bp), the size of which is compatible with that predicted from the restriction map of the *gerD* region (53), was purified with a Gene Clean Kit II (Bio 101) and then subjected to sequencing on an Applied Biosystems model 373 DNA sequencer with the M13 primer RV and Dscal primer.

***B. subtilis* transformation.** Conventional transformation of *B. subtilis* was performed by the procedure of Anagnostopoulos and Spizizen (4).

Plasmid construction. Plasmid pBT374 is a pGB223 (*orf1 cwlD mms223* Ap^r) derivative in which the *cwlD* was in vitro inactivated at the unique *Bsm*I (blunt-ended) site by inserting a chloramphenicol resistance (Cm^r) cassette (cloned as a 1.3-kb *Eco*RI (blunt-ended) DNA fragment) (2). The insertional inactivation of the *cwlD* gene took place between codons 127 and 128. pBT375 is also a pBG223 derivative in which the *mms223* gene was in vitro inactivated at the unique *Spe*I site by inserting the *cat* cassette. The insertion took place between codons 9 and 10 of the *mms223* gene. For the transfer of the *cwlD*::*cat* and *mms223*::*cat* insertion alleles from plasmids pBT374 and pBT375, respectively, into the *B. subtilis* chromosome, the plasmids were digested with *Ava*II and used to transform *B. subtilis* YB886 competent cells to Cm^r as described previously (3). Recombination between the incoming single-stranded linear pBT374 or pBT375 DNA and the chromosome by a double crossover generates the Cm^r strains BG305 (*cwlD*::*cat*) and BG307 (*mms223*::*cat*), respectively. The allele replacement through a double crossover was confirmed by Southern blot analysis (2). To construct a *cwlD*::*cat* strain isogenic with *B. subtilis* AC327, pBT374 DNA was digested with *Bam*HI and used to transform AC327 competent cells to Cm^r (the generated strain was termed ADD1).

pHYD containing the *cwlD* gene was constructed as follows. The 1.5-kb *Eco*RI-*Pst*I fragment containing the *orf1* and *cwlD* genes (Fig. 1) was inserted into the *Eco*RI and *Pst*I sites of pKP1500. The resultant plasmid, pKPEPD, was digested with *Eco*RI and *Sph*I, blunt ended with T4 DNA polymerase, and then self-ligated. A 1.0-kb *Eco*RI (regenerated)-*Pst*I fragment of the resultant plasmid was ligated with a *Eco*RI-*Pst*I fragment of pHY300PLK containing a tetracycline resistance cassette and ori-pAMα1 (a replication origin for *B. subtilis*), and then the resultant plasmid, pHYD, was introduced into *B. subtilis*.

Spore germination. *B. subtilis* AC327 and ADD1 were cultured in Schaeffer medium for 2 days at 37°C. Spores were purified in Urografin (33% metrizoic acid; Sigma) with centrifugation at 3,000 × *g* for 15 min basically as described by Nicholson and Setlow (34). Spores were diluted with a 0.1 M Tris-HCl (pH 8.4)–0.1 M KCl solution to give a final A_{580} of about 16. After heat activation at 80°C for 20 min, the solution was kept at 37°C for 10 min, and then germination was initiated by the addition of L-alanine at 10 mM. At appropriate times, the A_{580} of the mixture was measured, and a 1-ml sample was taken and centrifuged in a microcentrifuge. The supernatant was used for the measurement of released dipicolinic acid as described by Nicholson and Setlow (34) and then for measurement of released reducing group by a modification (47) 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 (34).

RNA analysis. Ten milliliters of a sporulating cell suspension in Schaeffer medium was centrifuged. Each pellet was suspended in 1 ml of SET buffer (25) containing 2 mg of lysozyme. The suspension was immediately centrifuged for 2 min in a microcentrifuge. The pellet was used for RNA preparation with Isogen (Nippon Gene) according to the manufacturer's instructions. Northern (RNA) blot analysis of RNAs fractionated by electrophoresis in agarose-formaldehyde gels was performed as described by Sambrook et al. (43). The 0.9-kb *Eco*RI-*Sac*I fragment containing *orf1* and the 5′ half of the *cwlD* gene, the 0.4-kb *Hin*dIII fragment containing the 5′ half of the *cwlD* gene, and the 1.0-kb *Pst*I-*Eco*RI fragment containing *mms223* and the 3′ terminus of the *gerD* gene were radioactively labeled with the Multiprime labeling system (Amersham) and [α-^{32}P] dATP (3000 Ci/mmol; Nippon Gene). Primer extension analysis was performed as described previously (25). Primers Dsca2 and DH2 were oligonucleotides complementary to nucleotides 407 to 424 and 893 to 910 in the sequence, respectively. The primers were 5′ labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) and T4 polynucleotide kinase (Nippon Gene) according to the manufacturers' instructions.

Nucleotide sequence accession numbers. The GSDB/DDBJ/EMBL/NCBI accession numbers for the *cwlD* operon and its upstream sequences are X74737 and D38374, respectively.

RESULTS

Nucleotide sequence of the region upstream of the *mms223* locus. The *mms223* gene, whose mutation confers a methyl methanesulfonate-sensitive phenotype, was located downstream of the *gerD* gene (53) in the opposite direction (Fig. 1). Nucleotide sequencing of the region upstream of the *mms223* gene revealed the presence of two open reading frames (Fig. 1 and 2). An upstream open reading frame (*orf1*) encoding a protein of 147 residues with a molecular mass of 17,653 Da is preceded by a putative Shine-Dalgarno sequence (AAAGG AG; $\Delta G = -13.5$ kcal [-56.4 kJ]/mol). An open reading frame further downstream (*orf2*) encoding a protein of 237 residues with a molecular mass of 27,006 Da is preceded by a putative Shine-Dalgarno sequence (GGAGG; $\Delta G = -13.8$ kcal (-57.7 kJ)/mol) and followed by a typical rho-independent terminator ($\Delta G = -21.6$ kcal [-90.3 kJ]/mol). Another typical rho-independent terminator ($\Delta G = -22.7$ kcal [-95.0 kJ]/mol) is present upstream of *orf1*. Therefore, *orf1* and *orf2* seem to form an operon.

A similarity search of protein databases showed that the *orf2* product has extensive similarity to the cell wall-lytic amidase of *B. subtilis*, CwlB (35% identity over 191 residues) (23, 28), the sporulation-specific cell wall hydrolase of *B. subtilis*, CwlC (27% identity over 193 residues) (20), the cell wall-lytic amidase of *Bacillus licheniformis*, CwlM (26% identity over 194 residues) (27), and the cell wall-lytic enzyme of *E. coli*, AmiB (28% identity over 116 residues) (50) (Fig. 3). We have previously reported that the N-terminal to central region of the

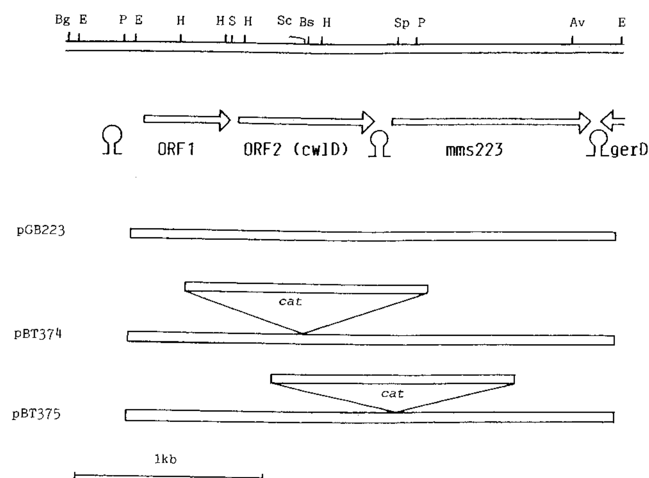

FIG. 1. Restriction map of the downstream region of the *gerD* gene of *B. subtilis*. Large unfilled arrows indicate the coding regions of the respective genes and their transcriptional direction. Stem-loop structures indicate the putative terminators. pGB223, pBT374, and pBT375 are pUC19 derivatives containing the indicated fragments. Abbreviations: Av, *Ava*II; Bg, *Bgl*II; Bs, *Bsm*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sph*I; Sc, *Sac*I; Sp, *Spe*I.

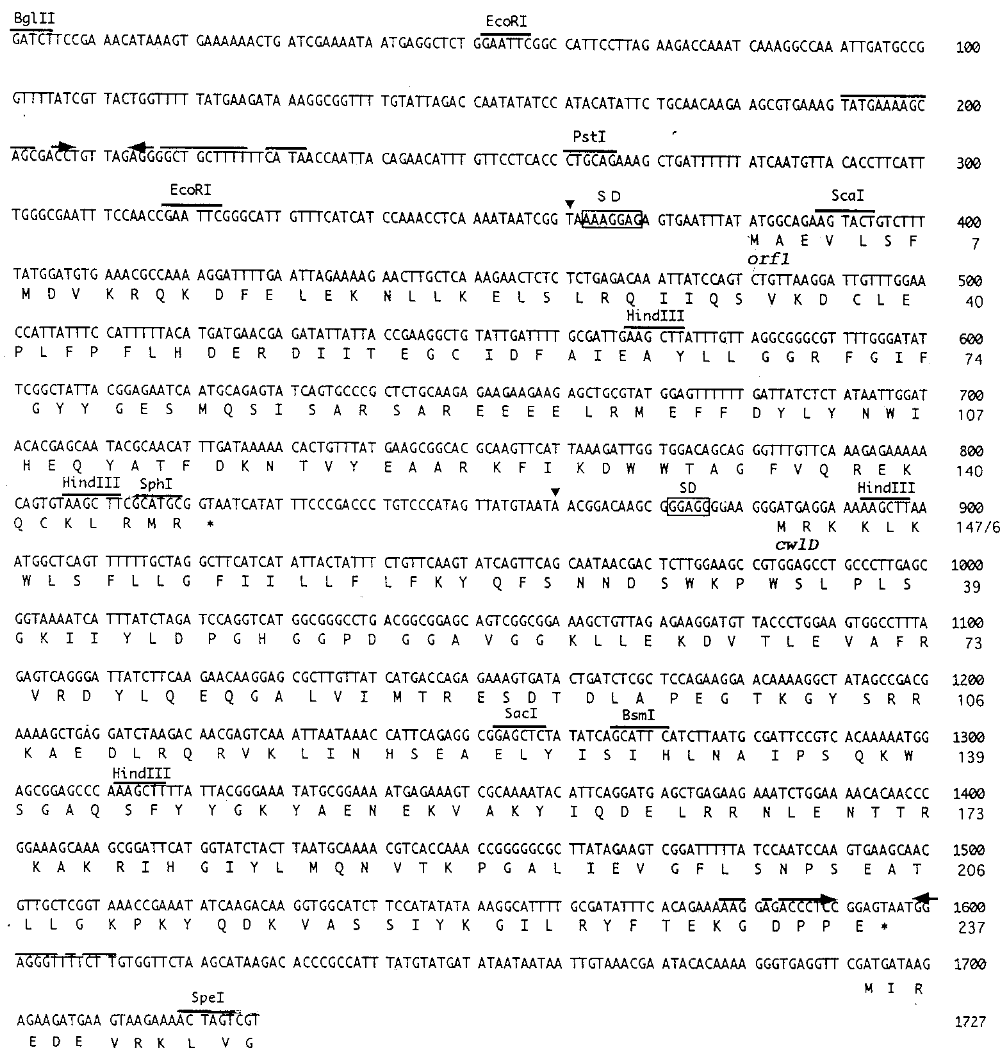

FIG. 2. Nucleotide sequence of *orf1* and *cwlD* genes of *B. subtilis*. Only the sequence of the nontranscribed DNA strand is shown, from positions 1 (*Bgl*II site) to +1727 (3 bp downstream of the *Spe*I site). The deduced amino acid sequences of *orf1* (nucleotides 381 to 821) and *cwlD* (884 to 1594) are given below the nucleotide sequence. Asterisks indicate stop codons. Putative Shine-Dalgarno sequences (SD; nucleotides 363 to 369 and 872 to 876) and putative rho-independent terminator sequences (nucleotides 190 to 232 and 1578 to 1611) are indicated. Arrowheads indicate the positions of the 5′ termini of transcripts determined by primer extension analysis. The nucleotide sequence of the 5′ region of *mms223* (1693 to 1727) and its deduced amino acid sequence are also shown. Nucleotide and amino acid numbers are shown on the right.

CwlM protein is a catalytic domain (27) and the C-terminal half of CwlB is probably involved in catalytic activity (23). Thus, it was tentatively concluded that *orf2* may encode a cell wall-lytic amidase, and its gene was designated *cwlD*. The CwlD protein contains a hydrophobic region (amino acid numbers 7 to 21) at the N terminus which is preceded by four positively charged amino acids. Therefore, the N-terminal sequence of the CwlD protein may act as a signal peptide or a membrane anchor (51). Amino acid sequence repetitions were found in the N-terminal region of the CwlB protein and in the C-terminal region of the CwlC and CwlM proteins, which are considered to recognize cell wall specificity (20, 27). On the contrary, the CwlD protein did not contain extensive direct repeats. Alignment of the five sequences reveals an insert of 14 amino acids (amino acid numbers 96 to 109) in the CwlD protein (Fig. 3). In particular, the amino acid sequence (93 TDLAPEG 99) around the insert was very similar to that of the germination-specific protease (GPR) cleavage site in the GPR zymogen (TDLAVEA) (18).

Insertional inactivation of the *cwlD* gene. To investigate a possible role of *cwlD*, we constructed a plasmid, pBT374, containing an insertionally inactivated *cwlD* gene by means of a *cat* gene (Fig. 1). The linearized pBT374 was used to transform *B. subtilis* AC327 to Cm^r. A transformant was designated *B. subtilis* ADD1. To confirm that the predicted insertion occurred in strain ADD1, Southern analysis was performed with a 2.5-kb *Eco*RI fragment as a probe. On *Hin*dIII digestion of the chromosomal DNA, four hybridizing bands at 3.5 kb (two overlapping fragments), 0.42 kb, and 0.24 kb for the parent AC327 and four bands at 3.5 kb (two overlapping), 1.7 kb, and 0.24 kb for the mutant ADD1 (fragments less than 0.12 kb were not analyzed) indicated the predicted double-crossover integration of the linearized pBT374 (26). *Eco*RI digestion of the chromosomes also supported these results (26).

Strain ADD1 showed normal growth, cell separation, and motility and gave bright refractile spores with about 87% efficiency. No significant change was found in the cell wall hydrolase profile as determined by electrophoresis of denaturing gels

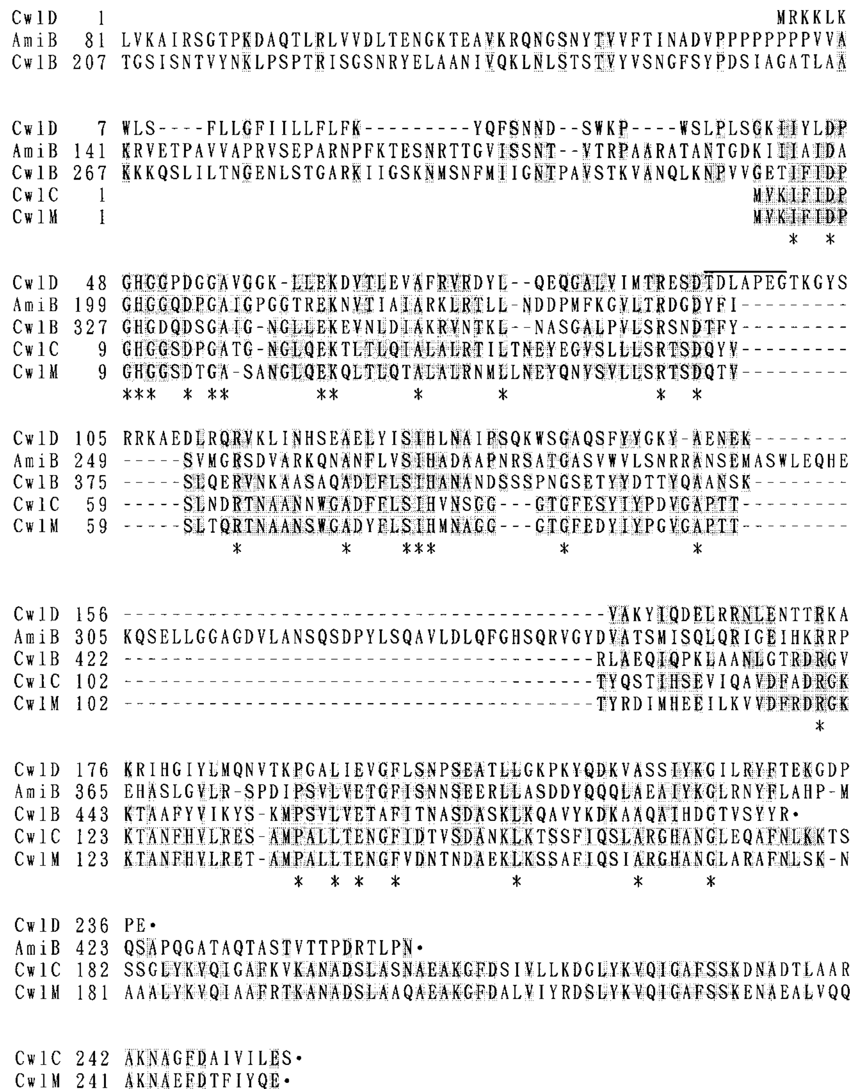

FIG. 3. Alignment of the deduced amino acid sequence of CwlD, AmiB (*E. coli*-amidase) (50), CwlB (*B. subtilis* major amidase at a vegetative phase) (23, 28), CwlC (*B. subtilis* sporulation-specific cell wall hydrolase) (20), and CwlM (*B. licheniformis* amidase) (27). Amino acid identities are indicated by two types of shading, and identical amino acids among the five proteins are indicated by asterisks. Amino acids are numbered from the N termini of the proteins, and dots indicate the C termini. Dashes indicate the introduction of gaps in the alignment, and an overline above the CwlD sequence indicates a sequence similar to the cleavage site of GPR (18).

containing *B. subtilis* vegetative cell wall, *Micrococcus luteus* cell wall, or *B. subtilis* spore cortex (26).

The colony-forming ability of spores of the *cwlD* mutant after heat treatment to kill vegetative cells (80°C, 20 min) indicated the germination of the *cwlD* mutant to be completely blocked (Table 2). In contrast, spores produced by *B. subtilis* BG307 (*mms223*::*cat*) germinate normally (26). Germination deficiency was also observed when the insertionally inactivated *cwlD* gene was introduced into a different wild-type strain (26). Interestingly, spores of the *cwlD* mutant treated with lysozyme (200 μg/ml) partially germinated (Table 2). After lysozyme treatment, spores which had been washed with 1% SDS solution and then four times with water retained the ability to germinate (Table 2). After heat activation of spores at 80°C for 20 min, germination was measured by monitoring the fall in the A_{580} of spore suspensions upon the addition of a germinant (10 mM L-alanine) (Fig. 4). Spores of strain ADD1 responded to L-alanine, and the A_{580} values of the spore suspensions slowly decreased by 30% as much as for the wild-type strain. During

TABLE 2. Effect of lysozyme treatment on spore germination and complementation analysis with a plasmid containing the *cwlD* gene

Strain	No. of spores	No. of colonies after heat treatment	Germination efficiency[a]
AC327	3.1×10^8	3.4×10^8	~1
ADD1	2.7×10^8	<10	$<3.7 \times 10^{-8}$
ADD1 (lysozyme)[b]	2.7×10^8	4.6×10^4	1.7×10^{-4}
ADD1 (lysozyme and SDS wash)[c]	2.7×10^8	1.3×10^4	4.8×10^{-5}
ADD1(pHYD)	8.0×10^6	2.1×10^6	2.6×10^{-1}

[a] Defined as the ratio of number of colonies on LB agar after heat treatment to that of spores counted under a microscope.

[b] Spores were suspended in a lysozyme solution (200 μg of lysozyme per ml in 10 mM potassium phosphate–50 mM KCl–1 mM $MgCl_2$) followed by incubation for 30 min at 37°C.

[c] After lysozyme treatment, spores were washed with a 1% SDS solution and then washed with water four times.

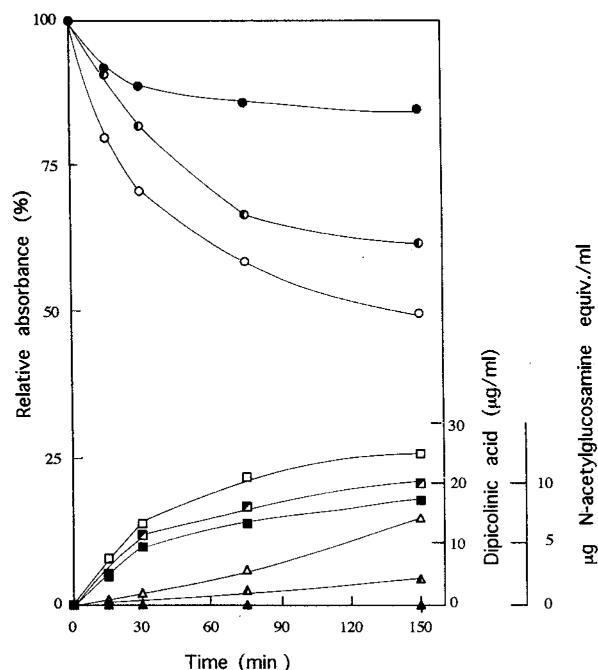

FIG. 4. Spore germination of *B. subtilis* AC327, ADD1, and AC327(pHYD). Germination of spores of *B. subtilis* strains was monitored at A_{580} at the indicated times after addition of L-alanine and is expressed as the relative absorbance (circles). Released dipicolinic acid (squares) and reducing groups (triangles) in the supernatant of spore suspensions were also measured. Unfilled, filled, and half-filled symbols indicate *B. subtilis* AC327, ADD1, and AC327(pHYD), respectively. equiv., equivalents.

the incubation, bright spores became darkened, and dipicolinic acid was released into the suspension in an amount not greatly different from that of the wild-type strain (18 μg/ml for *B. subtilis* ADD1 and 25 μg/ml for *B. subtilis* AC327 after a 2.5-h incubation). In contrast to the wild-type strain, however, the amount of reducing group released by ADD1 did not increase in the supernatant of the spore suspension, indicating that the ability to degrade cortex peptidoglycan and to release glycan strands containing free reducing groups was lacking. The *cwlD*-deficient spores did not produce any colonies on LB agar even when heat activation was omitted (26). Moreover, germination of spores without heat activation was monitored at 580 nm. Time courses of the A_{580} values of the AC327 and ADD1 spores in the germination solution were very similar to those with heat activation (26). Even if we treated the ADD1 spores with chloroform and then plated them on LB agar, the mutant spores did not germinate (less than 0.003% colonies per spore) (26).

Plasmid pHYD containing the *cwlD* gene and its σ^E promoter sequence (see below) was introduced into *B. subtilis* ADD1. The transformant ADD1(pHYD) showed rapid loss of absorbance (Fig. 4). Free reducing groups were released to 30% of the wild-type level (Fig. 4). The germination efficiency was also restored to 26% (Table 2). These results indicate that the gene product driven by the σ^E promoter is able to partially complement the defects in the *cwlD*-deficient strain.

Analysis of RNA transcripts of the *cwlD* gene. Northern blot analysis in Fig. 5A shows that two transcripts hybridized to a probe containing *orf1* and the 5′ half of the *cwlD* gene. For the wild-type strain, the smaller transcripts (RNA2) estimated to be 0.73 kb were detected 3 h (t_3) to 6 h (t_6) after initiation of sporulation (Fig. 5A). The larger transcript (RNA1) estimated to be 1.2 kb was detected 4.5 h ($t_{4.5}$) to 8.5 h ($t_{8.5}$) after sporulation. Neither of the transcripts was detected in RNA from cells at the vegetative growth phase (Fig. 5A). Other 1.1- and 1.4-kb transcripts were hybridized to a probe generated from a 1-kb *Pst*I-*Eco*RI fragment containing the *mms223* region (14, 15, 26), indicating that the two transcripts (RNA1 and RNA2) end at the putative terminator located between the *cwlD* operon and the *mms223* locus. Therefore, the 1.2-kb RNA1 may correspond to a polycistronic mRNA containing *orf1* and the *cwlD* loci. On the other hand, the 0.73-kb RNA2 may correspond to an mRNA containing the *cwlD* locus.

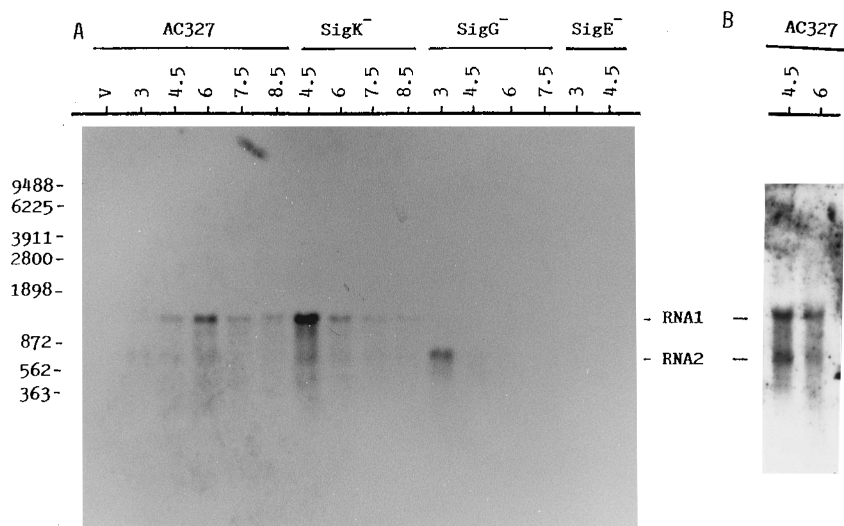

FIG. 5. Northern blot analysis of the *cwlD* region. Each lane contains 20 μg of RNA from *B. subtilis* AC327 during the vegetative growth phase (v) and at t_3, $t_{4.5}$, t_6, $t_{7.5}$, or $t_{8.5}$, *B. subtilis* 1S38 (*spoIIIC* SigK⁻) at $t_{4.5}$, t_6, $t_{7.5}$, or $t_{8.5}$, *B. subtilis* spoIIIGΔ1 (SigG⁻) at t_3, $t_{4.5}$, t_6, or $t_{7.5}$, or *B. subtilis* 1S60 (*spoIIG* SigE⁻) at t_3 or $t_{4.5}$, as indicated above the lanes. Northern hybridization was performed with a probe generated from a 0.94-kb *Eco*RI-*Sac*I fragment containing the *orf1* gene and the 5′ half of the *cwlD* gene, using a Multiprime labeling kit (Amersham) with [α-^{32}P]dATP (A), or from a 0.42-kb *Hin*dIII fragment containing the 5′ half of the *cwlD* gene labeled as described above (B). Hybridizing RNAs are indicated by bars. Sizes in base pairs of RNA standard marker (Seikagaku Kogyo, Tokyo, Japan) are indicated at the left of panel A.

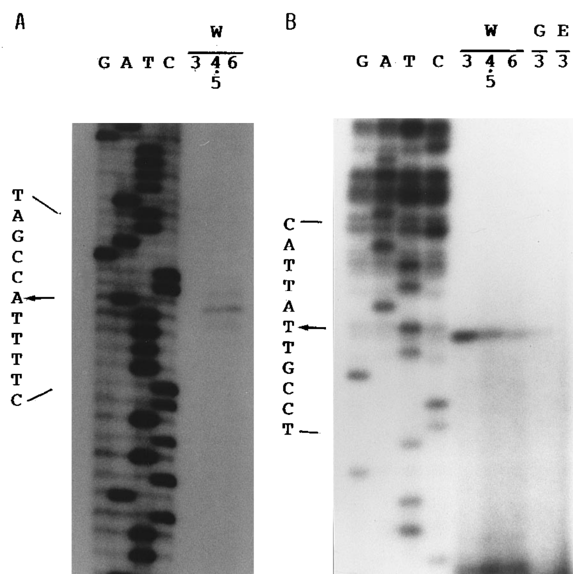

FIG. 6. Determination of transcriptional start sites by primer extension analysis. RNA (40 μg in panel A and 50 μg in panel B) from *B. subtilis* AC327 (W) at t_3 (lane 3), $t_{4.5}$ (lane 4.5), or t_6 (lane 6), *B. subtilis* spoIIIGΔ1 (G) at t_3 (lane 3), or *B. subtilis* IS60 (E) at t_3 (lane 3) was hybridized with a labeled Dsca2 primer, which is complementary to nucleotides 407 to 424 in Fig. 2 (A), or a labeled DH2 complementary to 893 to 910 (B). Primer-extended products obtained with reverse transcriptase were subjected to electrophoresis in 12% (wt/vol) polyacrylamide sequencing gels and then autoradiography. Dideoxy DNA sequencing reaction mixtures with the same primer (Dsca2 [A] or DH2 [B]) were electrophoresed in parallel (lanes G, A, T, and C). The positions of the products are indicated by arrows on the sequences.

RNA1 was missing in RNAs from the *spoIIG* (σ^E) and *spoIIIG* (σ^G) mutants but not the *spoIIIC* (σ^K) mutant (Fig. 5A), while RNA2 was missing in RNAs from *spoIIA* (σ^F) (26) and *spoIIG* (σ^E) mutants (Fig. 5A) but present in *spoIIIG* (σ^G) and *spoIIIC* (σ^K) mutants (Fig. 5A). These results indicate that RNA1 was driven from a σ^G-dependent promoter and that RNA2 was from a σ^E-dependent promoter.

Since the intensity of the signal of the RNA2 band was weaker than that of RNA1 (Fig. 5A), we changed the hybridizing probe from the 0.9-kb *Eco*RI-*Sac*I fragment containing *orf1* and the 5′ half of the *cwlD* gene to the 0.4-kb *Hin*dIII fragment containing only the 5′ half of the *cwlD* gene, followed by hybridization for *B. subtilis* AC327 (wild-type) RNAs at $t_{4.5}$ and t_6 (Fig. 5B). The 0.4-kb probe hybridized to RNA1 at $t_{4.5}$ and t_6 and to RNA2 at $t_{4.5}$ and weakly at t_6. The intensities of RNA1 and RNA2 at $t_{4.5}$ were about the same, suggesting similar contributions of RNA1 and RNA2 in producing the CwlD protein. A very weak band corresponding to a size of 0.92 kb was detected at t_6 (Fig. 5B).

Determination of the 5′ end of RNA1 and RNA2. From the sequence information and Northern blot analysis, it seemed likely that the 5′ end of RNA1 and RNA2 would be located upstream of the *orf1* gene and between *orf1* and *cwlD*, respectively. Primer extension analysis was performed with an oligonucleotide primer (Dsca2) that is complementary to the 5′ region of *orf1* (bases 407 to 424). A transcriptional signal was obtained with RNAs from cells at $t_{4.5}$ and t_6 but not at t_3 (Fig. 6A). Assuming that this transcript ends at the putative terminator, the length of the transcript calculated from the sequence would be 1.25 kb (Fig. 2). From the similarities in length and in the timing of the appearance of the transcript, the primer extension products seemed to correspond to the 5′ end of the 1.2-kb RNA1. The −35 (GGCATT) and −10 (CAAAATA) regions at a spacing of 18 bp for the apparent downstream start point were similar to those of the σ^G consensus sequence (TGAATA for the −35 region and CATACTA for the −10 region at a spacing of 17 to 18 bp; the underlined nucleotides are highly conserved [35]). The consensus sequences for σ^G- and σ^F-dependent promoters are rather similar, but Sun et al. demonstrated that good transcription by E-σ^F was correlated with G residues at positions −15 and −16, a purine residue at position −13, and a T residue at position −7 relative to the start site of transcription (46). The sequence of this promoter does not meet these requirements for a σ^F-dependent promoter except for a T at position −7. These results also suggest the dependence of RNA1 on E-σ^G.

Primer extension analysis was performed with an oligonucleotide primer (DH2) which is complementary to bases 893 to 910 in the 5′ region of the *cwlD* gene. A transcriptional signal was obtained with RNAs from wild-type cells at t_3, $t_{4.5}$, and t_6 (Fig. 6B). The signal was also obtained with RNA from the *spoIIIG* (σ^G) mutant, but not from the *spoIIG* (σ^E) mutant, at t_3 (Fig. 6B). The relative weakness of the signal in the *spoIIIG* mutant might be due to the difference in genetic background. These results indicate that *cwlD* is transcribed by E-σ^E. Assuming that this transcript ends at the putative terminator, the length of the transcript as calculated from the sequence would be 0.75 kb. From the similarities in length and the dependence of the transcript on E-σ^E, the primer extension products seem to correspond to the 5′ end of the 0.73-kb RNA2. The −35 (TCATATT) and −10 (CATAGTTA) regions at a spacing of 14 bp for the apparent downstream start point were very similar to those of the σ^E consensus sequence (kmATATT [k is G or T and m is A or C for the −35 region] and CATACA-T for the −10 region at a spacing of 14 to 15 bp [33, 41]). This result also agrees reasonably well with the dependence of RNA2 on E-σ^E.

These results suggest that at the sporulation stage, the *cwlD* gene is transcribed by E-σ^E, and then *orf1* and *cwlD* are transcribed polycistronically by E-σ^G in the forespore compartment.

DISCUSSION

Cell wall-lytic amidases in the genus *Bacillus* are classified into two groups; class I contains CwlA (8, 22), CwlL (36), and a lytic enzyme from PBSX (XlyA) (29), and class II contains CwlB (LytC) (23, 28) and CwlM (27). Furthermore, amino acid sequence homology among other autolytic enzymes suggests that a lytic enzyme of a *Bacillus* species (39) and a sporulation phase-specific lytic enzyme, CwlC (20), belong to class I and class II, respectively. In *E. coli* and *Salmonella typhimurium*, the gene product (amidase) of *amiB* upstream of the DNA repair gene (*mutL*) (50) and the deduced protein of *orf32* upstream of *hemF* (an oxygen-dependent coproporphyrinogen oxidase gene) (48, 49, 52) belong to the class II group. Recent reports suggest that the class I family may be lytic enzymes derived originally from phages, because the genes neighboring the lytic enzyme gene are functionally similar to phage genes (8, 10, 29, 36). However, in spite of extensive research on autolysins, their role in cell differentiation is almost unknown. CwbA (LytB) is a modifier protein which stimulates amidase activity, as measured by the decrease of cell wall turbidity, but does not stimulate activity measured by the increase of the N terminus of the L-alanine of the enzyme reaction products (17, 24). The *cwbA* gene is a second gene in the *cwlB* operon, and the amino acid sequences of the N-terminal and C-terminal regions of CwbA are highly homologous with those of the cell

wall binding domain of CwlB and the *spoIID* product, respectively (21, 28). However, disruption of the *cwbA* gene did not produce any morphological differences in cell growth and differentiation (26). We have shown here that a putative cell wall hydrolase gene, *cwlD*, is located upstream of the *mms223* gene near the *gerD* gene (mapped at 16°). Disruption of the *cwlD* gene led to deficiency in late germination. The predicted amino acid sequence of the CwlD protein showed high similarity with the sequences of proteins in the class II family but no similarity with those in class I. The central to C-terminal region of the CwlD protein may correspond to a catalytic domain. GPR, which digests small acid-soluble proteins, recognizes certain amino acid sequences. CwlD contains a similar sequence, which is not found in those of other amidases (Fig. 3) (18). Therefore, CwlD might be inactivated during germination. If this is the case, CwlD should be functional during the sporulation stage and/or in spores and may contribute to the formation of mature spores which are able to germinate.

Cell wall-lytic activity of the CwlD protein has not yet been detected in sporulating cell fractions by using SDS-polyacrylamide gel electrophoresis with substrate (cortex or vegetative cell wall)-containing gels. It is unlikely that this zymographic method will be able to identify all of the cell wall hydrolases because of possible heat and limited SDS sensitivity. Also, any protein composed of nonidentical subunits will be unable to renature. Moreover, Foster and Johnstone demonstrated that during the germination of *B. megaterium* KM spores, the 63-kDa pro form of a germination-specific cortex-lytic enzyme is processed to release the active 30-kDa enzyme (11, 12). Therefore, the possibility of modification of the CwlD protein to an active form during germination remains. On the other hand, the AmiA amidase of *E. coli* hydrolyzes only mucopeptides and not sacculi (38, 48). Moreover, Makino et al. reported the isolation from *B. cereus* of a spore-lytic 24-kDa enzyme which exhibits substrate specificity to the coat-stripped spore but not to the isolated spore cortex and vegetative cell wall (30). Therefore, the lack of success in detecting the CwlD enzyme may be due to the use of inadequate substrates.

The germination deficiency of the *cwlD* mutant was partially removed when the spores were treated with lysozyme. This result suggests that the CwlD protein could act as a cell wall hydrolase. Since spores washed with SDS solution after lysozyme treatment also recovered the ability to germinate (Table 2), the modification of spores is irreversible, indicating that the spore cortex is enzymatically modified with lysozyme. The spores of the wild-type strain were extremely resistant to lysozyme because the spore coat layers block lysozyme access to the peptidoglycan in the cortex and germ cell wall (34). However, adding excess lysozyme into a spore suspension of ADD1 might make lysozyme partially accessible to peptidoglycan of the cortex. Since the lysozyme and CwlD are muramidase and a predicted amidase, respectively, the low frequency of germination recovery of ADD1 spores with lysozyme may be due to a difference in the substrate specificities of the enzymes.

The *cwlD* promoters are parts of the σ^E and σ^G regulons, which are switched on in the mother cell and in the forespore, respectively. Since the CwlD protein has charged amino acids at the N-terminus, followed by the hydrophobic region, it might be located not on the cortex but on the spore membranes. We constructed a *B. subtilis* plasmid, pHYD, containing the σ^E-dependent promoter and the *cwlD* gene. The germination efficiency of the *B. subtilis* ADD1 harboring pHYD recovered considerably. Lysozyme-treated *cwlD*-deficient spores exhibited restored germination, albeit at low frequency. Therefore, it remains unknown whether the possible feature for the localization of the CwlD protein is important for germination. The role of the σ^G-dependent promoter for germination remains ambiguous. Although inactivation of the *orf1* gene did not lead to an apparent reduction of germination frequency (26), the σ^G-dependent promoter may be more important in producing the *orf1* product. Excess production of CwlD in the mother cell compartment may overcome the lack of CwlD transported from the forespore compartment. On the other hand, the sporulation efficiency (the ratio of the number of spores to that of vegetative cells observed under a microscope) of ADD1 harboring pHYD was reduced to about 1/15 that of ADD1 harboring pHY300PLK (26). It seems possible that the CwlD protein overexpressed in the mother cell inhibits sporulation.

The nucleotide sequence and deduced amino acid sequence of *orf1* showed no significant homology with sequences in the GSDB/DDBJ/EMBL/NCBI, Swiss, and PIR databases. The *orf1* gene is expressed polycistronically and only in the forespore from the σ^G-dependent promoter. The N-terminal amino acid sequence did not correspond to a signal sequence. Chou-Fasman analysis (7) of the amino acid sequence of ORF1 revealed that the region (amino acids 106 to 124) has the helix-turn-helix structure which may be required for protein-DNA interaction (37) and is followed by the C-terminal region, which contains higher amounts of positively charged amino acids.

This is the first report to describe a deduced cell wall hydrolase gene which affects germination. To clarify the effect, our present research is directed toward detecting the enzyme activity of the CwlD protein.

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