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Overexpression of *Bacillus thuringiensis* HknA, a Histidine Protein Kinase Homology, Bypasses Early Spo$^-$ Mutations That Result in CryIIIA Overproduction

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The *Bacillus thuringiensis* CryIIIA insecticidal crystal protein (ICP) is a vegetatively expressed protein that is toxic to coleopteran insect larvae. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the asporogenous *B. thuringiensis* subsp. *morrisoni* strain EG1351, which harbors the native *cryIIIA*-encoding 88-MDa plasmid, showed a 2.5-fold overproduction of the CryIIIA protein compared with that of an isogenic wild-type strain. Further studies showed that neither CryIIIA protein synthesis nor CryIIIA protein processing was affected in strain EG1351 during vegetative growth. In an attempt to characterize the EG1351 mutation by complementation of function, the *hknA* gene was identified and cloned from a *B. thuringiensis* cosmid library. Primer extension analysis of *hknA* mRNA in wild-type *B. thuringiensis* demonstrated that the *hknA* gene is transcribed during vegetative growth from a σ^A-like promoter. Multiple copies of either the *hknA* gene or the *Bacillus subtilis kinA* (*spoIIJ*) gene were shown to bypass the sporulation defect in strain EG1351 as well as a *spo0F* mutation in *B. thuringiensis* EG1634. Additional studies showed that the *hknA* gene was not defective in strain EG1351. The results of this study suggest that *hknA* encodes a novel histidine protein kinase involved in *B. thuringiensis* sporulation. We also propose that the CryIIIA-overproducing phenotype of strain EG1351 is most likely due to a defect in the phosphorylation of Spo0A and confirm that CryIIIA production is not dependent on sporulation.

The gram-positive bacterium *Bacillus thuringiensis* is the natural host for a variety of plasmid-encoded insecticidal crystal proteins (ICPs) that are toxic to important coleopteran, lepidopteran, and dipteran insect pests (11). Several classes of ICPs have been identified (CryI, CryII, CryIII, and CryIV, etc.) on the basis of their protein sequence similarities and their target insect specificities. Most ICP gene transcription is concomitant with sporulation, and several ICP genes that are dependent on the sporulation-specific sigma factors σ^{35} and σ^{28} have been identified (1, 6). In contrast, Sekar (28, 29) and De-Souza et al. (7) have demonstrated that the CryIIIA protein is expressed during vegetative growth, an observation confirmed in our study. Similarly, other members of the CryIII class of ICPs are produced during vegetative growth (19). This is likely a consequence of the σ^A-like promoter sequences identified by Donovan et al. (10) for the *cryIIIA*, *cryIIIB*, and *cryIIIB2* genes.

In this study, we describe CryIIIA-ICP production in the asporogenous *B. thuringiensis* strain EG1351. Our results show a 2.5-fold increase in CryIIIA protein production in EG1351 compared with that of the isogenic wild-type strain EG2158 (9). A *B. thuringiensis* cosmid library was constructed for complementation of function studies in order to investigate the nature of the EG1351 mutation. The *hknA* gene was identified from the cosmid library by complementation of the Spo$^-$ defect in strain EG7651, an acrystalliferous derivative of EG1351. However, further studies showed that *hknA* was not defective in EG7651 and that the Spo$^-$ phenotype of EG7651 was bypassed by multiple gene copies of *hknA*. The deduced HknA protein sequence was determined to be similar to that of the *B. subtilis* histidine kinase, KinA (SpoIIJ) (3, 26). The KinA protein functions early in the Spo0A phosphorylation cascade by directly phosphorylating the Spo0F protein (13). Sporulation is delayed in *kinA* mutants, while multiple copies of the *kinA* gene can suppress certain stage 0 mutations (26). The identification and characterization of the *hknA* gene, its role in *B. thuringiensis* sporulation, and the nature of the Spo$^-$ mutation in strain EG1351 are discussed.

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MATERIALS AND METHODS

Bacterial strains and media. *B. thuringiensis* strains that were used in this study are listed in Table 1. Strain EG1351 is a spontaneous asporogenous isolate of EG2158, a naturally occurring strain that harbors the *cryIIIA* gene on an 88-MDa plasmid (9). The acrystalliferous strain EG7651 is a cured derivative of strain EG1351. Strains EG1385(pEG1007), EG1386(pEG1020), EG1671(pEG1010), EG1672(pEG1011), EG1673(pEG1006), EG1674(pEG1019), and EG1676 (pDG580) were all derived by transforming EG7651 with the appropriate plasmid DNA as indicated in parentheses. Strain EG7566 is a plasmid-free derivative of HD73-26 and was used in the construction of a recombinant *B. thuringiensis* cosmid library. Strain EG10368 is a derivative of the acrystalliferous strain HD73-26 and is more readily transformed by plasmid DNA harvested from *Escherichia coli* (8). Strain EG1668 harbors a disruption of the *hknA* gene created by homologous recombination between pEG1024 and the *hknA* locus in EG10368 (see below). Strain EG1634 is derived from EG10368 and contains a disruption in the *B. thuringiensis spo0F* allele (20) resulting from a Tn*5401* (4) insertion. Strains EG1635(pEG1020) and EG1677(pDG580) were derived by transforming EG1634 with the appropriate plasmid DNA (indicated in parentheses).

B. thuringiensis was grown at 30°C in LB (1% Bacto Tryptone [Difco], 0.5% yeast extract [Difco], 0.5% NaCl [pH 7.0]), BHIG (3.8% brain heart infusion [Difco], 0.1% glycerol),

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

Strain	Genotype or phenotype[a]	Source or reference
HD73-26	Cry$^-$	11a
EG7566	Cry$^-$	Ecogen, Inc.
EG2158	88-MDa [*cryIIIA*$^+$] native ICP[b] plasmid	10
EG1351	Spontaneous Spo$^-$ mutant of EG2158	Ecogen, Inc.
EG7651	EG1351 cured of the 88-MDA [*cryIIIA*$^+$] plasmid	
EG1385	EG7651 (pEG1007)[c]	
EG1386	EG7651 (pEG1020 [*hknA*$^+$])[c]	
EG1671	EG7651 (pEG1010 [*hknA*$^+$])[c]	
EG1672	EG7651 (pEG1011)[c]	
EG1673	EG7651 (pEG1006 [Δ*hknA*])[c]	
EG1674	EG7651 (pEG1019 [*hknA*$^+$])[c]	
EG1676	EG7651 (pDG580 [*kinA*$^+$])	
EG10368	Uncharacterized derivative of HD73-26 with increased transformation efficiency	8
EG1668	EG10368 *hknA*::NotI linker insertion	
EG1634	EG10368 *spo0F*::Tn*5401*	20
EG1635	EG1634 (pEG1020 [*hknA*$^+$])[c]	
EG1677	EG1634 (pDG580 [*kinA*$^+$])	

[a] The *hknA* and *kinA* genes are derived from *B. thuringiensis* and *B. subtilis*, respectively.
[b] ICP, insecticidal crystal protein.
[c] See Fig. 3 for plasmid structures.

nutrient sporulation medium (NSM; 0.8% nutrient broth [Difco], 0.05 mM $MnCl_2 \cdot 0.7$ mM $CaCl_2$, 1.0 mM $MgCl_2$), or C2 [1% glucose, 0.2% peptone, 0.5% NZ amine-A casein hydrolysate (Sheffield Products), 0.2% yeast extract, 15 mM $(NH_4)2SO_4$, 23 mM KH_2PO_4, 27 mM K_2HPO_4, 1 mM $MgSO_4 \cdot 7H_2O$, 600 μM $CaCl_2$, 17 μM $ZnSO_4 \cdot 7H_2O$, 17 μM $CuSO_4 \cdot 5H_2O$, 2 μM $FeSO_4 \cdot 7H_2O$] medium each supplemented to either 3 μg of chloramphenicol (Sigma Chemical Co.) or 100 μg of streptomycin (Sigma Chemical Co.) per ml when appropriate. *E. coli* JM110 (GIBCO BRL) and XL1-Blue MR (Stratagene Cloning Systems) were used as the host strains for plasmid and cosmid propagation, respectively, and were grown at 37°C in LB or BHIG medium containing 50 μg of ampicillin (Sigma Chemical Co.) per ml when appropriate.

Plasmids. Plasmids pEG1019 and pEG1020 contain approximately 23 and 20 kb, respectively, of *B. thuringiensis* EG7566 chromosomal DNA and were identified from a *B. thuringiensis* cosmid library. Plasmid pEG1006 was derived from pEG1020 by deletion of a 3.2-kb *Bam*HI DNA fragment. Plasmid pEG1007 was derived from pEG1020 by excision and replacement of the 3.2-kb *Bam*HI DNA fragment in the opposite orientation. Plasmid pEG1010 contains the 6.5-kb *Pst*I-*Hin*dIII DNA fragment from pEG1020 ligated into the polylinker of pEG597 (5). Plasmid pEG1011 contains the 4.0-kb *Bam*HI-*Hin*dIII DNA fragment from pEG1020 ligated into the polylinker of pEG597. Plasmid pEG1004 contains the 3.2-kb *Bam*HI DNA fragment from pEG1020 cloned into the polylinker of pUC18. Plasmid pEG491 consists of a 6.5-kb *Bam*HI DNA fragment containing the temperature-sensitive origin of replication (rep^{ts}) and the chloramphenicol acetyltransferase gene (*cat*) isolated from pTV32^{ts} (35) that was cloned into the polylinker of pUC18. Plasmid pEG1024 was derived from pEG491 and contains a 1.0-kb *Cla*I-*Bam*HI internal DNA fragment of the *hknA* coding region (bp +33 to +1073) disrupted by the insertion of a 10-bp *Not*I linker (Stratagene Cloning Systems) at the *Eco*47III site (bp +347). The cosmid shuttle vector pEG886 was used for the construction of a *B. thuringiensis* genomic cosmid library. The vector pEG886 contains the *B. thuringiensis* replication origin *ori60*, the multiple cloning site, and the *cat* gene isolated as a *Not*I DNA fragment from pEG853 (5) and cloned into the *Not*I site of the Supercos I cosmid vector (Stratagene Cloning Systems). Plasmid pDG580 (30) contains the *Sac*I-*Sph*I DNA fragment of the *B. subtilis kinA* gene cloned into pHV33 and was generously provided by Issar Smith.

Sporulation measurements. The extent of *B. thuringiensis* sporulation was determined from 36-h NSM cultures. Because of the aggregation of many of the *B. thuringiensis* strains used in this study, we were unable to determine a total viable cell count. The number of spores per ml of culture was determined after heating each culture at 65°C for 40 min and plating for single colonies.

Transformation and DNA manipulations. *E. coli* transformations were performed using frozen competent cells as described by the manufacturer (GIBCO BRL or Stratagene Cloning Systems). *B. thuringiensis* was transformed as described by Mettus and Macaluso (23). Plasmid isolations from *E. coli* and *B. thuringiensis* were by the alkaline lysis procedure described by Maniatis et al. (21). Plasmid DNAs were dephosphorylated using calf intestinal alkaline phosphatase (Boehringer Mannheim Corp.). Restriction endonucleases (Promega Corp.) and T4 DNA ligase (Promega Corp.) were used according to protocols described by the manufacturer.

***B. thuringiensis* genomic cosmid library.** *B. thuringiensis* EG7566 chromosomal DNA was partially digested with *Sau*3AI. DNA fragments larger than 15 kb in size were excised from a 0.7% agarose gel and were isolated using the Geneclean DNA isolation kit (Bio 101) according to the manufacturer's recommendations. The cosmid shuttle vector pEG886 was prepared as recommended for the double *cos* vector SuperCosI (Stratagene Cloning Systems). The vector was digested with *Xba*I to cleave the DNA between the dual *cos* sites, treated with calf intestine alkaline phosphatase, and subsequently digested with *Bam*HI to provide a compatible cloning site for ligation to the *Sau*3A inserts. Packaging of the ligation mix, infection of *E. coli* XL1-Blue MR (Stratagene Cloning Systems), titration, and amplification of the cosmid library were conducted using the Gigapack II packaging kit (Stratagene Cloning Systems) according to the manufacturer's directions. Following the amplification of the cosmid library, the culture was split into two equal fractions. Aliquots (1 ml) of one fraction were frozen in 30% glycerol, and the remaining fraction was used for cosmid DNA preparation. The cosmid DNA was used to transform *B. thuringiensis* EG10368 and was plated on LB medium containing 3 μg of chloramphenicol per ml. All resulting *B. thuringiensis* transformants were harvested, pooled, and grown in LB liquid medium containing 3 μg of chloramphenicol per ml. The resulting culture was split into two equal fractions. One fraction was stored as 1-ml aliquots in 30% glycerol, and the remaining fraction was used to prepare cosmid DNA. Cosmid DNA derived from strain EG10368 was used to transform the Spo$^-$ strain EG7651. Complementation of the sporulation defect in EG7651 was scored visually on NSM plates containing 3 μg of chloramphenicol per ml.

RNA preparation and quantitative primer extension analysis. To determine the temporal expression of *hknA* mRNA, total RNA was harvested from 5-ml aliquots of cells withdrawn from a culture of *B. thuringiensis* EG1351 or EG2158 grown in NSM medium. RNA was isolated according to the method described by Zuber et al. (36). Quantitation of total RNA following glyoxal treatment (22) and resolution on a 1.0% agarose gel were determined on the basis of the relative

intensities of ethidium bromide-stained rRNA present in each sample. Equal amounts of total RNA per primer extension reaction mixture were used, and reactions were performed using the Primer Extension System (Promega Corp.) as described by the manufacturer. Synthetic oligonucleotide primers were synthesized on an Applied Biosystems model 380B DNA synthesizer or purchased from Integrated DNA Technologies Inc.

DNA sequencing and computer analysis. Double-stranded plasmid DNA template preparation and subsequent alkali denaturation are described elsewhere (34). The *hknA* DNA sequence was determined for both strands using the Sequenase DNA sequencing kit (U.S. Biochemical Corp.). Initial DNA sequence for the *hknA* gene was determined using pEG1004 and the forward and reverse M13-specific primers (GIBCO BRL). Additional synthetic oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer or were purchased from Integrated DNA Technologies, Inc.

Computer-assisted protein analysis and nucleotide and amino acid homology searches were performed using programs (FASTNSCN, FASTPSCN, PROSITE, RAOARGOS, CLUSTAL, and PALIGN) contained in the Intelligenetics PC/GENE sequence analysis package.

SDS-PAGE and Western analysis of CryIIIA proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western analysis procedures were essentially done as described by Towbin et al. (31), with the addition of protease inhibitors (50 μl of aprotinin [1 mg/ml], 50 μl of phenylmethylsulfonyl fluoride [10 mg/ml], 50 μl of leupeptin [1 mg/ml], 50 μl of a pepstatin A [1 mg/ml], and 10 μl of EDTA [0.5 M]) to 5 ml of the extraction buffer prior to protein extraction. In the case of asporogenous *B. thuringiensis* strains, cultures were passed three times through a French pressure cell press (SLM Aminco, SLM instruments, Inc.) to disrupt the non-lysed cells. SDS-PAGE gels were electrophoretically transferred to a nitrocellulose filter and blocked with nonfat milk (5%). The filter was probed with CryIIIA antibody (27) that was diluted 1:500 in Tris-buffered saline (pH 7.8). Washes were done using a Tris-buffered saline solution containing 0.05% Triton X-405 and 0.05% SDS. An alkaline phosphatase-linked goat anti-mouse second antibody was used to detect bound CryIIIA antibody and visualized with a 5-bromo-4-chloro-indolylphosphate–nitroblue tetrazolium substrate system.

Disruption of *hknA* in *B. thuringiensis* EG1668. The temperature-sensitive plasmid pEG1024 (see above), which contains an internal fragment of the *hknA* gene that was disrupted by the insertion of a *Not*I linker at the *Eco*47III site, was transformed into strain EG10368. Transformants were selected on BHIG medium under chloramphenicol selection at 30°C. Recombination between pEG1024 and the *hknA* locus on the bacterial chromosome was allowed to occur by growing a selected transformant at the permissive temperature of 30°C under chloramphenicol selection. Subsequent plating of the culture at the nonpermissive temperature of 41°C under chloramphenicol selection allowed for the selection and growth of cells in which the integration event had occurred. The resulting colonies were inoculated and grown without selection in 200 ml of BHIG at 30°C with subsequent passages and growth in 2× 200 ml of BHIG at 41°C to allow for the resolution and loss of pEG1024. The final culture was serially diluted and plated on LB medium. Random colonies were replica patched at 30°C to LB plates with and without 3 μg of chloramphenicol per ml to select for colonies which were chloramphenicol sensitive (Cm^s). The resulting Cm^s colonies represented clones in which pEG1024 had recombined out of the genome leaving either a wild-type *hknA* or a *Not*I-linker disrupted form of *hknA* (*hknA*::*Not*I-linker). In order to identify those clones containing the disrupted form of *hknA*, chromosomal DNA was prepared from six Cm^s colonies and each was used for PCRs along with two oppositely oriented *hknA*-specific primers. The primers selected were homologous to *hkna* DNA sequences located 5′ and 3′ to the internal *hknA* DNA sequence contained on pEG1024. Analysis of the resulting PCR products by *Not*I restriction digests showed that three of the six colonies were identical and contained the *hknA*::*Not*I-linker allele (determined by the presence of a *Not*I restriction site). One of the three strains, designated EG1668, was selected for subsequent studies (see below).

TABLE 2. The *hknA* and *kinA* genes suppress sporulation defects in EG7651 and EG1634[a]

Strain	Genotype or phenotype	No. of spores/ml
EG2158	88-MDa [*cryIIIA*$^+$] native ICP[b] plasmid	3.0×10^8
EG1351	Spontaneous Spo$^-$ mutant of EG2158	1.0×10^2
EG10368	Uncharacterized derivative of HD73-26 with increased transformation efficiency	5.8×10^8
EG1634	EG10368 *spo0F*::Tn*5401*	2.6×10^4
EG1668	EG10368 *hknA*::*Not*I linker insertion	5.5×10^8
EG1634	EG10368 *spo0F*::Tn*5401*	2.6×10^4
EG1635	EG1634 (pEG1020 [*hknA*$^+$])[c]	8.0×10^7
EG1677	EG1634 (pDG580 [*kinA*$^+$])	6.0×10^7
EG7651	EG1351 cured of the 88 MDa [*cryIIIA*$^+$] plasmid	1.4×10^4
EG1386	EG7651 (pEG1020 [*hknA*$^+$])[c]	7.0×10^7
EG1674	EG7651 (pEG1019 [*hknA*$^+$])[c]	1.0×10^8
EG1676	EG7651 (pDG580 [*kinA*$^+$])	3.0×10^7

[a] The *hknA* and *kinA* genes are derived from *B. thuringiensis* and *B. subtilis*, respectively.
[b] ICP, insecticidal crystal protein.
[c] See Fig. 3 for plasmid structures.

Nucleotide sequence accession number. The GenBank/EMBL nucleotide accession number for the *B. thuringiensis* *hknA* gene is U03552.

RESULTS

Increased production of CryIIIA protein in the Spo$^-$ *B. thuringiensis* strain EG1351. *B. thuringiensis* EG1351 (Table 2) was identified as a spontaneous asporogenous mutant of strain EG2158 (9). Microscopic evaluation of EG1351 cultures grown on sporulation medium showed a marked increase in CryIIIA crystal size compared with similarly grown EG2158 cultures. In addition to the unusually large rhomboid crystal, EG1351 cells are also characterized by the absence of a developing forespore. To quantitate the level of CryIIIA protein production, an SDS-PAGE gel was run on 3-day-old C2 cultures of EG2158 and EG1351. Densitometric analysis of the SDS-PAGE gel showed a 2.5-fold increase in CryIIIA production in EG1351 compared with that in EG2158 (Fig. 1; compare lanes b and d and lanes c and e).

Previous reports have shown that the *cryIIIA* gene contains a σ^A-like promoter (10) and is transcribed during vegetative growth (7, 28, 29). To assess whether the expression of CryIIIA protein is altered in vegetative EG1351 cells, a time course of CryIIIA protein production in EG1351 and EG2158 was determined by Western blot (immunoblot) analysis (Fig. 2). No difference in the temporal expression of CryIIIA protein was observed between EG2158 and EG1351 during the vege-

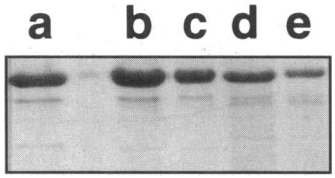

FIG. 1. SDS-PAGE of the CryIIIA proteins from the *B. thuringiensis* strains EG2158 and EG1351 (Spo$^-$). The CryIIIA proteins, harvested from 3-day-old C2 cultures, were resolved on a 12.0% polyacrylamide gel and visualized by Coomassie blue staining (see Materials and Methods). In the case of the asporogenous strain EG1351, the culture was passed twice through a French pressure cell press (SLM Aminco; SLM Instruments, Inc.) to ensure complete cell lysis. Lane a, CryIIIA standard (8.0 μg). Equal volumes of the cultures were loaded on the gel as follows: EG1351, 15.0 μl (lane b) and 10.0 μl (lane c); EG2158, 15.0 μl (lane d) and 10.0 μl (lane e). The amount of CryIIIA protein per sample was quantitated by densitometry on a Molecular Dynamics computing densitometer (model 300A).

tative phase. A distinct preprocessed form of the CryIIIA protein was detected in both strains as early as mid-log growth in sporulation medium (Fig. 2; t_{-4} NSM) and in the nutrient-rich BHIG medium (Fig. 2; t_{-4} BHIG). The processed form of the CryIIIA protein was detected at approximately stationary phase in both strains (t_{-1} NSM). The level of CryIIIA production during vegetative growth appears to be higher in EG2158, suggesting that the increased accumulation of CryIIIA in EG1351, as evident by SDS-PAGE (Fig. 1), occurs sometime after the onset of stationary phase.

Suppression of the Spo$^-$ defect in *B. thuringiensis* EG1351. We attempted to identify the Spo$^-$ mutation in EG1351 causing the CryIIIA overproduction phenotype by complementation of function using a genomic cosmid library derived from the plasmid-free *B. thuringiensis* strain EG7566 (see Materials and Methods). EG7651 (Table 2), an acrystalliferous derivative of EG1351, was transformed with cosmid DNA from the *B. thuringiensis* library, and the transformants were screened visually on NSM plates. On sporulation medium, asporogenous *B. thuringiensis* strains display a translucent colony morphology, while Spo$^+$ strains appear opaque. Approximately

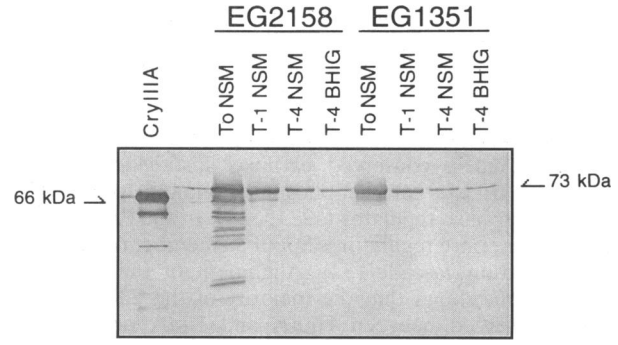

FIG. 2. Western blot of the CryIIIA proteins from *B. thuringiensis* EG2158 and EG1351. Aliquots of EG2158 and EG1351 (Spo$^-$) cultures were taken during vegetative (t_{-4} and t_{-1}) and early stationary (t_0) phases of growth. The CryIIIA protein was harvested from each aliquot by twice passing the samples through a French pressure cell press (SLM Aminco; SLM Instruments, Inc.). The preprocessed form of the CryIIIA protein is detected in all lanes and runs at approximately 73 kDa. The processed form of the CryIIIA protein is detected at 66 kDa.

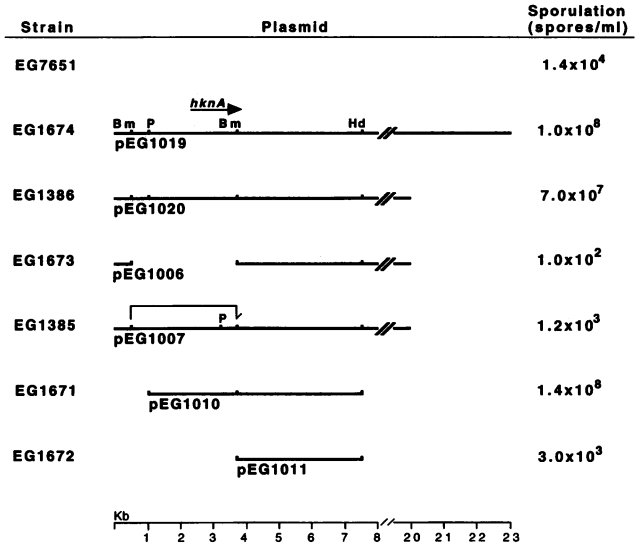

FIG. 3. Complementation of the EG7651 (Spo$^-$) sporulation defect by pEG1019 and pEG1020. Plasmids pEG1019 and pEG1020 were identified from a *B. thuringiensis* cosmid library (see text). Only the *B. thuringiensis* DNA fragments contained on each plasmid construction are shown in the figure. Also indicated are the position and orientation of the *hknA* open reading frame.

5,000 transformants were screened in this manner, and two colonies that displayed an opaque phenotype were recovered. The cosmids identified from the opaque colonies were designated pEG1019 and pEG1020. Endonuclease restriction analysis showed that the cosmids contained cloned *B. thuringiensis* DNA fragments that shared identical 5′ ends, while the cloned DNA fragment in pEG1019 contained an additional 3 kb of 3′ DNA sequences (Fig. 3).

Derivatives of pEG1020 were constructed and subsequently introduced into EG7651 to determine the location of the complementing DNA fragment (Fig. 3). Plasmids pEG1006 (in which the 3.2-kb *Bam*HI DNA fragment was deleted), pEG1007 (in which the 3.2-kb *Bam*HI DNA fragment was replaced in the opposite orientation), and pEG1011 (which contained the 4.0-kb *Bam*HI-*Hin*dIII DNA fragment) all failed to complement the EG7651 Spo$^-$ mutation. Plasmid pEG1010, which contained the 7.5-kb *Pst*I-*Hin*dIII DNA fragment, complemented the Spo$^-$ defect in EG7651. These data localized the gene that complemented the Spo$^-$ mutation in EG7651 as spanning the more rightward of the two *Bam*HI sites contained on pEG1020 (Fig. 3).

DNA sequencing and characterization of the *hknA* gene that suppresses the Spo$^-$ defect in strain EG7651 (EG1351). Initial sequence data for DNA contained on pEG1020 were obtained using plasmid pEG1004 and the forward and reverse M13 sequencing primers (GIBCO BRL). Additional DNA sequence analysis identified an open reading frame (*orf*) of 1,119 bp which spanned the *Bam*HI site in pEG1020 that was identified by complementation studies. The DNA and the deduced amino acid sequences of the *orf*, designated *hknA* (histidine kinase A) on the basis of protein similarities (discussed below), are shown in Fig. 4. Mapping of the 5′ end of the *hknA* mRNA by primer extension analysis (Fig. 5) detected a single transcript apparently originating at nucleotide position −35 (Fig. 4 [*]) relative to a putative initiation codon (+1). The proposed −10 (**TAATAT** and −35 (**TTAACG**) regions for

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-681 TTTTACGCTTCTAGTAACTATTTAATTAAGTGTAACTTTTACTTCATTTCTTGTTTGAAA
-621 ATAGTATCTTCTACATAAATTTGTTCTTTATCTACAGCAGTAGCATGAGTTAATACTAAA
-561 CCGATTGGTGTTTTTGTATCTTTATTTTGAACAATTGTAAAAGGAACATTTTTTTCGTTT
-501 GCTAATTTAATGTATTTTGATAAATGTGGGTAAGCAATGCCACCATTTAGGAGGAGTTGT
-441 AATGATTGAGAAGGGCGCATGCTACCCGCCACTTCAGAGTATACATTACTTTGCATTACT
-381 TGACCGATCGTTAAAGCGATTTCCACACGCTCACGTAATGTAGTTAAATACATATTACGT
-321 TCTTCTGGTTTGTTTTGCTTTTGGCCGTAAATGCCTTCTTGGAGATAATCTTCCACATTT
-261 TTATTTACCATATTTTTCACACCTTTCATTTTCTATTGTACGCAAGAATTAAAAATGATG
-201 CCAAAAAAGTCGTTTTGACTACGGAAATAATGAAAATATGTGAAGGAAAAAGGGGATTTG
-141 CATACAATTAGTTAAATAAGAAAGAAAAAATAATTTTATCAACAGAAATTAACAAAATGT

                                  *
 -81 GACAAAAATCTATTAACGAATCGTATTTTTTGTGTTAATATTCAAAATATAAAGGGTATT

 -21 CGTCGTATGGAAATGGAGGGA

     M  E  V  F  P  I  D  K  D  I  K  E  I  F  C  S  H  L  K  N
   1 ATGGAGGTTTTTCCAATCGATAAGGATATTAAAGAAATATTTTGTTCACACTTGAAAAAC

     N  R  H  Q  F  V  E  N  W  K  N  K  M  I  I  S  E  K  D  P
  61 AATAGGCACCAATTCGTAGAGAACTGGAAAAATAAAATGATAATTTCCGAAAAAGATCCA

     F  K  L  E  V  V  Q  N  G  E  D  L  L  E  L  I  I  E  L  T
 121 TTTAAACTAGAAGTAGTTCAAAATGGAGAGGATTTACTAGAGTTAATTATCGAACTTACT

     M  E  D  K  D  I  N  Y  L  Q  P  L  C  E  K  I  A  I  E  R
 181 ATGGAAGATAAAGATATAAATTATCTTCAACCGTTATGTGAGAAAATTGCTATTGAACGT

     A  G  A  D  A  N  I  G  D  F  V  Y  N  A  N  V  G  R  N  E
 241 GCTGGAGCAGATGCGAATATTGGAGATTTTGTTTATAACGCAAACGTGGGAAGAAATGAA

     L  F  E  A  M  C  E  L  D  V  S  A  R  E  L  K  P  I  M  A
 301 CTTTTCGAAGCGATGTGTGAATTAGATGTTAGCGCTCGTGAATTGAAACCAATTATGGCA

     K  I  H  T  C  F  D  K  L  I  Y  Y  T  V  L  K  Y  S  E  I
 361 AAAATACATACTTGTTTTGACAAATTAATTTATTATACCGTTTTAAAATACTCGGAAATT

     I  S  K  N  L  E  E  K  Q  Q  Y  I  N  E  T  H  K  E  R  L
 421 ATATCGAAGAATTTAGAGGAAAAACAGCAATATATTAACGAAACACATAAAGAAAGACTG

     T  I  L  G  Q  M  S  A  S  F  V  H  E  F  R  N  P  L  T  S
 481 ACGATTTTAGGACAAATGTCAGCTAGTTTTGTACATGAATTTCGTAATCCGCTTACTTCC

     I  M  G  F  V  K  L  L  K  A  D  H  P  S  L  S  Y  L  D  I
 541 ATTATGGGGTTTGTCAAATTATTAAAGGCAGATCATCCGAGTTTATCGTATTTAGATATT

     I  S  H  E  L  D  Q  L  N  F  R  I  S  Q  F  L  L  V  S  K
 601 ATTTCGCATGAATTAGATCAATTAAATTTTCGTATTTCGCAATTTTTACTCGTATCAAAA

     K  E  M  W  N  E  S  E  R  F  W  L  N  D  L  F  Q  D  I  I
 661 AAAGAAATGTGGAATGAATCGGAACGGTTTTGGCTTAATGACTTGTTTCAAGACATTATA

     Q  F  L  Y  P  S  L  V  N  A  N  V  L  I  E  K  N  L  P  Y
 721 CAATTCTTATATCCGAGTTTGGTCAATGCGAATGTTTTGATTGAAAAGAATTTACCGTAT

     P  I  P  L  V  G  Y  R  S  E  V  R  Q  V  F  L  N  I  L  M
 781 CCAATTCCGCTTGTTGGTTATCGGAGTGAAGTGAGACAAGTATTTTTAAACATATTAATG

     N  S  I  D  A  L  E  S  M  K  E  E  R  K  I  I  I  D  V  F
 841 AATTCAATTGATGCTCTTGAATCAATGAAAGAAGAACGAAAAATTATCATTGATGTATTT

     E  E  D  Q  A  I  R  I  V  I  K  N  N  G  P  M  I  P  A  E
 901 GAAGAAGATCAAGCTATTCGAATTGTGATAAAAAATAATGGACCAATGATTCCAGCTGAA

     N  V  E  T  I  F  E  P  F  V  T  T  K  K  L  G  T  G  I  G
 961 AATGTAGAAACGATTTTTGAACCATTTGTAACTACTAAAAAGTTAGGAACTGGTATTGGA

     L  F  V  C  K  Q  I  V  E  K  H  N  G  S  I  M  C  R  S  D
1021 TTGTTTGTATGTAAACAAATTGTGGAAAAACATAATGGATCCATTATGTGTCGATCAGAT

     N  D  W  T  E  F  Q  I  A  F  Q  K  -
1081 AACGATTGGACAGAATTTCAAATTGCATTTCAAAAATAA

1120 ACAGTCTACACCAAATGAATGGTGTAGACTGTTTTAATTTGTGAGTATAACGGCTTCTAA
1180 TTTAGGATCTTTAGTTGAATAACCTACTATTGAAATTGTATAAATAACATTTGGTTCAAC
1240 ATTGAATTTTCGGTATAGTCAATAAAACGTTTTTATTATCTGCGAGTGAAATTTCAATAT
1300 CTGCTGTACCAGGGCTAACTTGCAAAAAATCGGTTATTTGTTTAAAGAGTGCATTTTCAA
1360 ATAAATGATCTGGATCTTTTAAATCTACATTTACAACAGGGGTATCTGGAGAGAAATGTG
1420 CAAAGCGTATTTTTTGCTTGACCAGCTGGTAAATGTGTATTATCAAGTATAGGTTGTAAT
1480 TGTAGGTGATTATCACTATTTATTGCAGCAAGAGTATAAGTATGATTTCCCATTATTGGT
1540 ACCAACGCTGAAAAGATTGGAGTTTCATTTCCGACAGAACAATATCTATACGGTATTTAC
1600 CTTGCACTAATGATAAATAAGGGCTGAATTGCTTAAATGAAATATTTTTAATAACCTTTT
1660 GTCCGTTTACTAAAATGTCAACCGCCGGTATATTTGGATCGGCATGAAAAATTCTTATAT
1720 GTGATGGTAAAGCAGCTTCTTGTGAGTCTCTTGTTTCATACGCCTGTACAAGTTTTGATA
1780 GTGCATCGTAATATTTCATATATATTCTATATATTTTT
```

FIG. 4. The DNA and deduced protein sequences of the *hknA* gene from *B. thuringiensis*. The DNA and protein sequences of the *hknA* gene as well as the 3′ and 5′ flanking DNA sequences are shown. The transcription start site is indicated (*), along with the putative −10 (TAATAT) and −35 (TTAACG) promoter sequences. Also indicated are the *Eco*47III restriction site (double underline) that served as the disruption site for the *hknA* gene (see Materials and Methods) and the 13-bp inverted repeats (arrows) that are located immediately 3′ to the *hknA* coding region.

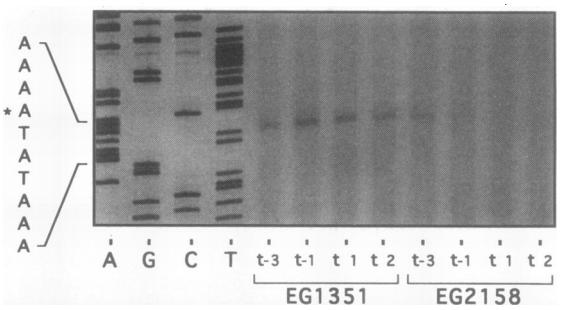

FIG. 5. Temporal expression and 5′ mapping of *hknA* mRNA in the *B. thuringiensis* strains EG1351 (Spo$^-$) and EG2158. Total RNA was harvested at the appropriate time points from NSM cultures of EG1351 and EG2158. An identical oligonucleotide primer was used in both the DNA sequence and primer extension reactions. A specific primer extension product is detected during both the vegetative (t_{-3} and t_{-1}) and poststationary (t_1 and t_2) phases of growth in EG1351. An identical product is detected only during vegetative growth in EG2158 (t_{-3} and t_{-1}). The transcription start site is indicated by an asterisk and resides 35 bp upstream of the putative ATG (Fig. 4).

the *hknA* gene resemble a σ^A-dependent promoter sequence, suggesting that the *hknA* gene is transcribed during vegetative growth. This was confirmed by analyzing *hknA*-specific mRNA production in NSM-grown cultures of EG2158 and EG1351 by primer extension analysis (Fig. 5). In the Spo$^+$ strain EG2158, *hknA* mRNA was not detected after t_0. In contrast, *hknA* mRNA was observed at all time points in EG1351, consistent with the expression of a σ^A-dependent gene in a sporulation-deficient background. These data show that *hknA* transcription in the Spo$^-$ strain EG1351 was prolonged compared with that of the isogenic Spo$^+$ strain EG2158. A set of 13-bp inverted repeats with a ΔG (25°C) of 25.2 kcal (ca. 105 kJ)/mol resides immediately 3′ to *hknA* (arrows in Fig. 4) and could function as a rho-independent terminator, suggesting that the *hknA* gene is transcribed as a monocistronic unit.

HknA is homologous to histidine kinases belonging to two-component signal transduction systems. The 43.6-kDa protein deduced from the *hknA* DNA sequence shows similarity to the histidine kinase, KinA (SpoIIJ), from *B. subtilis* (3, 26) as well as to other histidine kinases that belong to two-component signal transduction systems (24). Two-component regulatory systems are composed of a histidine kinase and a cognate response regulator. The majority of the sequence similarity observed between the histidine kinases occurs in the carboxyl domain. Signals, presumably received through the amino domain, result in the autophosphorylation of the histidine kinase at a conserved carboxyl histidyl residue and subsequent transfer of the phosphate moiety to the corresponding response regulator (12, 13, 24). In the case of KinA, the target response regulator is Spo0F, a member of the Spo0A phosphorylation cascade (32). Alignment of the HknA and KinA proteins shows that the majority of the 22% sequence identity observed between HknA and KinA occurs within conserved carboxyl domains that are shared among histidine protein kinases (Fig. 6).

Multiple copies of *hknA* or *kinA* suppress early sporulation defects in *B. thuringiensis*. The similarity of HknA to KinA suggested that HknA may function in a manner similar to that of KinA in early sporulation. In *B. subtilis*, increased gene copy of *kinA* has been shown to bypass *spo* mutations that occur in the *spo0K*, *spo0F*, and *spo0B* components of the Spo0A phosphorylation cascade (26). However, elevated gene levels

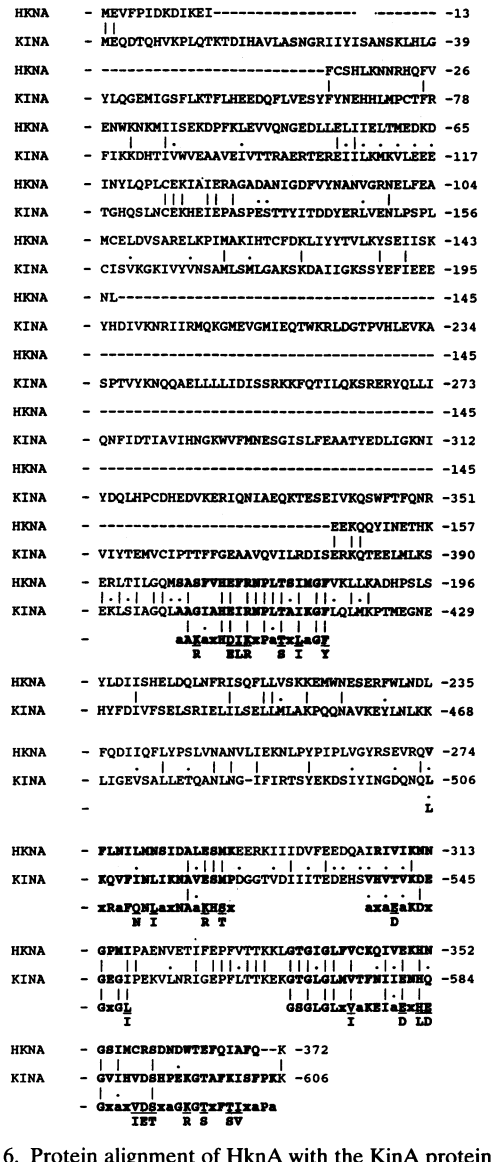

FIG. 6. Protein alignment of HknA with the KinA protein from *B. subtilis*. |, identical aligned residues; •, similar aligned residues. Residues that are considered similar are as follows: A, S, and T; D and E; N and Q; R and K; I, L, M, and V; F, Y, and W. The residues in boldface type indicate conserved regions found among the histidine kinases. The consensus sequences for these regions are shown below the HknA-KinA alignment and were adapted from T. Msadek et al. (24). a, I, L, or V; x, any amino acid.

of *kinA* are unable to bypass a defect in the *spo0A* gene (26). To evaluate the effect that multiple gene copies of *hknA* or *kinA* have on an early sporulation mutant in *B. thuringiensis*, strain EG1634 (*spo0F* [20]) was transformed with either the *hknA*-encoding pEG1020 (strain EG1635 [Table 2]) or the *kinA*-encoding pDG580 (strain EG1677 [Table 2]). The results of this experiment demonstrate that increased gene copies of either *hknA* or *kinA* were able to bypass the *spo0F* defect in EG1634 to the same extent (Table 2).

Similar experiments were conducted to test if multiple copies of the *kinA* gene could bypass the Spo$^-$ defect in *B. thuringiensis* EG7651 (Table 2). Strain EG7651 transformants containing either the *kinA* gene (strain EG1676 [Table 2]) or the *hknA* gene (strains EG1386 and EG1674 [Table 2]) showed a similar elevation in sporulation. These data demonstrate that HknA resembles KinA in its ability to suppress an early sporulation mutant in *B. thuringiensis* and suggest that HknA plays a role in *B. thuringiensis* sporulation similar to that observed for KinA in *B. subtilis*. The observation that multiple copies of *kinA* could increase sporulation in EG7651 (Spo$^-$) and EG1634 (*spo0F*) suggested that *hknA*, present in multiple copies, may not be complementing an *hknA* defect in EG7651 but may be suppressing another defect that results in the EG7651 Spo$^-$ phenotype.

The *hknA* gene is not defective in the Spo$^-$ *B. thuringiensis* strain EG1351. A mutation in *kinA* delays sporulation in *B. subtilis* (13). This is in contrast to the tight Spo$^-$ phenotype of EG1351 that was suppressed by multicopy *hknA* (Table 2). These observations, together with the relatedness of HknA to KinA and the ability of increased levels of *hknA* to suppress a *spo0F* mutation in *B. thuringiensis*, suggested that the *hknA* gene may not be responsible for the Spo$^-$ defect in EG1351. This was confirmed with *B. thuringiensis* EG1668, which harbors a disruption of the *hknA* gene (see Materials and Methods) and showed no significant effect on sporulation frequency (Table 2) or on CryIIIA protein production (19). To support this result, the *hknA* gene was PCR amplified from strains EG2158 and EG1351 and sequenced (19). No significant difference was observed between the *hknA* DNA sequences from EG1351 and EG2158.

DISCUSSION

Previous studies have shown that the *cryIIIA* gene is unique among ICP genes in that it is transcribed during vegetative growth from a σ^A-like promoter (7, 10, 28, 29). Like *cryIIIA*, the *cryIIIB* and *cryIIIB2* genes also possess σ^A-like promoter sequences and are expressed during vegetative growth (10, 19). In the present study, we have demonstrated that the CryIIIA protein is produced during vegetative and stationary phase and is overproduced in strain EG1351, an asporogenous variant of the *B. thuringiensis* subsp. *morrisoni* strain EG2158. These results and data presented elsewhere (20) show that CryIIIA protein production is not only not dependent on sporulation but can also be elevated in certain Spo$^-$ backgrounds. As a consequence of investigating the nature of the EG1351 mutation, we have cloned and characterized the gene (*hknA*) for a novel histidine kinase, HknA, from *B. thuringiensis*, which shows sequence similarity to the KinA protein from *B. subtilis*. However, we have also demonstrated that a defect in the *hknA* gene does not give rise to the Spo$^-$ EG1351 phenotype. We propose that when *hknA* is present in multiple copies, it bypasses the Spo$^-$ defect in EG1351.

In *B. subtilis*, multiple copies of *kinA* can bypass certain stage 0 mutations prior to *spo0A* (26). Our strongest evidence that HknA functions as a histidine protein kinase in a manner similar to KinA is the observation that increased levels of either HknA or KinA can bypass a *spo0F* mutation in *B. thuringiensis* (Table 2). The identification of *B. thuringiensis* homologs for the *B. subtilis spo0F* and *spo0A* (20) genes suggests that a similar if not identical Spo0A phosphorylation cascade functions in *B. thuringiensis*. Furthermore, Southern analysis of *B. subtilis* total DNA (14) using *hknA* as a radiolabeled DNA probe failed to detect any hybridizing DNA fragments under conditions of low stringency (19). Similar experiments on total *B. thuringiensis* DNA using *kinA* as a radiolabeled DNA probe detected multiple hybridizing DNA bands, none of which corresponded to *hknA* (19). These results suggest that although *B. subtilis* apparently does not contain an

hknA homolog, *kinA*-related DNA sequences appear to be present in *B. thuringiensis*, suggesting a similar sensing system for initiating sporulation.

Recently, two additional histidine protein kinases, KinB (33) and KinC (17), involved in the Spo0A phosphorylation cascade have been identified in *B. subtilis*. The deduced KinB protein contains a hydrophobic amino terminus with six putative membrane-spanning segments (33). Computer-aided protein alignment showed that HknA shared 21 and 23% sequence identity with KinB and KinC, respectively. The majority of the amino acid sequence similarity between HknA and KinB and KinC resides within the carboxyl halves of the proteins. Additional analysis showed that HknA does not possess a hydrophobic amino terminus and is probably not a membrane-associated protein (19).

The nature of the EG1351 mutation, as determined from experiments using the acrystalliferous isogenic strain EG7651, has not been resolved. However, the results of this study suggest that the mutation occurs early in sporulation and probably affects the Spo0A phosphorylation cascade. In support of this proposal, multiple copies of the *kinA* gene were shown to bypass the EG7651 Spo$^-$ defect and the *spo0F* mutation in *B. thuringiensis* (Table 2), just as increased *kinA* levels have been observed to bypass certain stage 0 mutations in *B. subtilis* (26). Likewise, multiple copies of the *hknA* gene were also shown to bypass the EG7651 defect as well as the *spo0F* mutation in *B. thuringiensis* (Table 2). Furthermore, we have demonstrated that the vegetative transcription of the *hknA* gene was down-regulated at t_0 in wild-type *B. thuringiensis*, while *hknA* mRNA was detected well into stationary phase in EG1351 (Fig. 5). Whether this down-regulation of *hknA* is due to the action of a transition state regulator (25) is not known. These observations suggest an early sporulation defect in EG1351, since defects that occur later in sporulation would be unable to support vegetative gene expression. Lastly, we have shown that the CryIIIA protein is overproduced in EG1351. Recent reports demonstrate that the CryIIIA protein is also overproduced in a *spo0F* mutant of *B. thuringiensis* (20) and that a *spo0A* mutant of *B. subtilis* supports increased *cryIIIA* transcription (2).

Since multiple copies of the *kinA* gene cannot bypass Spo$^-$ mutations in the *B. subtilis spo0A* gene (26), it is reasonable to expect that the EG1351 defect does not affect the recently identified *B. thuringiensis spo0A* gene (20). We have used the recently described *B. thuringiensis spo0F* gene (20) to PCR amplify and sequence *spo0F* from both EG1351 and its isogenic wild-type strain EG2158. The results have shown that *spo0F* is not defective in EG1351 (19). Of the remaining *spo0* genes that have been identified in *B. subtilis* (12, 13) and that are presumably represented in *B. thuringiensis*, defects in the *spo0K* or *spo0B* genes are likely candidates for the EG1351 mutation.

In regard to CryIIIA protein expression, two factors may account for the overproduction of CryIIIA in an asporogenous background. First, the increased accumulation of CryIIIA protein may be a consequence of the terminal stationary phase of an early Spo$^-$ mutant of *B. thuringiensis*. This prolonged stationary phase would allow additional time for gene transcription, protein synthesis, and crystal growth. Our studies seem to exclude altered CryIIIA protein expression during the vegetative phase, as demonstrated by our Western blot analysis of the temporal expression of CryIIIA protein in EG1351 and EG2158 (Fig. 2). Processing of the 73-MDa form of the CryIIIA protein to its 66-MDa form was not altered and occurred during early stationary phase in both strains. Second, induction of sporulation-specific proteases (15, 16, 18), which would not occur in early sporulation mutants, may affect the stability of the CryIIIA protein in wild-type *B. thuringiensis* strains. Our Western blot analysis of CryIIIA production showed a consistent breakdown pattern for the CryIIIA protein in the wild-type strain EG2158 (Fig. 2). In contrast, the CryIIIA protein from the Spo$^-$ strain EG1351 was detected primarily as a full-length protein (Fig. 2). Accordingly, both of these factors likely contribute to the increased accumulation of CryIIIA protein in strain EG1351.

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