Transcriptional Regulation and Characteristics of a Novel *N*-Acetylmuramoyl-L-Alanine Amidase Gene Involved in *Bacillus thuringiensis* Mother Cell Lysis

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In *Bacillus thuringiensis*, a novel *N*-acetylmuramoyl-L-alanine amidase gene (named *cwlB*) was detected, and the CwlB protein was purified and characterized. Reverse transcription-PCR (RT-PCR) results indicated that *cwlB* and an upstream gene (named *cwlA*) formed one transcriptional unit. 5' rapid amplification of cDNA ends (5'-RACE)-PCR and transcriptional fusions with the *lacZ* gene indicated that transcription of the operon was directed by a promoter, P_{cwlA}, which is located upstream from the *cwlA* gene and that the transcription start site is a single 5'-end nucleotide residue T located 25 nucleotides (bp) upstream from the *cwlA* translational start codon. Moreover, the activity of P_{cwlA} was controlled by σ^K . Morphological analysis suggested that the mutation of *cwlB* could delay spore release compared to the timing of spore release in the wild-type strain. Western blot assay demonstrated that purified CwlB bound to the *B. thuringiensis* cell wall. Observations with laser confocal microscopy and a green fluorescent protein-based reporter system demonstrated that the CwlB protein localizes to the cell envelope. All results suggest that the CwlB protein is involved in mother cell lysis in *B. thuringiensis*.

Bacillus thuringiensis is a Gram-positive, spore-forming bacterium belonging to the *Bacillus cereus* group that produces parasporal crystal proteins. These parasporal crystal proteins are encoded by the *cry* or *cyt* genes and possess highly specialized insecticidal activity against a large number of insect species (1). Because of the insecticidal properties of the crystal proteins, *B. thuringiensis* has been commercially used for many years as a pesticide in biocontrol applications (2). However, one of the problems encountered after applying *B. thuringiensis* in the field is a lack of stability of the crystals on plant leaves due to inactivation by environmental factors, such as UV light (3, 4). A previous study showed that the cell envelope was sufficient to protect against *B. thuringiensis* crystal degradation under UV sunlight and had no significant effect on insecticidal activity. The genetic construction with σ^K disruption did not sporulate, still produced toxins, and did not result in lysis of the mother cell (5). However, disruption of the σ^K factor can decrease both the transcription of some *cry* genes during late sporulation and the biomass (6–8). Thus, developing an alternative method that will block mother cell lysis but have no effect on sporulation and Cry protein production is important.

Peptidoglycan hydrolases, some of which are known as autolysins, play an essential role in mother cell lysis of *Bacillus* strains; these proteins form a vast and highly diverse group of enzymes capable of cleaving bonds in polymeric peptidoglycan (sacculi) and/or its soluble fragments (9, 10). Four different families have been defined according to the chemical bond cleaved in the peptidoglycan molecule: *N*-acetylmuramidase, *N*-acetylglucosaminidase, *N*-acetylmuramyl-L-alanine amidase, and endopeptidase (11). These enzymes participate in bacterial cell wall growth, the turnover of peptidoglycan during growth, the separation of daughter cells during cell division, and autolysis (12–15). Three autolysins were reported to be involved in the mother cell lysis of *Bacillus subtilis*. Two autolysins, CwlB and CwlC, are present in large amounts at the time of mother cell lysis (16–18). A third, sporulation-specific amidase (CwlH), has also been shown to be

required for mother cell lysis and acts in a compensatory manner with CwlC. Single inactivation of *cwlB*, *cwlC*, or *cwlH* has not been shown to affect mother cell lysis. However, mother cell lysis was found to be blocked in a mutant that had multiple inactivated genes (19). In the *B. cereus* group, the cell wall hydrolases that are responsible for mother cell lysis still remain unknown.

The roles of several peptidoglycan hydrolases from the *B. cereus* group have been studied. The spore-lytic enzymes cortical-fragment-lytic enzyme (CFLE; *sleL* gene) and spore cortex-lytic enzyme (SCLE; *sleB* gene) were found in the exudate of germinated *B. cereus* IFO 13597 spores. These enzymes differ from each other in the morphology of the substrates that they recognize; CFLE requires disrupted spore peptidoglycan for its activity, and SCLE preferentially hydrolyzes intact spore peptidoglycan (20, 21). The potential cell wall peptidase CwpFM from *B. cereus* ATCC 14579 was shown to be involved in bacterial shape, motility, adhesion to epithelial cells, biofilm formation, vacuolization of macrophages, and virulence (22). These peptidoglycan hydrolases have not been reported to be involved in mother cell lysis.

In this study, two open reading frames (ORFs) (*bt2493* and *bt2494*, both encoding putative peptidoglycan hydrolases) were identified in the *B. thuringiensis* HD73 genome, and we named these two genes *cwlA* and *cwlB*, respectively. We also confirmed that *cwlB* is a novel cell wall hydrolase gene expressed during *B.*

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
<i>E. coli</i> strains		
TG1	$\Delta(lac-proAB) supE thi hsd-5$ (F' <i>traD36 proA⁺ proB⁺ lacI^q lacZ</i> Δ M15)	23
SCS110	$\Delta(lac-proAB) rpsL(Str^r) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44$ (F' <i>traD36 proAB lacI^q lacZ</i> Δ M15)	24
BL21(DE3)	F [−] <i>dcm ompT hsdS</i> (r _B [−] m _B [−]) <i>gal</i> λ (DE3)	33
BL21(pETcwlB)	BL21(DE3) with pETcwlB plasmid	This study
BL21(pGEXgerE)	BL21(DE3) with pGEXgerE plasmid	This study
<i>B. thuringiensis</i> subsp. <i>kurstaki</i> strains		
HD73	Contains <i>cryIAc</i> gene	25, 26
HD($\Delta sigK$)	HD73 mutant, $\Delta sigK$	8
HD($\Delta gerE$)	HD73 mutant, $\Delta gerE$	This study
HD($\Delta cwlA$)	HD73 mutant, $\Delta cwlA$	This study
HD($\Delta cwlB$)	HD73 mutant, $\Delta cwlB$	This study
HD($\Delta cwlA\Delta cwlB$)	HD73 mutant, $\Delta cwlA \Delta cwlB$	This study
HD(P _{cwlA} [−] - <i>lacZ</i>)	HD73 strain containing plasmid pHTP _{cwlA}	This study
HD $\Delta sigK$ (P _{cwlA} [−] - <i>lacZ</i>)	<i>sigK</i> mutant containing plasmid pHTP _{cwlA}	This study
HD $\Delta gerE$ (P _{cwlA} [−] - <i>lacZ</i>)	<i>gerE</i> mutant containing plasmid pHTP _{cwlA}	This study
HD(pHT-gfp- <i>cwlB</i>)	HD73 strain containing plasmid pHT-gfp- <i>cwlB</i>	This study
HD($\Delta cwlB::cwlB$)	HD($\Delta cwlB$) genetic complementation strain carrying pHTHFcwlB plasmid; Erm ^r	This study
HD($\Delta cwlA\Delta cwlB::cwlB$)	HD($\Delta cwlA\Delta cwlB$) genetic complementation strain carrying pHTHFcwlB plasmid; Erm ^r Kan ^r	This study
Plasmids		
pHT304-18Z	Promoterless <i>lacZ</i> vector, Ery ^r Amp ^r , 9.7 kb	31
pET-21b	Expression vector, Amp ^r , 5.4 kb	This laboratory
pGEX-4T-1	Expression vector, Amp ^r , 4.9 kb	34
pHT315	<i>B. thuringiensis</i> - <i>E. coli</i> shuttle vector	41
pRN5101	Temp-sensitive plasmid, 8.0 kb	42
pHTP _{cwlA}	pHT304-18Z carrying P _{cwlA} , Amp ^r Erm ^r	This study
pETcwlB	pET-21b containing <i>cwlB</i> gene, Amp ^r	This study
pGEXgerE	Pgex-4T-1 containing <i>gerE</i> gene, Amp ^r	This study
pHT-gfp- <i>cwlB</i>	pHT315 containing P _{cwlA} -gfp- <i>cwlB</i>	This study
pRN5101 Ω cwlA	pRN5101 carrying partial <i>cwlA</i> deletion gene	This study
pRN5101 Ω cwlB	pRN5101 carrying partial <i>cwlB</i> deletion gene	This study
pRN5101 Ω AB	pRN5101 carrying partial <i>cwlA cwlB</i> deletion gene	This study
pHTHFcwlB	pHT315 containing P _{cwlA} - <i>cwlB</i>	This study

thuringiensis sporulation, characterized the activity of the enzyme it encodes, and determined the localization, role in mother cell lysis, and transcriptional pattern of the enzyme. Our findings will better elucidate the mechanism of mother cell lysis in *B. thuringiensis* and provide new insight into the development of biocontrol agents with better persistence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* TG1 was used as the host for the cloning experiments, while SCS110 was used to generate unmethylated plasmid DNA for *B. thuringiensis* transformations (23, 24). *B. thuringiensis* HD73 was used as the recipient strain to monitor promoter activity and clone the target gene (25, 26).

E. coli was cultured at 37°C in Luria-Bertani medium (1% NaCl, 1% tryptone, 0.5% yeast extract). *B. thuringiensis* strains were grown at 30°C in Schaeffer’s sporulation medium (SSM) (27). The antibiotic concentrations used for bacterial selection were as follows: 100 µg/ml ampicillin for *E. coli* and 50 µg/ml erythromycin for *B. thuringiensis*.

DNA manipulation and transformation. PCR was performed using Taq DNA polymerase and KOD DNA polymerase (New England BioLabs

Ltd., Beijing, China). Amplified fragments were purified using Axygen, Inc., purification kits (Axgen Biotechnology Corporation, Hangzhou, China). Chromosomal DNA was extracted from *E. coli* and *B. thuringiensis* as described previously (28). Restriction enzymes and T4 DNA ligase (TaKaRa Biotechnology Corporation, Dalian, China) were used according to the manufacturer’s instructions. Oligonucleotide primers (see Table S1 in the supplemental material) were synthesized by Sangon (Shanghai, China). Plasmid DNA was extracted from *E. coli* using the Axygen, Inc., plasmid extract kit (Axgen Biotechnology Corporation, Hangzhou, China). All constructs were confirmed by DNA sequencing (BGI, Beijing, China). Standard procedures were used for *E. coli* transformation. *B. thuringiensis* cells were transformed by electroporation as previously described (29).

Total RNA isolation and RT-PCR analysis. Total RNA extraction from *B. thuringiensis* HD73 and reverse transcription-PCR (RT-PCR) identification were performed as described previously by Du et al. (30) unless otherwise noted. For RT-PCR analysis, the primers used to detect the expression of the *cwlA* and *cwlB* genes are shown in Table S1 in the supplemental material.

Determination of transcriptional start sites. To determine the transcriptional start sites, we employed the SMARTer RACE (switching mechanism at the 5’ end of the RNA transcript-rapid amplification of cDNA

ends) cDNA amplification kit (Clontech, Mountain View, CA) following the manufacturer's instructions. Gene-specific primers, CwLAce, and universal primer mix (UPM) were used to amplify the 5' end of *cwlB* mRNA.

Construction of promoter P_{cwlA} fusions with the *lacZ* gene. The putative promoter fragment of P_{cwlA} (102 bp), which is located upstream from the *cwlA* gene, was cloned from *B. thuringiensis* HD73 genomic DNA using the specific primers P_{cwlA} -5 (with a PstI restriction site) and P_{cwlA} -3 (with a BamHI restriction site). The PstI-BamHI fragment of the P_{cwlA} promoter was then integrated into the vector pHT304-18Z, which harbors a promoterless *lacZ* gene (31). The recombinant plasmid pHTP $_{cwlA}$ was introduced into HD73 and the *sigK* mutant (8). The corresponding strains HD(P_{cwlA} -*lacZ*) and HD Δ *sigK*(P_{cwlA} -*lacZ*) were selected by erythromycin resistance and PCR identification.

β -Galactosidase assays. *B. thuringiensis* strains containing *lacZ* transcriptional fusions were cultured in SSM at 30°C and 220 rpm with appropriate antibiotics. Samples of 2.0 ml were taken from T_8 to T_{20} at 1-h intervals (T_0 is the end of the exponential phase; T_n is n hours after the end of the exponential phase). The cells were harvested, and the specific β -galactosidase activities of the samples were measured as previously described and expressed as Miller units per milligram of protein (32). The values reported are the means for at least three independent experiments.

Expression and purification of CwLB protein. CwLB protein with a His tag was purified from *E. coli*. The expression plasmid pETCwLB was constructed by PCR amplification of the *cwlB* sequence from the *B. thuringiensis* HD73 genome using the primer pair *cwlB*-5 (with a BamHI restriction site) and *cwlB*-3 (with a SalI restriction site). The DNA fragment was digested with BamHI and SalI, cloned into pET21b (Novagen, Bloemfontein, South Africa) digested with the same restriction enzymes, and transferred into *E. coli* BL21(DE3) (33).

The *E. coli* BL21 strain harboring pETCwLB was grown to log phase in LB medium with ampicillin at 37°C. Expression of the CwLB-His protein was induced by adding IPTG (isopropyl- β -D-thiogalactopyranoside) to a final concentration of 2 mM, and the cultures were incubated for 3 h at 37°C. The cells were harvested by centrifugation at $13,500 \times g$ for 10 min in 50-ml tubes and resuspended in 10 mM imidazole NPB buffer (10 mM imidazole, 1 M NaCl, 20 mM sodium phosphate, pH 7.4). Bacteria were lysed on ice by sonication using an ultrasonic cell disruption system. The bacterial lysate was centrifuged at $16,000 \times g$ for 10 min at 4°C, and the supernatant contained the solubilized CwLB-His protein. The supernatant was filtered through a 0.45- μ m-pore-size membrane filter (Nalgene) and loaded onto a HiTrap chelating column (1 ml; Pharmacia). After binding the protein, the column was washed with 10 mM imidazole NPB solution, and the target CwLB-His protein was eluted with NPB solution containing a stepwise gradient of imidazole from 100 to 500 mM.

Expression and purification of GerE. GerE protein with a glutathione S-transferase (GST) tag was purified from *E. coli*. The expression plasmid pGEXgerE was constructed by PCR amplification of the *gerE* sequence from the *B. thuringiensis* HD73 genome using the primer pair *gerE*-5 (with a BamHI restriction site) and *gerE*-3 (with a SalI restriction site). The DNA fragment was digested with BamHI and SalI, cloned into pGEX-4T-1 (34) digested with the same restriction enzymes, and transferred into *E. coli* BL21(DE3).

When the optical density at 600 nm (OD_{600}) reached 0.6, IPTG was added to a final concentration of 1 mM. After 4 h of induction at 37°C, bacterial cells were harvested by centrifuging the culture at $13,500 \times g$ for 10 min. The pellet was resuspended with phosphate-buffered saline (PBS) buffer and sonicated on ice. All subsequent procedures were carried out at 4°C. The supernatant was collected by centrifuging the lysate at $13,500 \times g$ for 20 min and loading it onto a glutathione-Sepharose 4B column previously equilibrated with PBS buffer. The column was washed with 50 mM Tris-HCl (containing 10 mM reduced glutathione, pH 8.0). The fractions were analyzed by SDS-PAGE. Fractions with the target protein were pooled and dialyzed against PBS buffer. The purified GST-GerE protein was analyzed by SDS-PAGE on a 12% polyacrylamide gel using a

protein molecular standard. All the steps described above were performed according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Gel mobility shift assays. GST-tagged GerE protein was purified from *E. coli* BL21(DE3) as described above. GerE- P_{cwlA} binding experiments were performed using a modified gel mobility shift assay described previously (35). The DNA probe (1 mg) was incubated with various concentrations of purified GerE at 25°C for 20 min in a buffer containing 20 mM Tris base (pH 7.5), 2 mM dithiothreitol (DTT), 5 mM MgCl₂, 0.5 mg ml⁻¹ calf bovine serum albumin (BSA), and 5% (vol/vol) glycerol in a total volume of 20 μ l. After incubation, nondenaturing 4% (wt/vol) polyacrylamide gels were stained with SYBR gold nucleic acid gel stain (Invitrogen) for 40 min in TBE (89 mM Tris-base, 89 mM boric acid, 1 mM EDTA, pH 8.0) buffer and photographed under UV transillumination using a Fuji X-5000.

Preparation of cell walls. *B. thuringiensis* HD73 cells from exponential-phase cultures (absorbance at 600 nm of 1.5 to 2.0) growing in LB medium were deposited by centrifuging ($16,000 \times g$ for 5 min) at 4°C. All subsequent treatments were at 0 to 4°C. The bacteria were resuspended in a small volume of TK buffer (0.1 M Tris-HCl, 0.1 M KCl, pH 8.0) and disrupted with a BeadBeater (Biospec) in a vessel containing glass beads (0.1-mm diameter). After low-speed centrifugation ($1,000 \times g$, 10 min) to remove the glass beads and unbroken cells, the crude cell walls were pelleted at $27,000 \times g$ for 5 min at 4°C. The pellets were washed three times with TK buffer and resuspended in TK buffer (36).

Cell wall binding ability test. The cell wall binding ability of the CwLB hydrolase enzyme was examined in distilled water containing the purified protein and *B. thuringiensis* cell walls. After 30 min of incubation at 0°C, the reaction mixture was centrifuged, and then the protein in the sediment was used for SDS-PAGE and Western blot analyses. Proteins in the samples were separated by electrophoresis (4% polyacrylamide stacking gel, 10% polyacrylamide separating gel) and then transferred onto polyvinylidene difluoride (PVDF) membranes before incubation in a 1:5,000 dilution of anti-His antibody (Proteintech Group, Inc., Chicago, IL, USA). Western blotting experiments were performed after the samples were separated by SDS-PAGE as described by Wang et al. (23).

Determination of the sites of cell wall cleavage by CwLB. *B. thuringiensis* cell walls were suspended in 0.1 M TK buffer (adjusted to pH 7.0) to yield a final absorbance of 0.3 at 540 nm. Purified CwLB-His was added to the solution, followed by incubation at 37°C for 0, 10, 20, 40, or 60 min. One unit of cell wall hydrolase activity was defined as the amount of enzyme necessary to reduce the absorbance at 540 nm by 0.001 per min (37).

The amino groups released were labeled with 1-fluoro-2,4-dinitrobenzene during enzyme digestion of *B. thuringiensis* cell walls (38). The hydrolyzed dinitrophenyl (DNP) compounds were analyzed on a reverse-phase column. The release of free reducing groups during the enzymatic reaction was assayed as described by Thompson and Shockman (39).

Construction of GFP gene fusion directed by the CwLA promoter. To determine the localization of the CwLB protein in *B. thuringiensis*, a fusion construct with the GFP gene was created by PCR amplification. A 669-bp DNA fragment of the *cwlB* ORF and 864 bp of the upstream sequence, including the 102-bp promoter, 714-bp GFP ORF, and 48-bp linker, were spliced in turn by overlapping PCR (see Fig. 7A). *cwlB* and promoter P_{cwlA} were amplified by PCR using genomic DNA from *B. thuringiensis* HD73 as the template. The GFP ORF and linker fragment were amplified by PCR using the Cry1Ac-GFP plasmid as the template (40). The PstI-EcoRI fragment of GFP-*cwlB* was then ligated into the shuttle vector pHT315 (41). Recombinant pHT-*gfp-cwlB* was introduced into the *B. thuringiensis* HD73 strain by electroporation to obtain the corresponding strain HD-(pHT-*gfp-cwlB*). The primers in this study are listed in Table S1 in the supplemental material.

Laser confocal microscopy. FM4-64, a vital membrane stain (Molecular Probes, Inc., Eugene, OR), was suspended in DMSO (dimethyl sulfoxide) at a final concentration of 100 μ M liter⁻¹. Bacterial cells were

stained with FM4-64 (100 μM liter⁻¹) for 1 min on ice. The stained cells were scanned using a confocal laser scanning microscope (Leica TCS SL; Leica Microsystems, Wetzlar, Germany).

GFP quantification in the *B. thuringiensis* cell walls. The cell walls from the *B. thuringiensis* HD (pHT-*gfp-cwlB*) strain were collected for GFP quantification at T_9 , T_{12} , T_{15} , and T_{18} , and the OD₆₀₀ was determined by spectrophotometry. The GFP fluorescence of the *B. thuringiensis* cell walls was measured at an excitation wavelength of 476 nm and an emission wavelength of 507 nm using a fluorescence spectrophotometer (F-4500; Hitachi, Japan). For each specific measurement, 400 μl of each culture was placed into a cuvette and measured immediately. The relative fluorescence units of GFP were obtained by dividing the fluorescence value by the OD₆₀₀ (40).

Construction of the *cwlA*, *cwlB*, and *cwlAB* deletion mutants. All primers for gene deletion were designed according to the *B. thuringiensis* HD73 genome sequence. The 322-bp fragment upstream from the start codon of *cwlA* (*cwlA* fragment A) was amplified by PCR using *B. thuringiensis* HD73 genomic DNA as the template and CwIA-a and CwIA-b as primers. The primers CwIA-c and CwIA-d were used to amplify the 335-bp fragment downstream from the 107th codon of *cwlA* (*cwlA* fragment B), and the primers AKm-a and AKm-b were used to amplify a 1,473-bp kanamycin resistance gene (*kan*) cassette which is directed by the *PaphA3* promoter from pDG780 (23). *cwlA* fragment A, *kan*, and *cwlA* fragment B were ligated together by overlapping PCR using primers CwIA-a and CwIA-d. The resulting fragment (2,150 bp) was inserted into the BamHI-SalI restriction sites of the erythromycin-resistant, temperature-sensitive suicide plasmid pRN5101 (42) to generate the pRN5101 Δ *cwlA* plasmid. The recombinant plasmid was electroporated into HD73. Transformants resistant to erythromycin and kanamycin were grown at 30°C and transferred to LB liquid medium with kanamycin at nonpermissive temperature (38°C) for about 10 generations. The bacterial cells were then plated onto LB agar plates supplemented with kanamycin or erythromycin, respectively. They were incubated for 12 h at 30°C. The transformants with kanamycin resistance but without erythromycin resistance were screened and identified by PCR; the final HD(Δ *cwlA*) strain, with a deletion from the start codon to the 107th codon of *cwlA* and insertion of the kanamycin resistance gene cassette at the same position, was verified by PCR; and DNA sequencing was performed (see Fig. S1 in the supplemental material).

The primers AB-a/AB-b/AB-c/AB-d and ABKm-a/ABKm-b were used to amplify the 382-bp fragment upstream from the start codon of *cwlA* (*cwlA* *cwlB* fragment A), the 1,473-bp *kan* gene cassette, and the 279-bp fragment downstream from the end codon of *cwlB* (*cwlA* *cwlB* fragment B). These fragments were ligated together by overlapping PCR using primers AB-a and AB-d. These were integrated into pRN5101 to generate pRN5101 Δ AB. The corresponding deletion-insertion mutant, strain HD(Δ *cwlA* Δ *cwlB*), with a deletion from the start codon of *cwlA* to the end codon of *cwlB* and the insertion of the kanamycin resistance gene cassette at the same position (see Fig. S1 in the supplemental material), was selected and confirmed by PCR, and DNA sequencing was performed.

The 788-bp fragment upstream from the 11th codon of *cwlB* (*cwlB* fragment A) was amplified by PCR using *B. thuringiensis* HD73 genomic DNA as the template and CwIB-a and CwIB-b as primers. The primers CwIB-c and CwIB-d were used to amplify the 882-bp fragment downstream from the end codon of *cwlB* (*cwlB* fragment B). Two fragments were ligated by overlapping PCR using the primers CwIB-a and CwIB-d. The PCR products were digested with BamHI-SalI restriction enzymes and then ligated with the plasmid pRN5101, which was digested with the same restriction enzymes, to generate the recombinant plasmid pRN5101 Δ *cwlB*. This recombinant plasmid was transformed into the HD73 strain by electroporation. The *Erm*^r transformants were verified by PCR using the CwIB-a and CwIB-d primers and cultured at the nonpermissive temperature of 38°C. Colonies with no erythromycin resistance were selected, and one deletion mutant, strain HD(Δ *cwlB*) (see Fig. S1 in the supplemental material), was verified by PCR and DNA sequencing.

Construction of the *sigK* and *gerE* disruption mutants. The primer pairs SigK-a/SigK-b, SigK-c/SigK-d, and KKm-a/KKm-b were used to construct *sigK* deletion mutation cassettes, including the 606-bp fragment upstream from the start codon of *sigK* (*sigK* fragment A), the 1,473-bp *kan* gene, and the 551-bp fragment downstream from the end codon of *sigK* (*sigK* fragment B). These fragments were ligated together by overlapping PCR using primers SigK-a and SigK-d. The PCR products were digested with the BamHI and SalI restriction enzymes, and the digested fragments were purified and ligated with pRN5101 to generate pRN5101 Δ SigK. The corresponding strain HD(Δ *sigK*), with a deletion from the start codon to the end codon of *sigK* and the insertion of the kanamycin resistance gene cassette at the same position, was selected and confirmed by PCR and DNA sequencing (8) (see Fig. S1 in the supplemental material).

The primers GerE-a/GerE-b and GerE-c/GerE-d were used to construct *gerE* deletion mutation cassettes, including the 1,064-bp fragment upstream from the start codon of *gerE* (*gerE* fragment A) and the 998-bp fragment downstream from the end codon of *gerE* (*gerE* fragment B). Two fragments were ligated by overlapping PCR using primers GerE-a and GerE-d. The PCR products were digested with the BamHI and HindIII restriction enzymes. The digested fragments were purified and ligated with pRN5101 to generate pRN5101 Δ gerE. The corresponding HD(Δ *gerE*) deletion mutants (see Fig. S1 in the supplemental material) were selected and confirmed by PCR and DNA sequencing.

Genetic complementation of the *cwlB* deletion mutant. The oligonucleotide primers cwlBF/cwlBR (with a SalI restriction site) and P_{cwlA}F (with a HindIII restriction site)/P_{cwlA}R were used to amplify the *cwlB* gene and its own promoter, P_{cwlA}, respectively. The *cwlB* and P_{cwlA} fragments were ligated by overlapping PCR using primers P_{cwlA}F and cwlBR. The resulting fragment was digested with HindIII and SalI and then integrated into shuttle vector pHT315 (41) to generate pHTHFcwlB. The genetically complemented mutant strains HD(Δ *cwlB*::*cwlB*) and HD(Δ *cwlA* Δ *cwlB*::*cwlB*) were obtained by introducing pHTHFcwlB into HD(Δ *cwlB*) and HD(Δ *cwlA* Δ *cwlB*), respectively.

Quantification of Cry1Ac protein production. The *B. thuringiensis* HD73 wild-type strain and the HD(Δ *cwlB*) mutant were grown in SSM medium at 30°C at 220 rpm. After complete autolysis, 1 ml of each sample was centrifuged, and the cells were suspended in 500 μl of Na₂CO₃ (50 mM, pH 8.0). The bacterial cells were then ruptured with a Mini-Bead-Beater (Biospec Products, Inc., Bartlesville, OK, USA), and the supernatant was mixed with 3 $\times$ protein-reducing sample buffer (32) and boiled for 5 min for subsequent total-protein quantitation and SDS-PAGE. The total protein quantitation was performed using the Pierce 660-nm protein assay reagent (Thermo Scientific). The same amounts of total protein were taken for SDS-PAGE of the HD73 and HD(Δ *cwlB*) mutants. Cry1Ac protein production was quantified with Image Master 1D Elite software (Amersham Biosciences).

Microscopic observation and determination of sporulation efficiency. Cells were cultured in a conical flask containing 50 ml of SSM medium at 30°C. Samples of 1 ml were taken at T_0 , T_{16} , T_{20} , and T_{24} and centrifuged; the sediment was suspended with 100 μl deionized water. Amounts of 0.5 μl of the samples were placed on glass slides, and then cell morphology was observed by optical microscopy.

Sporulation efficiency was measured in SSM. The total amount of cells at T_1 was determined. The spores released were collected at T_{24} , and the cell suspension was heated to 65°C for 20 min to inactivate vegetative cells and then plated onto LB agar medium. The sporulation frequency was defined as the ratio of the number of colonies after heat treatment to the number of colonies at T_1 .

RESULTS

Sequence analysis and transcriptional units in the *bt2492*–*bt2495* locus. Two open reading frames, *bt2493* and *bt2494*, in the *B. thuringiensis* HD73 genome were analyzed (Fig. 1A). The *bt2494* protein is encoded by a 669-bp DNA fragment that was annotated as a putative cell wall autolysin. The protein's second-

FIG 1 Analysis of the *cwlAB* operon in *B. thuringiensis* HD73. (A) Map of the *bt2492*–*bt2495* locus in *B. thuringiensis* strain HD73. The positions of the fragments deleted from the chromosome to disrupt various genes are indicated. The bent arrow represents the promoter. Dashed lines with small black arrows annotated by letters correspond to RT-PCR amplicons (see lanes in panel C). Solid lines below ORFs indicate operons. (B) Domain organization of the *B. thuringiensis* HD73 CwlB and *B. subtilis* CwlB, CwlC, and CwlH cell wall hydrolases. Gray boxes represent cell wall binding domains. Black boxes define the MurNAc-LAA domains. (C) RT-PCR analysis of the *bt2492*–*bt2495* locus in *B. thuringiensis* strain HD73. Negative controls consisted of RT-PCR with total RNA and 16S rRNA primers. Positive controls consisted of PCR with genomic DNA. Letters refer to the positions of the RT-PCR products in the locus as depicted in panel A. RNA was extracted from cells harvested at T_{12} in SSM.

ary structure (<http://www.ncbi.nlm.nih.gov>) indicates the existence of three predicted domains, two Src homology 3 (SH3) domains and a MurNAc-LAA family domain (Fig. 1B). This domain organization is very similar to that of *Bacillus subtilis* CwlC and CwlH (Fig. 1B), which have an N-terminal MurNAc-LAA domain and a C-terminal substrate-binding domain. *bt2493*, encoded by a 351-bp DNA fragment upstream from *bt2494*, contains only one domain of the MurNAc-LAA family and was also annotated as a putative cell wall autolysin. Thus, *bt2493* and *bt2494* were designated CwlA and CwlB. Orthologs of the *cwlAB* locus were found in many *B. cereus* and *B. thuringiensis* strains. The domains of CwlA and CwlB from *B. thuringiensis* HD73 were conserved in the genome sequences of *B. cereus* ATCC 14579, *B. cereus* B4264, *B. cereus* G9842, *B. thuringiensis* BMB171, *B. thuringiensis* 407, *B. thuringiensis* CT-43, *B. thuringiensis* HD-789, *B. thuringiensis* HD-771 (43–49), and *Bacillus weihenstephanensis* KBAB4 (50), and their *cwlAB* locus sequence similarities were above 87%. However, there was no occurrence of the *cwlAB* operon in *Bacillus anthracis*, *Bacillus mycoides*, or *Bacillus pseudomycoides*.

There is no potential stem-loop present between *cwlA* and *cwlB*. To determine whether *cwlB* and *cwlA* with their flanking genes form one operon, seven pairs of primers were designed according to the *bt2492*–*bt2495* gene sequences, including RT2492-5/RT2492-3, RTcwlA-5/RTcwlA-3, RTcwlB-5/RTcwlB-3, RT2495-5/RT2495-3,

RTA92-5/RTA92-3, RTAB-5/RTAB-3, and RTB95-5/RTB95-3 (Fig. 1A; see also Table S1 in the supplemental material). RT-PCR was carried out with total RNA extracted at T_{12} from *B. thuringiensis* HD73 cultures grown in SSM (Fig. 1C). The results showed that mRNAs for all four genes were present (Fig. 1C). A mRNA overlapping the *cwlA* and *cwlB* genes was detected by RT-PCR (Fig. 1C). However, no mRNA overlapping *cwlA* and *bt2492*, as well as no mRNA overlapping *cwlB* and *bt2495*, was transcribed (Fig. 1C). This suggests that the *cwlB* and *cwlA* genes were transcribed together and form an operon, *cwlAB*.

Regulation of the *cwlAB* operon. The transcriptional start site was confirmed to be a single 5'-end nucleotide residue T located 25 nucleotides (bp) upstream from the *cwlA* translational start codon according to the sequences of 12 random clones obtained by 5'-RACE-PCR (Fig. 2A).

To study the transcription and regulation of the promoter P_{cwlA} in the *cwlAB* operon, a P_{cwlA} -*lacZ* fusion was constructed and transformed into *B. thuringiensis* HD73, HD($\Delta sigK$), and HD($\Delta gerE$). A β -galactosidase assay indicated that P_{cwlA} promoter activity in the HD73 strain increased sharply after T_{10} , which suggested that it was expressed in the late stage of sporulation. The transcriptional activity of P_{cwlA} was demonstrated to be abolished in the *sigK* mutant strain HD($\Delta sigK$) (Fig. 2B). Typical σ^K -dependent sequences (HDCA and CATANNDD) (51) were also found

to exist in the -35 and -10 regions upstream from the transcriptional start site of the *cwlA* operon (Fig. 2A). All these results indicate that the promoter P_{cwlA} is controlled by the σ^K factor. Some σ^K -dependent genes were negatively or positively regulated by the GerE regulator in the late stage of sporulation. The activity of the P_{cwlA} -*lacZ* fusion in the *gerE* mutant HD($\Delta gerE$) was tested. The results showed that β -galactosidase activity in HD($\Delta gerE$) was significantly decreased compared to that in HD73 (Fig. 2B). An electrophoresis mobility shift assay (EMSA) to detect GerE protein and P_{cwlA} promoter complexes was performed; the results showed that no binding band was found on the gel. All these results reveal that the promoter P_{cwlA} is indirectly regulated by the GerE protein.

	T ₀	T ₁₆	T ₂₀	T ₂₄
HD73				
$\Delta cwIA$				
$\Delta cwIB$				
$\Delta cwIA$ $\Delta cwIB$				

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FIG 4 Optical micrographs of the genetically complemented strains HD($\Delta cwlB::cwlB$) and HD($\Delta cwlA\Delta cwlB::cwlB$) at T_0 , T_{16} , and T_{20} of incubation in SSM medium at 30°C. Bar, 10 μ m.

HD($\Delta cwlB$) and HD($\Delta cwlA\Delta cwlB$) completely released their spores at T_{24} , compared with T_{20} for HD73 and HD($\Delta cwlA$) (Fig. 3), which means that the mutation of *cwlB* delays spore release and *cwlB* plays a role in mother cell lysis.

In order to verify that the morphology resulted from *cwlB* gene disruption, genetically complemented strains of the *cwlB* deletion mutant were constructed. The morphologies of vegetative cells of the HD($\Delta cwlB::cwlB$) and HD($\Delta cwlA\Delta cwlB::cwlB$) strains were not significantly different from that of HD73 at T_0 (Fig. 4). However, although a few spores of the complemented strains were released at T_{16} , no spores of the wild-type strain HD73 were released at that time, and almost all of the spores of the complemented strains were released at T_{20} , the same as for HD73. The results confirmed that the delay of spore release resulted from the disruption of the *cwlB* gene.

The effects of *cwlB* mutation on sporulation and Cry protein production at T_{24} were analyzed in *B. thuringiensis*. The results showed that the sporulation rate of HD($\Delta cwlB$) was slightly decreased compared to that of HD73 (Fig. 5A). Cry protein production was determined in various strains by SDS-PAGE after crystal and spore release. The Cry protein production in HD($\Delta cwlB$) was similar to that in HD73 (Fig. 5B).

Characterization of the CwlB protein. CwlB contains a MurNAc-LAA family domain that acts on the amide bond between

MurNAc and the N-terminal L-alanine residue of the stem peptide (Fig. 6A), and it is consequently annotated as an N-acetylmuramoyl-L-alanine amidase (MurNAc-LAA). It was shown to play a role in mother cell lysis in *B. thuringiensis*, as described above. To confirm its cell wall binding ability and amidase activity *in vitro*, a CwlB-His tag fusion protein, which consisted of 233 amino acid residues, was expressed and purified. SDS-PAGE analysis showed that a single protein band with a molecular mass of 24.5 kDa, purified by nickel affinity chromatography, was found on the gel (Fig. 6B).

To examine the cell wall binding ability of CwlB, the purified CwlB-His protein and purified *B. thuringiensis* Cry1Ie-His protein (49, 50), which is an endotoxin with no ability to bind with cell walls and was used as the negative control, were added into *B. thuringiensis* cell wall buffer with a final absorbance of 0.3 at 540 nm. CwlB was also used in the absence of cell walls as another control to test whether CwlB can sediment. The reaction mixture was centrifuged at 0°C as described in Materials and Methods. Western blot analysis showed that a single protein band for CwlB was found in sediment using a His antibody, but no band was found for Cry1Ie-His or CwlB-His in sediment without cell walls (Fig. 6C). This means that the CwlB protein was bound to the *B. thuringiensis* cell walls.

As described above, the mixture containing CwlB protein and cell walls was incubated at 37°C, and the OD₅₄₀ values were calculated. The results demonstrated that cell wall density decreased after CwlB-His was added (Fig. 6D). This suggested that CwlB-His digested the *B. thuringiensis* cell walls. To determine the peptidoglycan cleavage site, the free amino groups of the digested sample were investigated by labeling free amino groups with 1-fluoro-2,4-dinitrobenzene, followed by hydrolysis of glycoside and amido linkages of the cell walls with HCl. Finally, the dinitrophenyl (DNP)-amino acids were separated by reverse-phase high-performance liquid chromatography. After digesting for 60 min, only the amount of DNP-L-alanine increased during the enzyme reaction (Fig. 6D). All of these results indicate that CwlB is an N-acetylmuramoyl-L-alanine amidase.

FIG 5 Comparisons of sporulation frequency and the production of crystal proteins between the HD($\Delta cwlB$) mutant and wild-type strain HD73. (A) Graph depicting sporulation frequency. Error bars show standard deviations. (B) Analysis of Cry1Ac crystal production in the wild-type strain HD73 and the mutant HD($\Delta cwlB$), as described in Materials and Methods.

FIG 6 Characteristics of the CwlB protein. (A) Schematic diagram of the amide bonds cleaved by amidase. (B) SDS-PAGE analysis of CwlB protein (6 mg) expressed in *E. coli* (pETCwlB) and purified using nickel affinity column chromatography. M, protein marker. (C) Western blot analysis of cell wall binding. H, His tag. (D) Digestion of *B. thuringiensis* cell walls by purified CwlB protein. *B. thuringiensis* cell walls (2.5 mg) and purified CwlB (6 mg) were mixed in 5 ml TK buffer (pH 7.0) and then incubated at 37°C. Aliquots (500 μ l of each) were removed at various intervals to determine turbidity at 540 nm ($\blacktriangle$); the cell walls without protein were used for the negative control ($\blacklozenge$); DNP-L-alanine ($\bullet$) and DNP-D-alanine ($\blacksquare$) were separately measured in reaction mixture with the cell walls and CwlB.

Localization of the CwlB protein. The CwlB protein, which is composed of two C-terminal SH3 domains, was shown to act on the cell wall peptidoglycan of *B. thuringiensis*. It was necessary to determine the localization of the CwlB protein in *B. thuringiensis* cells to elucidate the role of the CwlB protein in mother cell lysis. A fusion of the GFP gene with the *cwlB* gene at the 5' terminus, GFP-*cwlB*, was constructed in the vector pHT315 to obtain pHT-*gfp-cwlB* and then transformed into *B. thuringiensis* HD73 (Fig. 7A). The resulting strain, HD(pHT-*gfp-cwlB*), was cultured in SSM at 30°C and 220 rpm with appropriate antibiotics. The vital cell membrane of *B. thuringiensis* was stained with FM4-64. Cells were observed via laser confocal microscopy. A red fluorescent signal indicated the membrane of a bacterial cell, and a green fluorescent signal indicated the location of the Gfp-CwlB fusion protein.

At stage T_9 , bipyramidal crystals and spores were observed but no green fluorescent signal was detected in *B. thuringiensis* cells. At stage T_{12} , the cell walls were still intact and weak green fluorescent signals were observed in most of the mother cells of HD(pHT-*gfp-cwlB*). At stage T_{15} , some cells had undergone autolysis and the green fluorescent signal intensities had increased significantly and were spread over the cell envelopes of nonautolyzed cells only (Fig. 7B, white arrows). Moreover, no green fluorescence was detected in lysed cells (Fig. 7B, yellow arrows). However, at stage T_{18} , many crystals and spores were released and, although green fluorescence

intensity was not diminished, no green fluorescence was detected in the crystals and spores (Fig. 7B, blue arrows). Additionally, we obtained the cell walls of *B. thuringiensis* HD73(pHT-*gfp-cwlB*) in SSM at T_9 , T_{12} , T_{15} , and T_{18} and calculated the relative fluorescence units (Fig. 7C). Fluorescence intensity was not evaluated at T_9 , but it was tremendously increased at T_{15} compared with that in the HD73 strain. These results suggest that the CwlB protein is localized on the cell envelope.

DISCUSSION

We identified a novel *N*-acetylmuramoyl-L-alanine amidase gene involved in *B. thuringiensis* mother cell lysis, which was named *cwlB* in this study. In *B. subtilis*, the results of prior studies of the involvement of hydrolase genes in mother cell lysis have been clear and definite. Three autolysins, CwlB, CwlC, and CwlH, were present at the time of mother cell lysis (16–19). We tried to search for the orthologs of CwlB, CwlC, and CwlH in *B. thuringiensis*. No amidase was found to share more than 41.7% sequence similarity with them or have the same domain organization as CwlB, CwlC and CwlH in *B. thuringiensis*. Sequence analysis showed that CwlB of *B. thuringiensis* in this study shares low sequence similarity (below 10.0%) with CwlB, CwlC, and CwlH of *B. subtilis*. However, they all consist of a MurNAc-LAA family domain and peptidoglycan binding domains which are different from each other (Fig. 1B). This suggests that CwlB of *B. thuringiensis* is a novel

FIG 7 Localization of the CwlB protein. (A) Construction strategy for the GFP gene fusion directed by the CwlA promoter. (B) Images of HD(pHT-*gfp-cwlB*) strain cells in SSM at T₉, T₁₂, T₁₅, and T₁₈. The white arrows, yellow arrows, and blue arrows indicate nonautolyzed cells, lysed cells, and crystal proteins, respectively. GFP, green fluorescent protein signal in the bacterial cells; FM4-64, red fluorescent signal of FM4-64 stain; overlay, green and red fluorescent signals; PC, phase-contrast microscopy. Bar, 7.5 μ m. (C) GFP quantification analysis of cell walls from the HD(pHT-*gfp-cwlB*) strain and the wild-type HD73 strain. Error bars show standard deviations.

hydrolase involved in mother cell lysis. The *cwlC* and *cwlH* genes were σ^K dependent and expressed only in the late stage of sporulation in *B. subtilis*. The *cwlB* from *B. thuringiensis* showed a similar transcription pattern. Although single inactivation of *cwlB*, *cwlC*, and *cwlH* in *B. subtilis* has no effect on mother cell lysis (52), CwlB in *B. thuringiensis* was shown to play a role in mother cell lysis and delay spore release, even though CwlB shows low sequence similarity to the three autolysins in *B. subtilis*. This is the first report in which an autolysin gene in the *B. cereus* group is shown to be involved in mother cell lysis.

Although the disruption of *cwlB* was demonstrated to delay spore and crystal release in *B. thuringiensis*, mother cells still lysed after T₂₄. This suggests that other hydrolases exist and participate in this cellular process. The combined effects of hydrolases have been shown to be required for many cellular functions. In *E. coli*, the endopeptidases penicillin-binding protein 4 (PBP 4), PBP 7, and MepA contribute to cleavage of the septum. However, when deletions in amidases are combined with deletions in lytic transglycosylases or endopeptidases, bacterial chains become very long and may contain up to 100 cells during cell division (53, 54). In *B. subtilis*, cell separation is greatly affected by a combination of two

endopeptidases (38, 55, 56). Vegetative cell lysis is affected by the major autolysin, CwlB (57, 58), and greatly by four autolysins, including minor ones (CwlF [LytE] and CwlE [LytF]), in *B. subtilis* (38). Motility and cell wall turnover were also found to be affected by combinations of cell wall hydrolases in *B. subtilis* (58–60), and germination was found to be completely blocked in the absence of two deduced class II amidases (61). The combined effect of hydrolase deletion on mother cell lysis has been observed, and lysis was blocked in a mutant with the *cwlB*, *cwlC*, and *cwlH* genes inactivated (52). Therefore, to elucidate the mechanism of mother cell lysis in *B. thuringiensis*, we need to search for more cell wall hydrolases that combine with CwlB to act at the late stage of sporulation.

In this study, we reported that a novel cell wall hydrolase delays spore release and has no significant effect on sporulation and Cry protein production. It is a good candidate gene for disruption to develop a novel *B. thuringiensis* agent with greater persistence. This finding helps us to better understand the mechanism of mother cell lysis in *B. thuringiensis* and provides a foundation for developing a novel *B. thuringiensis* agent with protection of the insecticidal crystal proteins from UV degradation to increase their persistence.

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