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Deletion of *cwlC* delayed *Bti* mother cell lysis

Characterization of CwlC, an autolysin, and its role in mother cell lysis of *Bacillus thuringiensis subsp. israelensis*

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Significance and Impact

The deletion of the cell wall hydrolase gene cwlC delayed, but didn’t prevent, mother cell lysis of *Bacillus thuringiensis* subsp. *israelensis* (*Bti*), while both cwlB and cwlE were expressed at high levels in a cwlC mutant strain. Cell lysis was also delayed in a sigK knockout strain, a strain in which expression of cwlB and cwlC were reduced. Our findings indicate that cell lysis in Bti requires both the SigK-controlled CwlB and CwlC hydrolases as well as the separately controlled, and plasmid encoded, CwlE enzyme. This understanding of the complex process will be of great importance both for the study of bacterial autolysis and for the construction of engineered *Bt* products with increased persistence.

Abstract

*Bacillus thuringiensis* subsp. *israelensis* (*Bti*) has been proven to efficiently control mosquitoes, of which many species are important vectors of human disease. The larvicidal action is attributed to the parasporal crystals formed in the sporulating cells, and released upon cell autolysis. In this study, a sporulation-specific cwlC gene that encodes an N-acetylmuramoyl-l-alanine amidase was characterized in *Bti* strain Bt-59. CwlC was the only cell wall hydrolase in *Bti* found to contain both MurNAc-LAA and Amidase02_C domains. A recombinant CwlC-His protein was able to digest the *Bacillus* cell wall. Deletion of the cwlC gene delayed *Bti* mother cell lysis without impacting vegetative growth or insecticidal efficacy. Transcriptional analyses indicated that cwlC was expressed at the late sporulation stage and was controlled by SigK. Two other cell wall hydrolase genes, cwlB and cwlE, with high expression levels at $T_{14}$ in Bt-59, were also identified. Like cwlC, cwlB expression was controlled by SigK, in contrast cwlE was found not to be under the control of this sigma factor and unlike the other two, its gene was found to be plasmid encoded.

Key words
\textit{Bacillus thuringiensis} subsp. \textit{israelensis}, CwIC, cell wall hydrolase, mother cell lysis, transcriptional regulation

\textbf{Introduction}

\textit{Bacillus thuringiensis} (\textit{Bt}) is a ubiquitous spore-forming, Gram-positive bacterium that forms parasporal crystals during the stationary phase of its growth cycle (Schnepf et al. 1998). Due to its insecticidal activity against a variety of insect larvae, it is considered an insect pathogen. The insecticidal activity is attributed largely to protein toxins found within this parasporal crystal (Gill et al. 1992; Hofmann et al. 1988; Sanahuja et al. 2011). \textit{B. thuringiensis} subsp. \textit{israelensis} (\textit{Bti}) was the first subspecies of \textit{Bt} found to be toxic to dipteran larvae (Ben-Dov 2014). Due to its remarkable killing effect and harmlessness to humans and animals, \textit{Bti} has been successfully used worldwide as a microbial agent for the control of mosquitoes, including vectors of diseases such as malaria, filariasis, dengue and yellow fever (Gyapong and Twum-Danso 2006; Harrington et al. 2005; Reisen 2010; Sinka et al. 2012).

The toxicity \textit{Bti} is due to the expression of five different plasmid-encoded toxins (Berry et al. 2002; Monnerat et al. 2014; Zhang et al. 2017). At the late stage of sporulation, the mother cell is lysed and the spore and parasporal crystal are released (Ben-Dov 2014). The released crystal proteins are challenged by many environmental factors (e.g. solar irradiation and temperature), which can result in a short residual action and stability under field conditions (Becker et al. 1992; Patel et al. 1996; Zogo et al. 2019). Various measures have been taken to achieve protection from solar inactivation including encapsulation (Dunkel and Shasha 1989; Ramirez-Lepe et al. 2003), sustained-release formulation (Zhang et al. 2016), and addition of a variety of UV screens (Jalali et al. 2020; Jallouli et al. 2014; Maghsoudi and Jalali 2017). Using molecular techniques to block cell lysis, studies have shown that crystal proteins encased in the mother cell can effectively reduce their sensitivity to UV light (Chen et al. 2018; Sanchis et al. 1999).
Mother cell lysis is the final morphological process that occurs during sporulation in spore-forming Gram-positive bacteria to release the mature endospore. This process is performed by a group of cell wall hydrolases, which are also known as autolysins. *Bacillus subtilis* is a well-established model for mother cell lysis with CwIB, CwIC and CwIH being the main autolysins (Foster 1992; Nugroho et al. 1999). These hydrolases play important roles in mother cell lysis during the sporulation phase. Based on studies with *Bt* subsp. *kurstaki* (*Btk*) strain HD73, the mutation of cwlC in HD73 completely blocked mother cell lysis, leading to crystal proteins encapsulated in the mother cell, and increased the insecticidal persistence of HD73 (Chen et al. 2018; Lv et al. 2019). SigK is the primary mother cell-specific sigma factor and plays an important role in the late stage of sporulation. The transcription of cwlC in HD73 was found to depend on SigK. Deletion of sigK of HD73 also lead to completely blocked mother cell lysis during sporulation (Chen et al. 2018). In a previous study, a sigK mutation strain of *Bti* was constructed from *Bti* strain Bt-59. However, the deletion of sigK just delayed and not completely blocked the lysis of mother cells (Xu et al. 2020). We have though observed that a sigK knockout of a variant of Bti lacking the toxin-encoding plasmid is protected from cell lysis (unpublished data). In the present study, cwlC, a cell wall hydrolase gene was identified from the Bt-59 genome. The characterization of this enzyme and its role in cell lysis will help inform the design of Bti strains with improved persistence.

Results and Discussion

Bioinformatic analysis of CwlC and other putative cell wall hydrolases from *Bti*

In our previous study, the complete genome of Bt-59 was sequenced and was found to consist of one chromosome (CP039721) and five plasmids (CP039722–CP039726) (Xu et al. 2020). A total of 12 putative cell wall hydrolases were found according to the gene annotation information (Table S1). The cwlE gene was located on plasmid CP039724, while all the other cell wall hydrolase genes were on the chromosome. Conservative domain analysis indicated that a hydrolase named as CwID contained a spore_cwlD domain (TIGR02883), five putative cell wall
hydrolases contained the Amidase_2 domain (pfam01510) and five contained the MurNac-LAA domain (cd02696). One hydrolase named as CwlC contained both MurNac-LAA and Amidase02_C domains (pfam12123) (Fig. S1a). All of these hydrolases are N-acetylmuramoyl-L-alanine amidases, which hydrolyze the amide bond between N-acetylmuramoyl and L-amino acids in cell wall glycopeptides (Shida et al. 2001).

CwlC is an essential cell wall hydrolase present in *Bacillus* bacteria (Chen et al. 2018). CwlC from Bt-59 and HD73 show 97% amino acid sequence identity and include the N-terminal MurNac-LAA domain and the C-terminal amidase02_C domain (Fig. S1b). Two critical catalytic residues (E24 and E140) are also conserved. Mutations in either of the two glutamate residues caused the loss of CwlC catalytic activity in HD73 (Chen et al. 2018). The bioinformatic analysis of critical catalytic sites and highly similar conserved domains suggested that CwlC of Bt-59 could have similar function to that of its HD73 homologue.

**Deletion of the cwlC gene delayed mother cell lysis**

To determine the role of CwlC in mother cell lysis of *Bti*, a *cwlC* deletion in Bt-59 (*ΔcwlC*), was constructed and verified (Fig. S2 and S3a). The deletion of *cwlC* did not affect the vegetative growth of Bt-59 cells (Fig. S3b). When the morphology of Bt-59 and Bt-59 (*ΔcwlC*) grown in SSM medium to different growth phases were examined by optical microscopy (Fig. 1), the results showed that Bt-59 and Bt-59 (*ΔcwlC*) had similar cell morphology at *T*14. After another eight hours of culture, most of Bt-59 cells had lyses, while only a very small number of Bt-59 (*ΔcwlC*) cells had. At *T*25, Bt-59 cells were completely lysed, whereas some cells of Bt-59 (*ΔcwlC*) remained unlysed. However even Bt-59 (*ΔcwlC*) cells had completely autolyzed at *T*32. When the *ΔcwlC* knockout strain was transformed with a plasmid expressing CwlC (pHTHF*cwlC*) complementation was observed and the cells were almost completely autolyzed at *T*25, similar to the non-mutated parent.

While mutation of *cwlC* could completely block HD73 mother cell lysis, a *cwlH-cwlC-cwlB* triple deletion mutant
was required to significantly affect mother cell lysis in *B. subtilis*. This indicated that the mechanism underlying mother cell lysis differed between *Bt* and *B. subtilis* (Chen et al. 2018; Nugroho et al. 1999). Although CwlC from *Bt*-59 showed the same critical catalytic residues and conserved domains to that from HD73, the deletion of *cwlC* just delayed rather than blocked lysis in *Bt*-59 suggesting that as in *B. subtilis* multiple enzymes might be required for lysis in *Bti*.

![Observation of mother cell lysis by optical microscopy](image)

**Fig. 1 Observation of mother cell lysis by optical microscopy.** Mother cell lysis of *Bt*-59, *Bt*-59 (*ΔcwlC*) and *Bt*-59 (*HFcwlC*) was observed by optical microscopy at the indicated time points. Scale bars represent 10 μm.

**Hydrolytic activity of CwlC against the bacterial cell wall**

Recombinant CwlC-His proteins with a molecular weight of approximately 28 kDa were created from both *Bt*-59 and HD73 and purified by nickel column affinity chromatography. For the protein from *Bt*-59 two protein bands with a molecular weight around 28 kDa were observed by SDS-PAGE, and both were identified as recombinant CwlC protein by mass spectrometry (Fig. 2a). To determine the hydrolytic activity of the CwlC enzymes on cell walls from *Bt*-59 and HD73, the recombinant protein was mixed with cell wall preparations and incubated at 37°C for 60 minutes. Both CwlC hydrolases were found to be active against both cell walls (Fig. 2b).
To further characterize the CwlC enzyme from Bt-59, plasmids containing the Bt-59 *cwlC* gene under the control of its promoter from either Bt59 (P_BcwlC and HFcwlC) or HD73 (P_HcwlC) were introduced into HD73 (ΔcwlC). After culturing in SSM medium, cells containing any of the recombinant plasmids showed complete lysis at T24, whereas the HD73 (ΔcwlC) cells remained unlysed. This indicated that CwlC of Bt-59 could hydrolyze the HD73 cell wall. These results indicated that there were no significant differences between the activity of the two CwlC enzymes and therefore that there may be other factors causing the phenotypic differences between Bt-59 (ΔcwlC) and HD73 (ΔcwlC).

![Sequence Coverage: 79%](image.png)

**Fig. 2** Expression of CwlC and its hydrolytic activity of cell wall. (a) SDS-PAGE analysis and mass spectrometry identification of recombinant Bt-59 CwlC protein purified by nickel column affinity chromatography.
Peptides obtained by mass spectrometry are marked in red. M is protein molecular size marker. (b) Digestion of Bt-59 and HD73 cell wall by purified CwlC proteins from Bt-59 (squares) and HD73 (triangles). Cell wall incubated without addition of CwlC proteins was used as a negative control (circles). Each value represents the mean of at least three independent replicates. Error bars show standard deviations. Statistically significant difference analysis was performed by ordinary one-way ANOVA (P<0.05).

(c) Mother cell lysis of HD73 (ΔcwlC), HD73 (HF-cwlC), HD73 (P_{BcwlC}), and HD73 (P_{HcwlC}) was observed by optical microscopy at T_{24}. Scale bars, 10 μm.

**Transcriptional regulation of the cwlC gene**

Compared to the cwlC gene of HD73, a 20-bp fragment and an 80-bp fragment were missing in the promoter region of the Bt-59 cwlC gene according to the sequence alignment (Fig. 3a). Although there were a few other base differences in the remaining sequences, both contained the SigK -35 and -10 recognition regions, located upstream of the cwlC transcription start site (G) (Fig. 3a).

The expression of cwlC was analyzed in Bt-59 and Bt-59 (ΔsigK) at T_0, T_8 and T_{14}. The expression of cwlC in Bt-59 increased significantly over time, but there was no significant difference in the expression of cwlC in Bt-59 (ΔsigK) (Fig. 3b). In a sigK-restored strain, the expression pattern of cwlC was similar to that in Bt-59 (Fig. 3b).

This result indicated that the lack of sigK inhibited the expression of cwlC in Bt-59.

To further determine whether the expression of cwlC is regulated by SigK, four strains; Bt-59 (P_{BcwlC}-lacZ), Bt-59 (ΔsigK) (P_{BcwlC}-lacZ), Bt-59 (P_{HcwlC}-lacZ), and Bt-59 (ΔsigK) (P_{HcwlC}-lacZ) were created containing plasmids in which LacZ was placed under the control the cwlC promoter from either Bt-59 or HD73. β-Galactosidase activity assays showed that both promoters were active in Bt-59, but not in the sigK mutant strain (Fig. 3c). This result supported the idea that expression of Bt-59 cwlC was controlled by SigK. In order to determine whether
there was a difference in transcription levels between Bt-59 and HD73 the expression of the two LacZ constructs in the two strains was compared. β-Galactosidase activity assays showed that the expression of two reporter vectors started at $T_9$, reached a maximum at $T_{15}$, and then decreased in the four strains (Fig. 3d). The promoter used made no difference to the level of expression in a given strain, however there was significantly more expression in HD73 than in Bt-59 (Fig. 3d).

As a typical sigma factor, SigK plays an important role in the late stage of sporulation in bacteria (Piggot and Hilbert 2004). The main hydrolase genes associated with mother cell lysis, $cwlC$ and $cwlH$, are all regulated by SigK in *B. subtilis* (Kuroda et al. 1993; Nugroho et al. 1999). Previous studies have shown that *sigK* deletion mutants of Bt 407 and HD73 blocked mother cell lysis and produced crystals encapsulated within the mother cells (Du et al. 2012; Sanchis et al. 1999). Our results confirm that although $cwlC$ from *Bti* and *Btk* are both regulated by SigK, the deletion of *sigK* to inhibit the expression of $cwlC$ could not completely block mother cell lysis in *Bti* (Xu et al. 2020).
Fig. 3 Analysis of promoter sequence and transcriptional regulation of cwlC gene. (a) Comparison of nucleotide sequences of promoter regions from Bt-59 cwlC and HD73 cwlC. Different nucleotide bases between the two sequences are highlighted with gray shading. The predicted transcription start site G (+1) and the putative -35 and -10 motifs are marked with red arrow and red boxes, respectively. The translation start codon of cwlC is underlined. (b) Expression levels of cwlC gene in Bt-59, Bt-59 (ΔsigK), and Bt-59 (HFsigK) at T0, T8, and T14. Error bars represent the standard error of the mean. Statistically significant difference analysis was performed by ordinary one-way ANOVA (P<0.05). (c) Assays of β-galactosidase activity were performed to compare the activities of Bt-59 and HD73 cwlC promoters in Bt-59 and Bt-59 (ΔsigK). Tn, n hours after T0 (the end of the exponential growth phase). Each value represents the mean of at least three independent replicates. Error bars
show standard deviations. (d) Assays of β-galactosidase activity were performed to compare the activities of Bt-59 and HD73 cwlc promoters in Bt-59 and HD73. T

n hours after T

0. Each value represents the mean of at least three independent replicates. Error bars show standard deviations.

Expression levels of putative cell wall hydrolase genes

According to our bioinformatic analysis there are 12 putative cell wall hydrolases in Bt-59. Since the absence of CwlC only delayed the lysis of Bt-59 mother cells it is likely that other cell wall hydrolases are involved. qPCR analysis revealed that the three hydrolase genes, cwlB, cwlC, and cwlE, were highly expressed in Bt-59 at T

14 with relative gene expression values of more than 100 (Fig. 4) and so were selected for further study. We found that deletion of cwlC did not significantly inhibit the expression of either cwlB or cwlE (Fig. 4). When the sigK knockout strain was used expression of cwlB and cwlC was inhibited but not that of cwlE (Fig. 4).

CwlB and CwlE might therefore be the autolytic enzymes performing cell wall lysis in the cwlC mutant strain of Bt-59. Bt-59 CwlB shows 98% sequence identity and 67% sequence coverage with HD73 CwlB. In HD73, mutation of cwlB only delayed mother cell lysis during sporulation according to a previous study (Yang et al. 2013). The cwlE encoded protein contains the MurNAc-LAA domain yet only three amino acid sequences with more than 60% sequence identity with Bt-59 CwlE were found by NCBI Blast. Furthermore, there is no hydrolase gene similar to cwlE in HD73. Since cell lysis in Bt-59 is not completely blocked in the sigK knockout strain, and since cwlE is the only hydrolase gene with high expression in this strain it seemed reasonable to hypothesize that CwlE is a new and critical autolysin leading to mother cells lysis in Bti.
Fig. 4 Expression level of hydrolase genes in *B. thuringiensis* subsp. *israelensis* strains. Expression levels of *cwlB*, *cwlC*, and *cwlE* were determined in Bt-59, Bt-59 (Δ*cwlC*), and Bt-59 (Δ*sigK*) at *T₀* and *T₁₄*. Expression levels of *cwlD*, and *hydrolases1-8* were determined in Bt-59 at *T₀* and *T₁₄*. Three technical replicates and three biological replicates were performed. Error bars represent one standard error of the mean.

Insecticidal activity against *C. pipiens*

After 43 hours of culture in the fermentation medium, Bt-59 mother cells had completely lysed, but a small number of Bt-59 (Δ*cwlC*) mother cells had not lysed (Fig. S4). The LC₅₀ values of Bt-59 and Bt-59 (Δ*cwlC*) against *C. pipiens* were 0.144 µL/L and 0.173 µL/L, respectively (Table 1). The hypothesis of equality and parallelism were
both valid (P>0.05), indicating that the two toxicity regression curves were parallel and equal. Furthermore, the LCR value was 1.197 and the 95% confidence interval of LCR was 0.988-1.450 (Table 1). The value 1 is between the 95% confidence interval of LCR, indicating that there is no significant difference between the two LC50 values (P<0.05). Therefore, although deletion of cwlC delayed mother cells lysis of Bt-59, it did not affect its toxicity to C. pipiens.

Table 1 Insecticidal activities of B. thuringiensis subsp. israelensis strains against C. pipiens.

<table>
<thead>
<tr>
<th>Strain</th>
<th>LC50 (95% CI)</th>
<th>Slope ± SE</th>
<th>χ² (df)</th>
<th>LCR50 (95% CI)</th>
<th>Hypothesis of equality</th>
<th>Hypothesis of parallelism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bt-59</td>
<td>0.144 (0.123- 2.539±0.270</td>
<td>1.693 (4)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.165</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bt-59 (ΔcwlC)</td>
<td>0.173 (0.142- 2.821±0.281</td>
<td>4.209 (4)</td>
<td>1.197</td>
<td>(0.988- 3.63, 0.163</td>
<td>0.52, 0.469</td>
<td>1.450</td>
</tr>
<tr>
<td></td>
<td>0.206</td>
<td></td>
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</table>

In conclusion, the differentiation process of sporulation involves a number of events of cell wall rearrangement, in which peptidoglycan degradative enzymes play a key role (Waldemar et al. 2010). These cell wall lytic enzymes appear to be functionally redundant because none of the single autolysin gene deletions affected mother cell lysis in B. subtilis (Nugroho et al. 1999; Smith and Foster 1995). An engineered Bt HD73 with a cwlC deletion, which allowed parasporal crystal encapsulation in the mother cell, illustrated the potential to perform more effective biological control (Chen et al. 2018). Though CwlC proved to be essential and sufficient for Btk mother cell lysis, mutation of cwlC did not block Bti mother cell lysis completely. As with B. subtilis it appears that multiple hydrolases are required for cell lysis in Bti, with one of these (CwlE) being plasmid-borne and not under the control of the sporulation sigma factor SigK. It remains to be seen whether this reflects an evolutionary adaptation.
of Bt linking cell lysis with crystal production - as the crystal proteins are plasmid-encoded. A more thorough understanding of Bti mother cell lysis will be of great importance, both for providing a new perspective for the study of bacterial autolysis but also for the construction of engineered bacteria whose parasporal crystal is still encapsulated in the mother cell at the end of sporulation to increase the insecticidal persistence of Bti.

**Materials and Methods**

**Bacterial strains, plasmids, and culture conditions**

The strains and plasmids used in this study are shown in Table S2. Molecular cloning and protein expression were performed with Escherichia coli strains DH5α and BL21 (DE3), respectively. E. coli strain SCS110 was used to generate unmethylated plasmid DNA for transformation of Bt cells (Wang et al. 2006). Bt-59 (CGMCC strain number 16821) and HD73 (BGSC strain number BGSC 4D4) were used to clone the target gene and monitor promoter activity. E. coli strains were cultured at 37°C in Luria-Bertani (LB) broth medium (1% tryptone, 0.5% yeast extract, and 0.5%NaCl) or LB agar plates (LB broth medium, 1.5% agar). Bt strains were cultured at 30°C in Schaeffer’s sporulation Medium (SSM) (8 g of nutrient broth, 0.12 g of MgSO$_4$, 1 g of KCl, 0.5 mM NaOH per liter of broth) or LB agar plates (Schaeffer et al. 1965). The antibiotic concentrations used for bacterial selection were 100 μg/mL ampicillin (Amp) for E. coli strains and 25 μg/mL erythromycin (Ery) for Bt strains (Zhou et al. 2014).

**DNA manipulation**

PCR was performed with PrimeSTAR HS DNA polymerase (TaKaRa, Beijing, China). The amplified fragments and plasmid DNA were purified with the TIANgel Midi Purification Kit (TIANGEN, Beijing, China) and TIANprep Mini Plasmid Kit (TIANGEN, Beijing, China), respectively. Restriction enzymes (TaKaRa, Beijing, China) and ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China) were used according to the
manufacturer’s instructions. Oligonucleotide primers (Table S3 and S2) were synthesized and all constructs were confirmed by DNA sequencing in GENERAL BIOL (Hefei, China). Heat shock was used for *E. coli* transformation, and *Bt* cells were transformed by electroporation as previously described (Lereclus et al. 1989).

**Strain construction**

All primers for constructing recombinant plasmids were designed according to the *Bt*-59 and HD73 genome sequence (Table S3). The *cwlC* deletion mutant *Bt*-59 (Δ*cwlC*) was constructed as shown in the schematic diagram (Fig. S2). The fragment containing both the *cwlC* promoter and ORF was cloned into the pHT315 shuttle vector to generate pHTHFcwlC. After sequencing and demethylation, the recombinant plasmid pHTHFcwlC was introduced into *Bt*-59 (Δ*cwlC*) to generate strain Bt-59 (HFcwlC).

To further verify the hydrolytic activity of CwlC, a 1423-bp fragment containing the *cwlC* promoter region of HD73 (P_H) and the *cwlC* gene of *Bt*-59 (B_cwlC) was obtained and cloned into pHT315 (pHTP_B-B_cwlC). Then, the recombinant plasmids pHTHFcwlC and pHTP_B-B_cwlC were introduced into HD73 (Δ*cwlC*) to generate strains HD73 (P_B-B_cwlC) and HD73 (P_H-B_cwlC).

In order to construct a *cwlC* reporter vector, a 617-bp promoter region located upstream of the *cwlC* gene (P_BcwlC) was cloned into the linearized vector pHT304-18Z. Then, the recombinant plasmids pHTP_BcwlC and pHTP_HcwlC (designed in previous experiments and also called pHTP_cwlC) (Chen et al. 2018) were introduced into cells of *Bt*-59, *Bt*-59 (ΔsigK) and HD73, generating *Bt*-59 (P_BcwlC-lacZ), *Bt*-59 (ΔsigK) (P_BcwlC-lacZ), *Bt*-59 (ΔcwlC-lacZ), *Bt*-59 (ΔsigK) (P_BcwlC-lacZ), HD73 (P_BcwlC-lacZ), and HD73 (P_HcwlC-lacZ), respectively.

**Cell wall hydrolysis by recombinant CwlC proteins**

To analyze the activity of CwlC, a 732-bp *cwlC* gene without the stop codon TAA was cloned into pET21b for protein expression. Then, the recombinant CwlC proteins with a His tag were purified from *E. coli* strain BL21.
(pETcwLC). Bt cell wall was prepared as previously described (Yang et al. 2013). The purified recombinant CwIC proteins were added to the cell wall of Bt-59 and HD73, respectively. The mixtures were incubated at 37°C and the OD$_{540}$ of the mixtures was measured at the designated time points (Nugroho et al. 1999).

β-Galactosidase assays

Bt strains containing lacZ transcriptional fusions were cultured in SSM (Ery, 25 μg/mL) at 30°C with shaking at 220 rpm. Two-milliliter samples of cells were taken from $T_0$ to $T_{20}$ at 1-h intervals. $T_0$ is the end of the exponential phase and $T_n$ 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 (Zhang et al. 2018). The values reported are the means for at least three independent experiments.

Total RNA isolation and qPCR

Total RNA was extracted from Bt strains cultured in SSM at $T_0$, $T_8$ and $T_{14}$ with TRIzol reagent (Invitrogen, USA). The purified RNA was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Beijing, China). RpsU and gatB were chosen as reference genes (Reiter et al. 2011). Quantitative real-time PCR (qPCR) was performed using specific primers (Table S4) and TB Green Fast qPCR Mix (TaKaRa, Beijing, China) with a StepOnePlus Real-Time PCR System (ABI, California, USA). Three technical replicates and three biological replicates were designed, and the specificity and qualified amplification efficiency of the specific primers were verified. The relative expression level of each hydrolase gene was calculated according to the $2^{-\Delta\Delta CT}$ method.

Microscopic analysis

Bt strains were cultured in a conical flask containing SSM medium at 30°C with shaking at 220 rpm. Samples
were collected at certain time points and centrifuged, then sediments were suspended with appropriate volume of deionized water. Half microliter of the samples were placed on glass slides, and then cell morphology was observed by optical microscopy (Olympus, Japan).

**Bioassay**

Bt-59 and Bt-59 (ΔcwlC) were cultured in a fermentation medium (2% starch, 4.5% soybean cake powder, 2.0% corn steep liquor, 0.1% MgSO₄, CaCO₄, 0.1% KH₂PO₄ per liter of broth) for 44 h. Then, the fermentation broth were serially diluted with water to final concentrations of 0.0625, 0.125, 0.188, 0.250, 0.375, and 0.500 μL/L and used for the bioassay. Twenty larvae of *Culex pipiens* in the early fourth instar were collected for bioassay in each treatment (Xu et al. 2020). The experiment was performed with three replicates per concentration at 28°C in a biochemical incubator for 24 h, and distilled water was used as control.

**Sequence and data analysis**

Sequence analysis was performed by NCBI (https://www.ncbi.nlm.nih.gov/), Vector NTI 11, and GENEDOC. Primer Premier 5.0 was used for primer design, and the phylogenetic tree was constructed with MEGA Data analysis was performed using Microsoft Office Excel, GraphPad Prism 6. Adobe Photoshop CS6 was used for figure processing. The LC₅₀ values and 95% confidence intervals (CIs) were estimated with PoloPlus statistical software (LeOra Software Company 2002) (Straus 2008). Lethal concentration ratios (LC₅₀ ratio, LCR) were calculated to compare natural variation between different treatments, and the LCR value was considered to be significant (P<0.05) when the confidence intervals did not encompass the value 1 (Straus 2008).

**Author Contribution Statement**

JX and FS conceived and designed research. LH and LX conducted experiments. NC contributed new experimental ideas. LH and GH analyzed data. LH wrote the manuscript. All authors read and approved the
Compliance with Ethical Standards

The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

Data Availability Statements

All data generated or analysed during this study are included in this published article (and its supplementary information files).

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