Weak Transcription of the cry1Ac Gene in Nonsporulating Bacillus thuringiensis Cells

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The cry1Ac gene of Bacillus thuringiensis subsp. kurstaki HD-73 (B. thuringiensis HD-73) is a typical example of a sporulation-dependent crystal gene and is controlled by sigma E and sigma K during sporulation. To monitor the production and accumulation of Cry1Ac at the cellular level, we developed a green fluorescent protein-based reporter system. The production of Cry1Ac was monitored in spo0A, sigE, and sigK mutants, and these mutants were able to express the Cry1Ac-green fluorescent protein fusion protein. In nonsporulating B. thuringiensis HD-73 cells, low-level expression of cry1Ac was also observed. Reverse transcription-PCR and Western blotting results confirmed that the cry1Ac promoter has low activity in nonsporulating B. thuringiensis cells. A beta-galactosidase assay demonstrated that the transcription of the cry1Ac gene during exponential and transition phases is positively regulated by Spo0A. Additional bioassay results indicated that spo0A and sigE mutants containing the cry1Ac-gfp fusion exhibited insecticidal activity against Plutella xylostella larvae.

MATERIALS AND METHODS

Bacillus thuringiensis, a Gram-positive bacterium, is widely known for the production of parasporal crystals in mother cells (29). Because of the insecticidal properties of the crystal proteins, B. thuringiensis has been commercially used for many years as a pesticide in biocontrol applications (33). The parasporal crystal consists mainly of proteins encoded by cry or cyt genes that are classified according to amino acid sequence identity among their respective Cry proteins (14). These cry genes are classified as sporulation-dependent or sporulation independent based on their transcriptional mechanisms (9, 35). The cry3A gene, a typical example of a sporulation-independent cry gene, is controlled by σ^K and expressed during the stationary phase (1). Overexpression of this gene and high levels of insecticidal toxin production can be achieved in sporulation-deficient B. thuringiensis strains (25). In contrast, most cry genes (e.g., cry1A, cry1B, cry1C, cry2A, cry4A, cry4B, cry4D, cry11A, cry18Aa, cry3A, and cry40) are sporulation-dependent genes controlled by either σ^K or both (2, 9, 10, 36, 44, 45).

Spo0A is the master regulator of sporulation initiation (21). A high concentration of phosphorylated Spo0A (Spo0A-P) activates a series of sigma factors that temporally and compartmentally regulate genes during the sporulation process (20). In B. thuringiensis, the cry4Aa, cry4Ba, and cry11Aa genes are mainly transcribed from σ^K- and σ^K-dependent promoters (10, 16). However, the genes are transcribed at a low level during the transition phase that separates the end of exponential growth and the onset of sporulation, and it has been shown that this weak transcription is repressed by Spo0A (7, 30, 35, 44). However, the biological function of this transcriptional activity is not known, and no data are available regarding the putative transition state expression of other cry genes. For example, B. thuringiensis subsp. kurstaki HD-73 (here referred to as B. thuringiensis HD-73) is well known for containing only one cry gene, cry1Ac. The gene is a typical sporulation-dependent cry gene whose promoter is recognized by σ^K and σ^K factors during sporulation (40, 41). Analysis of cry1Ac gene transcription in Escherichia coli indicates that a transcription initiation site recognized by σ^K exists in the cry1Ac gene promoter (37), but the transcriptional regulation of the cry1Ac gene during the exponential and transition phases of B. thuringiensis remains unclear.

In this paper, we focus on analysis of the transcription of the cry1Ac gene during the exponential and transition phases in different genetic backgrounds of B. thuringiensis using a green fluorescent protein (GFP)-based reporter system. We demonstrate that the cry1Ac gene is transcribed in nonsporulating B. thuringiensis cells.

Bacterial strains, plasmids, and media. The B. thuringiensis strains and plasmids used in this study are listed in Table 1. All B. thuringiensis strains were grown at 30°C with 220-rpm rotary agitation in Schaeffer’s sporulation medium (SSM) (34) or in LB (Luria-Bertani) medium (1% NaCl, 1% tryptone, and 0.5% yeast extract).

For synchronous growth of B. thuringiensis strains, the cells were cultured as described by Noack et al. and Dori-Bachash et al. (15, 28), with slight modifications. The bacterial cells were cultured on LB plates overnight. A colony was taken from the plate to inoculate fresh liquid LB. After two consecutive overnight subcultures (1:1,000), the cells were in synchronized growth.

Construction of a cry1Ac-gfp fusion directed by cry1Ac promoter. To visualize the expression of the cry1Ac gene in B. thuringiensis, a cry1Ac-gfp fusion under the control of the cry1Ac promoter was constructed and introduced into the crystal-negative B. thuringiensis strain HD-73. The 3,534-bp DNA fragment of the cry1Ac ORF (opening reading frame) with its 387-bp promoter was amplified by PCR using genomic DNA from B. thuringiensis HD-73 as the template along with 1Ac-gfp-a and 1Ac-gfp-b as primers (Table 2). The 717-bp DNA fragment of the gfp ORF was

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amplified by PCR, using the pKEN(gfp) plasmid as the template and 1Ac-gfp-c and 1Ac-gfp-f as primers (13). The corresponding DNA fragments of the cry1Acp-cry1Ac-gfp fusion gene were amplified using overlapping PCR with 1Ac-gfp-a and 1Ac-gfp-f as primers. The overlapping PCR products were digested with BamHI-SphI and ligated into the BamHI and SphI sites of the temperature-sensitive shuttle vector pHT315 to produce the recombinant plasmid pH7-Tcry1Ac-gfp. The plasmid was transformed into the B. thuringiensis HD-73 strain (Cry R. B. thuringiensis HD-73) strain by electroporation (Gene Pulser at 2,200 V, 100 μF, and 25 μF; Bio-Rad) to produce the strain HD (cry1Ac-gfp).

The primers 1Ac-gfp-d and 1Ac-gfp-f were used to construct a pHTcry1Ac-gfp plasmid harboring the 3,534-bp cry1Ac ORF and a 717-bp gfp ORF. The resulting PCR products were used as the template, and spo0A-a/spo0A-d were used to amplify the downstream fragment of gfp. The two plasmids were introduced into the B. thuringiensis HD-73 strain to produce the HD (cry1Ac-gfp) and HD (gfp) strains, respectively.

**Construction of a sigE deletion mutant.** All primers for gene deletion were designed using B. thuringiensis 97-27 sequence as a base sequence (23). Using genomic DNA from B. thuringiensis HD-73 as a template, the upstream and downstream sequences from sigE were amplified using the primers sigE-a/sigE-b and sigE-c/sigE-d. The resulting amplified fragments were fused by overlapping PCR using the sigE-a and sigE-d primers. The resulting PCR products were then digested with BamHI and Sall and inserted between the BamHI and Sall sites of the temperature-sensitive suicide plasmid pMAD, producing the plasmid pMADsigE. The recombinant plasmid was transformed into the HD-73 strain by electroporation. The Erm’ transformants were verified by PCR using sigE-a and sigE-d as primers and cultured at 42°C for 3 h. The colonies sensitive to erythromycin were then selected, and the HD (ΔsigE) mutant strain was verified by Southern blotting (Fig. 1A).

The recombinant plasmid pH7-Tcry1Ac-gfp was transformed into the HD (ΔsigE) mutant strain by electroporation. The fusion protein-expressing mutants of the HD (ΔsigE) plasmid were selected using erythromycin (5 μg/ml) and subsequently analyzed by PCR amplification using the 1Ac-gfp-e and 1Ac-gfp-f primers.

**Construction of spo0A deletion mutant.** The upstream fragments of spo0A were PCR amplified using B. thuringiensis HD-73 genomic DNA as a template and spo0A-a and spo0A-b as primers. The primers spo0A-c and spo0A-d were used to amplify the downstream fragment of spo0A, and the primers Kan-a and Kan-b were used to amplify a kanamycin resistance gene from pDG780 (39). The deletion-insertion mutant cassette was amplified by two-step overlapping PCR. In the first step, the upstream fragments of the spo0A and Kan resistance genes were used as the templates, with spo0A-a/kan-b serving as primers. In the second step, the first-step products were used as the template, and spo0A-a/ spo0A-d were used as primers. The deletion-insertion mutant cassette was inserted into the BamHI and SalI sites of the pMAD plasmid to generate the recombinant plasmid pMADΔspo0A. The corresponding recombinant plasmid was then electroporated into HD-73. The confirmed transformants were incubated at 42°C. The colonies were screened for Kan’-Erm’, and the final HD (Δspo0A) mutant strain was verified by Southern blotting (Fig. 1B).
The recombinant plasmid pHT-P-cry1Ac-gfp was transformed into HD(spo0A) and HD(sigK) (17) by electroporation. The fusion protein-expressing mutant strains, HD(spo0A)(P-cry1Ac-gfp) and HD(sigK)(P-cry1Ac-gfp), were selected using an erythromycin plate (5 μg/ml) and further verified using PCR amplification with the primers 1Ac-gfp-e and 1Ac-gfp-f.

Construction of cry1Ac promoter fusion with lacZ. To analyze the transcriptional activity of the cry1Ac promoter in nonsporulating B. thuringiensis cells, the fragment located from the cry1Ac gene from B. thuringiensis HD-73 was amplified by PCR using cry1AcR and cry1AcF as primers and inserted into the PstI and BamHI sites of the B. thuringiensis-E. coli shuttle vector pHT304-18Z, producing the plasmid pHTcry1Acp. The pHTcry1Acp plasmid was transformed into various genetic backgrounds to generate the HD(cry1Acp-lacZ), HD(spo0A)(cry1Acp-lacZ), and HDΔsigK(cry1Acp-lacZ) strains. The HD(18Z) strain, containing pHT304-18Z, was used as a negative control.

Laser confocal imaging. FM4-64, a vital membrane stain (Molecular Probes Inc., Eugene, OR), was suspended in DMSO (dimethyl sulfoxide) at a final concentration of 100 μM/liter. Bacterial cells were stained with FM4-64 (100 μM/liter) for 1 min on ice. The stained cells were scanned using laser confocal microscopy (Leica TCS SL; Leica Microsystems, Wetzlar, Germany).

GFP quantification in B. thuringiensis cells. The samples were collected for GFP quantification, and the optical density at 600 nm (OD600) was determined by spectrophotometry. The GFP fluorescence of the B. thuringiensis strains was measured at an excitation wavelength of 481 nm and an emission wavelength of emission 507 nm using a fluorescence spectrophotometer (F-4500; Hitachi, Japan). For each specific measure-

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**TABLE 2 Primers and sequences**

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<tr>
<th>Primer</th>
<th>Sequence (‘5’→3’)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Restriction site</th>
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<td>BamHI</td>
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</tr>
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</tr>
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</tr>
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<tr>
<td>1Ac-gfp-f</td>
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<tr>
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</tr>
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<td>spo0AF</td>
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</table>

<sup>a</sup> Restriction enzyme sites are underlined.

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**FIG 1** Construction of sigE and spo0A deletion mutants.
ment, 2 ml of each culture was placed into a cuvette, and the samples were immediately measured. The relative fluorescence units of GFP were obtained by dividing the fluorescence value by the OD$_{600}$.

RNA isolation and reverse transcription-PCR (RT-PCR) of the cry$_{1}$Ac gene. RNA extraction was performed using TRIzol reagent (Invitrogen, San Diego, CA) according to the manufacturer’s instructions. The remaining genomic DNA was sequentially treated with RNase-free DNase I (TaKaRa, Tokyo, Japan) at 37°C for 15 min. RNA integrity was electrophoretically verified (Eppendorf, Hamburg, Germany).

The cDNA was synthesized using 500 ng of purified total RNA for 60 min at 50°C using Superscript III reverse transcriptase according to the manufacturer’s protocol (Invitrogen, San Diego, CA). PCR was performed with the 1Acp-orfR/1Acp-orfF primers to quantify the expression of the spoIID gene, which was normally regulated by the sigma E factor (31) (Table 2).

Western blotting. The HD-73 strain was grown in LB. At $T_{o}$, $T_{0}$, and $T_{i}$ with the strain grown in LB ($T_{o}$ is the end of the exponential growth phase and $T_{i}$ was n hours after the exponential growth phase), samples were taken and centrifuged, and the cells were resuspended in Na$_2$CO$_3$ (50 mmol/liter; pH 10) at equal cell densities. The resuspended cell lystate was further processed using a Mini-Beadbeater (Biospec Products Inc., Bartlesville, OK). The proteins of the supernatant were separated by SDS-PAGE and were electrotransferred to a PVDF (polyvinylidene difluoride) membrane. The protein-laden PVDF membranes were incubated with the anti-cry$_{1}$Ac antibody (Abcam Inc., Cambridge, MA) at a 1:1,000 dilution. The membranes were then washed, and secondary antibody visualization was performed as previously described (42).

β-Galactosidase assays. B. thuringiensis strains carrying lacZ transcriptional fusions were cultured in LB medium at 30°C. After culturing, 2.0-ml samples were harvested by centrifugation from $T_{i}$ to $T_{o}$ at 1-h intervals. The cells were resuspended in 0.5 ml of Z buffer (0.06 mol/liter Na$_2$HPO$_4$, 0.04 mol/liter Na$_2$PO$_4$, 0.01 mol/liter KCl, 0.001 mol/liter MgSO$_4$, 0.05 mol/liter β-mercaptoethanol, pH 7.0). The cells were then treated with a Mini-Beadbeater. The β-galactosidase activity was determined as previously described (8).

Bioassays with insect larvae. B. thuringiensis strains carrying cry$_{1}$Acp-cry$_{1}$Acp fusions were cultured in LB medium at 30°C. At $T_{o}$, the cells were centrifuged and resuspended in sterile water and enumerated using the viable count method (32). The samples and cabbage leaves were mixed together and fed to Plutella xylostella larvae being kept at 25°C. Twenty 2nd-instar larvae were placed on a leaf disk. The number of surviving larvae was recorded after 96 h. Each treatment was performed in triplicate. The negative controls were leaves treated with sterile water. The LC$_{50}$ (50% lethal concentration) was calculated using Probit analysis (19).

RESULTS

Monitoring the expression and accumulation of Cry$_{1}$Ac-GFP in B. thuringiensis HD-73. The HD$^{-}$ (cry$_{1}$Acp-cry$_{1}$Acp) strain was used to monitor the expression of the cry$_{1}$Ac gene during sporulation. The HD$^{-}$ (cry$_{1}$Acp-cry$_{1}$Acp) strain was used as a negative control. B. thuringiensis cells were observed via laser confocal microscopy. A red fluorescent signal indicated the membrane of a bacterial cell, and a green fluorescent signal indicated production of the Cry$_{1}$Ac-GFP fusion protein (Fig. 2). No green fluorescent signal was detected in the HD$^{-}$ (cry$_{1}$Acp-cry$_{1}$Acp) strain. T$_{o}$, end of exponential growth; T$_{3}$, asymmetric septum formation; T$_{4}$, pre-spore engulfment; T$_{8}$, spore wall development. GFP, green fluorescent protein signal in the bacterial cells. FM4-64, red fluorescent signal of the FM4-64 stain. The overlay shows green and red fluorescent signals. PC, phase-contrast microscopy. Bar, 7.5 μm.
HD strain was detected using laser confocal microscopy and a fluorescence spectrophotometer at T0, T3, T4, and T8 in SSM as depicted in Fig. 2A. At stage T0, no green fluorescent signal was detected in B. thuringiensis cells during the vegetative cell cycle. At stage T3 (initial asymmetric septum formation), the green fluorescent signals were observed in mother cells of HD strain (P-cry1Ac-gfp). At stage T4, the prespore started engulfment, and the green fluorescent signal intensities increased significantly. At T8 (spore wall developments), green fluorescence intensity was very high in the mother cell. These results indicated that the Cry-GFP fusion allowed monitoring of the accumulation of the Cry1Ac protein in B. thuringiensis mother cells during sporulation. Remarkably, GFP does not impair the formation of the parasporal inclusion.

Relative fluorescence units were calculated for the HD strain grown in SSM (Fig. 2C). The fluorescence intensity was evaluated at T3. The fluorescent intensity was slightly increased at T4 and enormously increased at T8. These results indicated that B. thuringiensis cells had already entered into sporulation at T8.

Expression and production of the Cry1Ac-GFP fusion in spo0A, sigE, and sigK mutants. Production of the Cry1Ac-GFP protein fusion occurred in the HDΔsigE(P-cry1Ac-gfp) strain (Fig. 3A). Double asymmetric septum formation, but not engulfment, was observed in the sigE mutant of B. thuringiensis during sporulation in SSM at T8 and T19. Surprisingly, a GFP fluorescence signal was detected in HDΔsigE(P-cry1Ac-gfp) cells at T8. At stage T19, the GFP fluorescence intensity was relatively high, thus suggesting that the cry1Ac promoter has σ-independent transcription activity.

To test whether this activity was completely independent of sporulation, the production of the Cry1Ac-GFP fusion protein was assessed in HDΔspo0A(P-cry1Ac-gfp) cells (Fig. 3B). No asymmetric septum was observed in HDΔspo0A(P-cry1Ac-gfp) cells, and the cells did not initiate sporulation. However, the green fluorescent signal was also detected at T8. At T19, the GFP fluorescence intensity was relatively high. These results suggested that the cry1Ac promoter had transcription activity independent of sporulation and was regulated by other factors in nonsporulating cells.

Further study of the expression of Cry1Ac-GFP fusion protein was performed in HDΔsigK(P-cry1Ac-gfp) mutant cells which had the ability to produce Cry protein and the inability to finish sporulation (Fig. 3C). The green fluorescent signal was observed at T8 and T19. The intensity of green fluorescence in HDΔsigK(P-cry1Ac-gfp) mutant cells was much lower than in HD strain (P-cry1Ac-gfp) cells at T8 but much stronger than in HDΔspo0A(P-cry1Ac-gfp) and HDΔsigE(P-cry1Ac-gfp) cells at T19. These results suggested that the transcription of the cry gene depended mainly on sigma E and sigma K during sporulation.

Analysis of the fluorescence intensity of the HDΔspo0A(P-cry1Ac-gfp), HDΔsigE(P-cry1Ac-gfp), and HDΔsigK(P-cry1Ac-gfp) mutant cells indicated that the Cry1Ac-GFP fusion protein expression is increased from T8 to T19, and the expression levels of the fusion proteins in HDΔsigE(P-cry1Ac-gfp) cells were approximately 2-fold higher than in the HDΔspo0A(P-cry1Ac-gfp) strain and 3-fold lower than in the HDΔsigK(P-cry1Ac-gfp) cells at T19 (Fig. 3D).

These results indicated that other regulator regulated the expression of crystal proteins in the HDΔspo0A(P-cry1Ac-gfp) mutant, Spo0A regulated the expression of crystal proteins in...
Hn, the main regulatory factor for crystal protein expression in HDΔsigK(P-cry1Ac-gfp) mutants, and sigma E was the main regulatory factor for crystal protein expression in HDΔsigK(P-cry1Ac-gfp). 

**Production of Cry1Ac-GFP in nonsporulating B. thuringiensis cells.** The forespore septum was formed at T3 (3 h after the onset of sporulation) in the strains cultured in SSM (Fig. 4A) and at T13 in the strains cultured in the LB medium (Fig. 4B). To lengthen the vegetative and transition phases, LB medium was used for analyzing the production of the Cry1Ac-GFP fusion protein in nonsporulating B. thuringiensis cells.

At T6, GFP fluorescence signals were observed in HDΔcry1Ac-gfp, HDΔsigK(P-cry1Ac-gfp), and HDΔsigK(P-cry1Ac-gfp) cells by laser confocal microscopy at a similar intensity, but the intensity was lower than that in HDΔcry1Ac-gfp cells at T13 (Fig. 4C). These images suggested that the expression of the Cry1Ac-GFP fusion proteins had already started during the transition phase in HDΔcry1Ac-gfp, HDΔsigK(P-cry1Ac-gfp), and HDΔsigK(P-cry1Ac-gfp) cells.

The fluorescence intensities in HDΔcry1Ac-gfp, HDΔsigE(P-cry1Ac-gfp), HDΔspo0A(P-cry1Ac-gfp), and HDΔsigK(P-cry1Ac-gfp) cells growing in LB medium were determined (Fig. 5). The relative fluorescence units among the HDΔcry1Ac-gfp, HDΔsigE(P-cry1Ac-gfp), HDΔspo0A(P-cry1Ac-gfp), and HDΔsigK(P-cry1Ac-gfp) cells all were higher than that in negative-control HDΔcry1Ac-gfp cells from T1 to T17. These results suggested that the cry1Ac promoter had activity in nonsporulating B. thuringiensis cells during the transition phase.

The green fluorescent intensity in HDΔcry1Ac-gfp and HDΔsigK(P-cry1Ac-gfp) cells was not significantly different from T1 to T17 and increased faster than that in HDΔsigE(P-cry1Ac-gfp) and HDΔspo0A(P-cry1Ac-gfp) cells from T13. The relative fluorescence intensity in HDΔcry1Ac-gfp, HDΔsigK(P-cry1Ac-gfp), and HDΔsigE(P-cry1Ac-gfp) cells were similar until T13. However, the fluorescence intensity in HDΔspo0A(P-cry1Ac-gfp) cells was lower than what was observed in HDΔcry1Ac-gfp, HDΔsigE(P-cry1Ac-gfp), and HDΔsigK(P-cry1Ac-gfp) cells after T3. These results suggested that Spo0A and other regulators are involved in the regulation of cry1Ac promoter activity in nonsporulating B. thuringiensis cells. Such an interpretation was consistent with the SSM frontal analysis.

The fluorescence intensities of HDΔcry1Ac-gfp and HDΔsigK(P-cry1Ac-gfp) cells were greatly increased after T12 compared to HDΔsigK(P-cry1Ac-gfp) cells. These results indicated that the SigE regulator is activated in B. thuringiensis cells at T12 in LB medium.

**FIG 4** Analysis of the production of Cry1Ac-GFP in nonsporulating B. thuringiensis cells. (A) The growth curve of the HDΔcry1Ac-gfp strain in SSM. (B) The growth curve of the HDΔcry1Ac-gfp strain in LB. (C) Images of the Cry1Ac-GFP fusion protein expression in mutants during exponential-growth and transition phases in LB medium.

**FIG 5** GFP quantification curves in mutant strains. Vertical bars represent the means ± standard deviations from 3 observations.
Expression of cry1Ac gene in B. thuringiensis cells. The RT-PCR method was used to confirm the transcription and expression of the cry1Ac gene in nonsporulating cells of wild-type strain B. thuringiensis HD-73 (Fig. 6A) and the HD(∆sigE) mutant (Fig. 6B) in LB medium. RT-PCR results indicated that the cry1Ac gene in B. thuringiensis HD-73 and HD(∆sigE) cells were transcribed at T₀, T₁, and T₃ in LB. To clearly show that cry1Ac transcriptional activity was not dependent on sporulation factors in nonsporulating cells, the spoIID gene regulated by the sigma E factor was used as a control. The results showed that the transcription of the spoIID gene in strain HD-73 was found at T₃ in LB but not in the HD(∆sigE) strain.

Western blotting using an anti-Cry1Ac antibody indicated the production of the Cry1Ac protein at T₀, T₁₀, and T₃ (Fig. 6C). These results, taken together, proved that the cry1Ac gene was transcribed in nonsporulating B. thuringiensis cells.

Transcriptional analysis of cry1Ac promoter. β-Galactosidase activity was not observed in HD(18Z) cells but was noted in HD(cry1Ac- lacZ), HD∆sigE(cry1Ac- lacZ), HD∆sp00A(cry1Ac- lacZ), and HD∆sigK(cry1Ac- lacZ) cells (Fig. 7). In LB, the β-galactosidase activities of the HD(cry1Ac- lacZ) and HD∆sigK(cry1Ac- lacZ) mutants were very low before T₉ and sharply increased after T₉, although the increasing rate of β-galactosidase activity in the HD∆sigK(cry1Ac- lacZ) strain was lower than that in the HD(cry1Ac- lacZ) strain. The β-galactosidase activity in spo0A and sigE mutants slowly increased after T₉ and maintained a low level thereafter. In agreement with the results of fluorescence analysis, β-galactosidase activity was lower in the spo0A mutant than in the sigE mutant after T₉ (Fig. 7A). We obtained the same tendency of β-galactosidase activity in SSM as in LB (Fig. 7B). The different results in LB and SSM were due to the different Tₚ values, which depended on the different growth phases in different media (Fig. 2 and 4).

These results once again indicated that Spo0A and other regulators had an effect on the transcriptional activity of the cry1Ac promoter in nonsporulating B. thuringiensis cells.

Bioassays on insect larvae. The insecticidal activity of HD-73, HD⁺(P-cry1Ac-gfp), HD∆sigE(P-cry1Ac-gfp), and HD∆sp00A(P-cry1Ac-gfp) mutants and the HD-73 wild strain against Plutella xylostella larvae was tested. The samples were prepared at T₀ in LB medium. At T₀, the insecticidal activity of the HD⁺(P-cry1Ac-gfp) strain, with an LC₅₀ of 1.29 × 10¹⁰ CFU/ml, was not significantly different from the LC₅₀ of the HD∆sigE(P-cry1Ac-gfp) strain (1.41 × 10¹⁰ CFU/ml) and HD-73 (1.17 × 10¹⁰), however, the strain

![FIG 6](image-url) Analysis of cry1Ac and spoIID expression in HD-73 wild-type strain and HD(∆sigE) mutant in LB medium. (A) RT-PCR detection of mRNA transcription of the cry1Ac and spoIID genes at different time points of HD-73 bacterial cells in LB medium. The product of the cDNA of cry1Ac was produced by using 1Acp-orfR and 1Acp-orfF primers. The product of cDNA of spoIID amplified using spoIID-R and spoIID-F primers was used as a negative control. The cDNA product of the 16S gene amplified using 16SrRANR and 16SrRANF primers was used as a positive control. M, molecular size marker (2,000, 1,000, 750, 500, 250, and 100 bp). (B) RT-PCR detection of mRNA transcription of the cry1Ac and spoIID genes at different time points of HD(∆sigE) bacterial cells in LB medium. (C) Western blot analysis of Cry1Ac protein expression in the HD-73 wild-type strain using the Cry1Ac antibody during exponential-growth and transition phases in LB medium.

![FIG 7](image-url) β-Galactosidase activity of cry1Ac promoter in HD(cry1Ac- lacZ), HD∆sigE(cry1Ac- lacZ), HD∆sp00A(cry1Ac- lacZ), HD∆sigK(cry1Ac- lacZ), and HD(18Z) strains in LB and SSM. (A) β-Galactosidase activity of cry1Ac promoter activity analysis in LB medium. (B) β-Galactosidase activity of cry1Ac promoter activity analysis in SSM. The HD(18Z) strain was used as a negative control.
TABLE 3 Insecticidal assay results with Cry1Ac-GFP

<table>
<thead>
<tr>
<th>Strain</th>
<th>T₉₀ (in LB medium)</th>
<th>LD₅₀ (CFU/ml)</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD-73</td>
<td>T₉₀</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HD⁻¹(P-cry1Ac-gfp)</td>
<td>T₉₀</td>
<td>1.29 × 10¹⁰</td>
<td>0.74—1.83</td>
</tr>
<tr>
<td>HDΔsigE(P-cry1Ac-gfp)</td>
<td>T₉₀</td>
<td>1.41 × 10¹⁰</td>
<td>0.86—1.76</td>
</tr>
<tr>
<td>HDΔspo0A(P-cry1Ac-gfp)</td>
<td>T₉₀</td>
<td>3.42 × 10¹⁰</td>
<td>2.56—4.73</td>
</tr>
<tr>
<td>HD-73</td>
<td>T₉₀</td>
<td>1.17 × 10¹⁰</td>
<td>0.99—1.21</td>
</tr>
<tr>
<td>HD-73</td>
<td>T₉₀</td>
<td>1.79 × 10⁸</td>
<td>0.89—2.57</td>
</tr>
</tbody>
</table>

*NA, no activity.

HDΔspo0A(P-cry1Ac-gfp) LD₅₀ of 3.42 × 10¹⁰ CFU/ml was higher than the HD-73 LD₅₀. However, the HD-73 LD₅₀ at T₉₀ (1.79 × 10⁸) was much lower than the HD-73 LD₅₀ at T₉₀ (Table 3).

**DISCUSSION**

A combination of laser confocal microscopy and a fluorescent reporter gene assay was used to monitor gene expression and the localization of gene products during cell development (11, 12, 43).

In this study, we successfully established this system to monitor the production and accumulation of Cry protein in *B. thuringiensis* mutants. The cry1Ac-gfp fusion controlled by a typical sporulation-dependent promoter, the cry1Ac promoter, was constructed (9). Our results indicated that a fluorescence signal could be observed in the spo0A, sigE, and sigK mutants harboring the cry1Ac-gfp fusion by use of laser confocal microscopy. The results also suggested that the cry1Ac promoter has low-level sporulation-independent activity in nonsporulating *B. thuringiensis* cells of spo0A, sigE, and sigK mutants, although the cry1A promoters BtI and BtII were previously reported to be controlled by sporulation-related sigma factors σ⁶⁸ and σ⁶⁹ (9). RT-PCR and Western blot evaluation of cry1Ac expression in nonsporulating *B. thuringiensis* HD-73 cells strengthened this hypothesis. Although previous studies have indicated that the transcription of a cry1A gene in *E. coli* initiated from the Ec site located between BtI and BtII, which is recognized by a σ⁷⁰-containing *E. coli* RNA polymerase (37), this is the first report providing clear evidence that the cry1A promoter exhibits transcriptional activity in nonsporulating *B. thuringiensis* cells.

Among sporulation-dependent cry genes, only cry4A, cry4B, and cry1A of *B. thuringiensis* subsp. *israelensis* have been proven to have low-level transcriptional activity during the transition phase, which might be controlled by σ⁹⁴ RNA polymerase and repressed by Spo0A (7, 30, 35, 44). The role of σ⁹⁴ in *B. subtilis* is essential for sporulation in the postexponential phase. There is a feedback loop among σ⁹⁴, AbrB, and Spo0A. During the early and mid-exponential phases, the transcription of the sigH gene is directly repressed by the transition state regulator AbrB. During the post-exponential phase, σ⁹⁴ and σ⁹⁴ activate the expression of spo0A. Spo0A–P represses the transcription of abrb and indirectly activates sigH. The increase of Spo0A–P activity enhances the level of σ⁹⁴ transcription, which is subsequently repressed by σ⁹⁴ itself (6, 9, 22). In our case, a β-galactosidase assay monitoring the expression of the cry1Ac-lacZ fusion demonstrated that cry1Ac promoter activity in the spo0A mutant was lower than what was observed in the sigE mutant. This is different from the cry genes of *B. thuringiensis* subsp. *israelensis*. These results suggested that in addition to sigma E and sigma K positively regulating cry1Ac transcription during the sporulation phase, Spo0A and other factors were involved in cry1Ac regulation during the transition phase. In addition, the β-galactosidase assay monitoring cry1Ac-lacZ fusion in the sigH deletion mutant illustrated the effects of sigma H on cry1Ac expression. The results indicated that the transcription activity of cry1Ac in the sigH deletion mutant was much higher than that in spo0A, sigE mutants and the HD-73 wild-type strain (data not show). This result suggested that sigma H is a negative regulator in regulating cry1Ac transcription during the transition phase. However, direct regulation of cry1Ac transcription by Spo0A or sigma H is inconsistent with the apparent absence of spo0A or a sigma H binding site in the cry1A promoter region (27). Future studies will need to focus on Spo0A, sigma H, and other regulators that act upon cry1Ac transcription in the nonsporulating *B. thuringiensis* cells.

Our previous study proved that there was no significant difference in the toxicity of the GFP-Cry fusion protein and the Cry1Ac protein (43). In this study, we demonstrated that the cells of spo0A and sigE mutants containing the cry1Ac-gfp fusion possessed insecticidal activity against *Plutella xylostella* larvae, similar to the nonsporulating cells of the cry-negative HD-73 strain did not possess such an activity. In contrast to the enormous increase in the transcription and expression levels of the cry1Ac gene during sporulation, as directly regulated by sporulation of the sigma factors σ⁶⁸ and σ⁶⁹ (9) and associated with insecticidal ability, the rather low level of cry1Ac expression in nonsporulating *B. thuringiensis* cells also confers insecticidal activity. *B. thuringiensis*, as an insect pathogen, belongs to the *B. cereus* group. Some virulence genes of *B. cereus*, such as the PlcR-regulated genes, expressed during the transition phase have been shown to be important for virulence in insects (18, 24, 38). However, they are not sufficient for virulence in the absence of the Cry proteins (24). Thus, the low-level transcription of cry1Ac during the transition stage allows the invasive nonsporulating cell to display toxicity against insect larvae.

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