Expression in *Bacillus subtilis* of the *Bacillus thuringiensis* *cryIIIA* Toxin Gene Is Not Dependent on a Sporulation-Specific Sigma Factor and Is Increased in a *spo0A* Mutant

HERVÉ AGAISSE^{1*} AND DIDIER LERECLUS^{1,2}

Unité de Biochimie Microbienne, Centre National de la Recherche Scientifique URA 1300, Institut Pasteur, 75724 Paris Cedex 15,¹ and Station de Recherches de Lutte Biologique, Institut National de la Recherche Agronomique, La Minière, 78285 Guyancourt Cedex,² France

Received 12 January 1994/Accepted 27 May 1994

Expression of the *Bacillus thuringiensis* *cryIIIA* gene encoding a *Coleoptera*-specific toxin is weak during vegetative growth and is activated at the onset of the stationary phase. *cryIIIA*'-'*lacZ* fusions and primer extension analysis show that the regulation of *cryIIIA* expression is similar in *Bacillus subtilis* and in *B. thuringiensis*. Activation of *cryIIIA* expression was not altered in *B. subtilis* mutant strains deficient for the σ^H and σ^E sporulation-specific sigma factors or for minor sigma factors such as σ^B , σ^D , or σ^L . This result and the nucleotide sequence of the -35 and -10 regions of the *cryIIIA* promoter suggest that *cryIIIA* expression might be directed by the $E\sigma^A$ form of RNA polymerase. Expression of the *cryIIIA*'-'*lacZ* fusion is shut off after t_2 (2 h after time zero) of sporulation in the *B. subtilis* wild-type strain grown on nutrient broth sporulation medium. However, no decrease in *cryIIIA*-directed β -galactosidase activity occurred in σ^H , *kinA*, or *spo0A* mutant strains. Moreover, β -galactosidase activity was higher and remained elevated after t_2 in the *spo0A* mutant strain. β -Galactosidase activity was weak in *abrB* and *spo0A* *abrB* mutant strains, suggesting that *AbrB* is responsible for the higher level of *cryIIIA* expression observed in a *spo0A* mutant. However, both in *spo0A* and *spo0A* *abrB* mutant strains, β -galactosidase activity remained elevated after t_2 , suggesting that even in the absence of *AbrB*, *cryIIIA* expression is controlled through modulation of the phosphorylated form of *Spo0A*. When the *cryIIIA* gene is expressed in a *B. subtilis* *spo0A* mutant strain or in the 168 wild-type strain, large amounts of toxins are produced and accumulate to form a flat rectangular crystal characteristic of the coleopteran-specific *B. thuringiensis* strains.

For a microbial cell, continued survival in conditions of nutrient stress requires either the expression of alternative metabolic pathways that allow the maintenance of a semiquiescent state or the development of a dormant form such as a spore. In response to such unfavorable conditions, bacteria of the genus *Bacillus* undergo a complex differentiation process that culminates in the formation of endospores. At the onset of sporulation, *Bacillus* spp. produce a wide variety of secondary metabolites, including degradative enzymes, antibiotics, and insecticides. Frequently, the appearance of secondary metabolites is subject to the same regulatory mechanisms that trigger spore formation.

Bacillus thuringiensis is a gram-positive sporulating bacterium producing secondary metabolites (insecticides). During sporulation, most strains of *B. thuringiensis* produce crystal proteins, which are toxic to insects and classified as CryI, -II, -III, and -IV, etc., according to their activity spectra (16, 23). Most *cry* genes are typical examples of sporulation genes specifically expressed in the mother cell compartment. Kinetic studies during sporulation in a *B. thuringiensis* lepidoptera-specific strain, carrying *cryI* genes, indicated that the parasporal inclusion appears in the mother cell at t_2 (2 h after time zero) of sporulation and increases in size until t_{12} (33). Studies of sporulation-negative mutants clearly show that the synthesis of the crystal is developmentally regulated and is dependent on sporulation; *Spo*[−] mutants blocked at t_0 fail to produce crystals

(34). Moreover, expression of the *cryI* gene in *Bacillus subtilis* is sporulation specific (18).

More recently, it was shown that a lepidoptera-specific *cryIA* gene was transcribed by an alternative form of RNA polymerase containing the sigma factor σ^{35} or σ^{28} (7, 8), which are homologous to the *B. subtilis* sporulation-stage-specific sigma factors σ^E and σ^K , respectively (1). In *B. subtilis*, the regulation of transcription of sporulation genes is governed principally by the sequential appearance of four sigma factors: σ^E and σ^K in the mother cell and σ^F and σ^G in the forespore (41). This sigma factor cascade regulates the spatial and temporal expression of the genes implicated in spore formation.

Various *B. thuringiensis* toxin gene promoters have been identified, and their sequences have been determined. Most, including those for the genes *cryIB* (6), *cryIIA* (47), *cryIVA* (49), and *cytA* (44), are similar to the promoters recognized by σ^{35} or σ^{28} (or by σ^E or σ^K , respectively). However, the promoter regions which direct the transcription of the *cryIII* genes differ from those of the other crystal protein genes (2, 10, 11, 37, 38). Using *lacZ* fusions, we have previously shown that the *cryIIIA* gene, unlike the other crystal protein genes, is expressed during vegetative growth and is activated at the onset of sporulation (10). These data suggest that regulation of *cryIIIA* expression could be different from that of the *cry* genes transcribed by $E\sigma^{35}$ and/or $E\sigma^{28}$. Unfortunately, σ^{35} and σ^{28} *B. thuringiensis* mutants are not available, and it is therefore not possible to determine directly whether *cryIIIA* gene expression is dependent on these sigma factors in this organism.

Therefore, we studied the regulation of *cryIIIA* expression in *B. subtilis* (in which many *spo* genes have been characterized).

* Corresponding author. Phone: (1) 45 68 88 12. Fax: (1) 45 68 89 38.

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

Strain	Genotype	Source or reference
168	<i>trpC2</i>	Laboratory stock
MO403	<i>trpC2 pheA1 ΔsigE</i>	40
IS233	<i>trpC2 pheA1 ΔsigH</i>	45
MO712	<i>trpC2 pheA1 spo0A::kan</i>	15
MO845	<i>trpC2 pheA1 kinA::(Tn917 erm)</i>	4
JH12586	<i>trpC2 pheA1 abrB::cat</i>	31
HC1	<i>trpC2 amyE::(cryIIIAH3-P1'-lacZ cat)</i>	pHC1 ^a →168
HC2	<i>trpC2 amyE::(cryIIIAH2-P1'-lacZ cat)</i>	pHC2 ^a →168
HC12E	<i>trpC2 pheA1 amyE::(cryIIIAH2-P1'-lacZ cat) ΔsigE</i>	pHC2 ^a →MO403
HC2H	<i>trpC2 pheA1 amyE::(cryIIIAH2-P1'-lacZ cat) ΔsigH</i>	pHC2 ^a →IS233
HC20A	<i>trpC2 amyE::(cryIIIAH2-P1'-lacZ cat) spo0A::kan</i>	MO712→HC2
HC2kinA	<i>trpC2 amyE::(cryIIIAH2-P1'-lacZ cat) kinA::Tn917 erm</i>	MO845→HC2
HC3	<i>trpC2 amyE::(cryIIIAH2-P1'-lacZ spc)</i>	pCm::Sp ^a →HC2
HC30A	<i>trpC2 amyE::(cryIIIAH2-P1'-lacZ spc) spo0A::kan</i>	MO712→HC3
HC3AbrB	<i>trpC2 amyE::(cryIIIAH2-P1'-lacZ spc) abrB::cat</i>	JH12586→HC3
HC3AbrB0A	<i>trpC2 amyE::(cryIIIAH2-P1'-lacZ spc) spo0A::kan abrB::cat</i>	MO712→HC3AbrB
168P	<i>trpC2 (pHT305P)</i>	pHT305P→168
168P0A	<i>trpC2 spo0A::kan (pHT305P)</i>	MO712→168P

^a Described in text. Arrows indicate construction by transformation.

Using *lacZ* fusion and primer extension analysis, we show that the *cryIIIA* gene displays the same temporally regulated expression pattern in *B. subtilis* as it does in *B. thuringiensis*. The analysis of the regulation of this gene in various *B. subtilis* sporulation mutants is also described.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* K-12 MC1061 [*hsdR mcrB araD139 Δ(araABC-leu)7679 ΔlacX74 galU galK rpsL thi*] (27) was used as the host for plasmid construction. The *B. subtilis* strains used in this study are listed in Table 1. *B. subtilis* was transformed as previously described by using plasmid or chromosomal DNA (3, 19). *cryIIIA*'-'*lacZ* fusions were introduced into *B. subtilis* 168 by integration of plasmids pHC1 or pHC2 (see Fig. 1) at the *amyE* locus. Transformant clones were selected by plating for resistance to chloramphenicol and screening for loss of amylase activity. *B. subtilis* isogenic strains were constructed by transforming strain 168 harboring the *cryIIIA*'-'*lacZ* fusion (HC2) with chromosomal DNA of the sporulation mutants with selection using the appropriate antibiotics (Table 1). *E. coli* strains were grown at 37°C in Luria Broth and were transformed as previously described (21). *B. subtilis* strains were grown at 37°C on HC medium or on nutrient broth sporulation medium (SP medium). HC is a minimal medium (3) supplemented after sterilization with the following nutrients: 0.5% casein hydrolysate, 0.5 mM CaCl₂, 10 μM MnCl₂, and 4.4 mg of ferric ammonium citrate per liter. SP medium contained 8 g of nutrient broth (Difco) per liter, 1 mM MgSO₄, 13 mM KCl, and 10 μM MnCl₂; after sterilization, 4.4 mg of ferric ammonium citrate per liter and 0.5 mM CaCl₂ were added. Antibiotic concentrations for bacterial selection were as follows: ampicillin, 100 μg/ml (for *E. coli* cells); erythromycin, 5 μg/ml; kanamycin, 10 μg/ml; chloramphenicol, 5 μg/ml; and spectinomycin 60 μg/ml (for *B. subtilis* cells).

Plasmids. Plasmid pCm::Sp used to change chloramphenicol resistance into spectinomycin resistance was kindly provided by M. Steinmetz (39). Plasmid pAF1 used for gene replacement at the *amyE* locus of *B. subtilis* was kindly provided by A. Fouet (14). Plasmid pHT7902'*lacZ* (10) was used as the source of DNA fragments carrying the *cryIIIA* expression regions (see Fig. 1). The plasmid vector pHT304 (5) and plasmid pHT305P

carrying the entire *cryIIIA* gene and its expression region (10) were used to assay toxin production in *B. subtilis* (see Fig. 5).

DNA manipulation. Plasmid DNA was extracted from *E. coli* by the standard alkaline lysis procedure. Restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were used as recommended by the manufacturers. The DNA restriction fragments were purified from agarose gels by using the Prep A Gene DNA Purification Matrix kit (Bio-Rad). Chromosomal DNA was extracted from *B. subtilis* cells and was purified as previously described (28). Nucleotide sequences were determined using the dideoxy-chain termination method (35) with double-stranded DNA as the template, a Sequenase version 2.0 kit (U.S. Biochemical Corporation, Cleveland, Ohio), and α-³⁵S-dATP (15 TBq; Amersham, United Kingdom).

RNA extraction and primer extension. *B. subtilis* HC2 (harboring the *cryIIIA*'-'*lacZ* fusion) was grown in HC medium at 37°C with shaking, and the cells were harvested at *t*₃ of sporulation. RNA extraction and primer extension were performed as previously described (10). For the first primer extension experiment (see Fig. 3A), a 39-mer oligonucleotide (3'-CTTAGGCTTGTTAGCTTCACTTGTAATGTTATT TTTG-5') complementary to the 5'-end of the *cryIIIA* gene was synthesized and 5' end labeled with [γ-³²P]ATP (110 TBq/mmol) using T4 polynucleotide kinase. To detect the upstream transcription start site (see Fig. 3B), a second primer extension was performed using a 32-mer oligonucleotide (3'-CAATCTATTCGTAACTCCATCTCAGGCAGGC-5') complementary to the DNA sequence from positions -452 to -420 with respect to the translation start site of *cryIIIA* (nucleotide position +1).

β-Galactosidase assay. *E. coli* and *B. subtilis* strains containing *lacZ* transcriptional fusions were detected by plating on solid medium containing 40 μg of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml and appropriate antibiotics. The cells were cultured in HC or SP medium, and β-galactosidase assays were performed as previously described (28). The specific activities presented (expressed in Miller units) are the means of at least two independent experiments.

Crystal preparation and analysis. *B. subtilis* cells were grown on SP medium at 37°C for 72 h, and the crystals were prepared as previously described (20).

FIG. 1. Construction of plasmids for introducing *cryIIIA'*-*lacZ* fusions at the *amyE* locus of *B. subtilis*. Plasmid pHT7902'*lacZ* (10) was digested to completion with *SacI* and was digested partially with *HindIII*. Appropriate DNA fragments (H3-*SacI* and H2-*SacI*) were purified from agarose gels and subcloned in pAF1 (14) digested with *SacI* and *HindIII* to give plasmids pHCl and pHc2, respectively. Arrows indicate the directions of transcription of the different genes. H2 and H3 indicate the positions of the *HindIII* sites in the expression region of *cryIIIA* as previously described (10), and the black boxes represent the upstream regions of *cryIIIA*.

RESULTS

***cryIIIA* expression is identical in *B. subtilis* and in *B. thuringiensis*.** To study the temporal regulation of the *cryIIIA* gene in *B. subtilis*, a plasmid containing the *cryIIIA'*-*lacZ* promoter fusion (pHc2) was constructed (Fig. 1) and introduced into *B. subtilis* by transformation of competent cells. The *B. thuringiensis* DNA included in this fusion extends from a *HindIII* site located 1542 bp upstream of the transcriptional start to a *PstI* site located at codon 50 of *cryIIIA*. Integration of the fusion at the *amyE* locus, giving the strain *B. subtilis* HC2, was obtained by selecting for chloramphenicol resistance and by screening for loss of amylase activity. Cells were grown in HC medium, and β -galactosidase activity was measured at various stages of growth (Fig. 2). Enzyme production was relatively low during the vegetative phase of growth and rose from about 10,000 U/mg of protein at t_0 to about 40,000 U/mg of protein at t_6 . These levels of production and the kinetics of expression in strain HC2 are very similar to those in *B. thuringiensis* strains harboring the same fusion on a low-copy-number plasmid (10), suggesting that the temporal regulation of *cryIIIA* is similar in *B. thuringiensis* and in *B. subtilis*.

It was previously shown that the 1-kb H2-H3 fragment located 400 bp upstream of the *cryIIIA* promoter is required for high-level production of CryIIIA in sporulating *B. thuringiensis* cells (10). Deletion of this DNA fragment from the *cryIIIA'*-*lacZ* fusion resulted in a decrease of β -galactosidase activity of about 50-fold. To confirm this effect in *B. subtilis*, plasmid pHCl harboring the *cryIIIA'*-*lacZ* promoter fusion without the H2-H3 fragment was constructed (Fig. 1) and introduced into *B. subtilis*, giving strain HC1. In this case, the upstream end of *B. thuringiensis* DNA corresponds to the *HindIII* site located 563 bp upstream of the translational start point. The cells were grown in HC medium, and β -galactosidase activity was measured at various stages of growth (Fig. 2); activity was very low during vegetative growth and rose from about 100 U/mg of protein at t_0 to about 600 U/mg of protein at t_6 , confirming that the transcriptional enhancing effect of the

FIG. 2. β -Galactosidase activities of *B. subtilis* 168 strains carrying *cryIIIA'*-*lacZ* fusions in the absence (strain HC1) or presence (strain HC2) of the H2-H3 DNA fragment. *B. subtilis* cells were grown in HC medium at 37°C. t_0 indicates the end of the exponential phase. t_n is the number of hours before (–) or after time zero.

H2-H3 fragment was similar in *B. subtilis* and in *B. thuringiensis*.

The 5' end of the *cryIIIA* mRNA was previously determined by S1 mapping and primer extension analysis and was mapped to a site located 129 bp upstream of the translation start site, apparently defining the *cryIIIA* promoter (10, 38). However, we have recently provided evidence that the 5' end of this transcript (nucleotide position –129) is not a transcriptional start site and is probably generated through a posttranscriptional event (2). Furthermore, we have shown that the essential promoter involved in full expression is located in the H2-H3 fragment. This promoter is responsible for the initiation of a transcript with a 5' end at nucleotide position –558 (2).

To determine whether this promoter is also active in *B. subtilis*, total RNAs isolated at t_3 from strain HC2 were subjected to primer extension using synthetic oligonucleotide primers corresponding to positions +3 to +41 or –452 to –420 (see Materials and Methods). Consistent with our previous results obtained using *cryIIIA* mRNA from *B. thuringiensis*, 5' ends corresponding to positions –129 and –558 were detected (Fig. 3A and B). As in *B. thuringiensis*, the major signal was the one at nucleotide position –129 and minor upstream signals (Fig. 3A) corresponded to processed forms of the long transcript initiated at nucleotide position –558 (Fig. 3B).

Taken together, these data indicate that *cryIIIA* gene expression and regulation are similar in *B. thuringiensis* and in *B. subtilis*, justifying the use of this model system to investigate the mechanisms governing postexponential expression of *cryIIIA*.

***cryIIIA* transcription is not dependent on sporulation-specific sigma factors in *B. subtilis*.** Two sigma factors homologous to the sporulation-stage-specific σ^E and σ^K of *B. subtilis* were recently isolated from *B. thuringiensis* and characterized (1). Whiteley et al. have demonstrated by genetic analysis in *B. subtilis* that *cryIA(a)* is efficiently transcribed by $E\sigma^E$ and $E\sigma^K$ in *B. subtilis* (46).

The wild-type strain HC2 was allowed to sporulate in SP medium. The *cryIIIA*-directed synthesis of β -galactosidase was relatively low during the vegetative phase, increased at t_0 to reach a maximum of about 12,000 U/mg of protein at t_2 , and decreased thereafter. This temporal pattern of expression is

FIG. 3. Determination of the transcription start sites of *cryIII α* in *B. subtilis*. *B. subtilis* HC2 was grown in HC medium, and total RNA was extracted at t_3 . RNA was subjected to primer extension as described in Materials and Methods and analyzed by electrophoresis (lanes 1). The same synthetic oligonucleotides were used to prime dideoxy sequencing reactions from a single-stranded DNA template which contained the region of interest (lanes A, C, G, and T). Arrows indicate the 5' extremities numbered with respect to the translational start site of *cryIII α* . (A) Primer extension from the 39-mer oligonucleotide complementary to the 5' end of the *cryIII α* gene; (B) primer extension from the 32-mer oligonucleotide complementary to the DNA sequence from nucleotide positions -452 to -420.

consistent with transient, sporulation-specific activation. To test whether activation of the *cryIII α* gene at the onset of sporulation was dependent on the sporulation-stage-specific σ^E factor (like the *cryI* genes), the *cryIII α '-lacZ* fusion (pHC2) was introduced into the σ^E mutant strain MO403, giving strain HC2E. The level and the kinetics of expression were identical in the mutant strain HC2E and in the wild-type strain HC2 (Fig. 4A), suggesting that *cryIII α* is not transcribed by $E\sigma^E$.

An alternative form of RNA polymerase containing the σ^H sigma factor directs transcription of several genes activated at the onset of sporulation (Fig. 4A). The *cryIII α '-lacZ* fusion (pHC2) was therefore introduced into the σ^H mutant strain MO806, giving strain HC2H. Expression of *cryIII α '-lacZ* fusion in this strain showed that activation of *cryIII α* transcription was not dependent on *spo0H* (Fig. 4A). Moreover, in

strain HC2H, β -galactosidase activity was elevated and prolonged during the stationary phase after t_2 .

Furthermore, similar patterns of *cryIII α '-lacZ* fusion expression were observed in the wild-type strain HC2 and in σ^B , σ^D , and σ^L mutants (data not shown). Transcription of *cryIII α* is therefore not directed uniquely by a form of RNA polymerase containing any of these alternative sigma factors in *B. subtilis*.

Activation of *cryIII α* does not require Spo0A. Spo0A is a key transcriptional factor controlling the transcription of genes involved in the initial stages of sporulation. To examine whether the activation of *cryIII α* expression is dependent on Spo0A, the disrupted *spo0A::kan* gene was introduced into strain HC2, giving strain HC20A. Strain HC20A was grown in SP medium and assayed for *cryIII α* -directed β -galactosidase activity at various stages of growth (Fig. 4B). Activation of *cryIII α* expression was not dependent on *spo0A*. Expression increased at the same stage as that in the wild-type cells; however, the level was two- to threefold higher. The β -galactosidase activity remained high during the stationary phase, in contrast to the wild-type strain, in which production decreased after t_2 .

The activated form of Spo0A is the phosphorylated form, Spo0A~P, and phosphorylation of Spo0A is accomplished through the phosphorelay initiated by the activity of two kinases, KinA and KinB (9, 43). To test whether the *kinA* gene is implicated in the regulation of *cryIII α* expression, the disrupted *kinA::(Tn917 erm)* was introduced into strain HC2 to give strain HC2kinA. β -Galactosidase production by strain HC2kinA was assayed in SP medium at various stages of growth (Fig. 4B). β -Galactosidase production in strain HC2kinA was not higher than that in the wild-type strain HC2; however, it did not decrease after t_2 .

Effect of AbrB on *cryIII α* expression in wild-type and *spo0A* mutant strains. To examine the role of AbrB in regulating *cryIII α* expression, we first replaced the Cm^r cassette of the *cryIII α '-lacZ* fusion with a Sp^r cassette conferring resistance to spectinomycin (the selectable marker for the *abrB* insertional mutant, Cm^r , was identical to the selectable marker of the *cryIII α '-lacZ* fusion previously used).

Steinmetz and Richter constructed the pCm::Sp plasmid by inserting a Sp^r cassette into the pC194 Cm^r gene of plasmid pC177 (39). This vector can be used to replace in vivo a pre-existing chloramphenicol resistance gene with a spectinomycin resistance gene. Linearized pCm::Sp was introduced into strain HC2 by transformation. Replacement of the Cm^r cassette was obtained by selecting for spectinomycin resistance and screening for loss of chloramphenicol resistance. The resulting *B. subtilis* strain bearing a chromosomal *cryIII α '-lacZ* was designated HC3. The level of *cryIII α* -directed β -galactosidase synthesis was lower in strain HC3 than in strain HC2 (Fig. 4C), probably because of day-to-day variations in the preparation of SP medium. However, the kinetics of activation were identical in both strains; synthesis of β -galactosidase was relatively low during the vegetative phase, increased at t_0 to reach a maximum at t_2 , and decreased thereafter. To confirm the effect of Spo0A, the disrupted *spo0A::kan* gene was introduced in strain HC3, giving strain HC30A. As expected, *cryIII α* -directed β -galactosidase production in a *spo0A* mutant increased at the same stage as that in the wild-type cells (HC3); however, the level was two- to threefold higher and remained high after t_2 (Fig. 4C).

The disrupted *abrB::cat* gene was introduced into strain HC3, giving strain HC3AbrB. The strain HC3AbrB displayed an overall β -galactosidase activity that was twofold lower than that of the wild-type HC3 strain, suggesting a positive role of

FIG. 4. β -Galactosidase activities of *B. subtilis* mutants carrying the *cryIIIA'*-*lacZ* fusion. *B. subtilis* cells were grown in SP medium at 37°C. Time zero indicates the end of the exponential phase. t_n is the number of hours before (–) or after time zero. The β -galactosidase activity profiles of wild-type *B. subtilis* 168 carrying the *cryIIIA'*-*lacZ* fusion with the *cat* gene as selectable marker (strain HC2) are shown in panels A and B for comparison. (A) β -Galactosidase activity profiles of sigma factor-deficient strains (Δ *sigE* strain HC2E; Δ *sigH* strain HC2H); (B) β -galactosidase activity profiles of sporulation-deficient strains [*spo0A::kan* strain HC20A; *kinA::*(Tn917 *erm*) strain HC2kinA]; (C) β -galactosidase activity profiles of wild-type *B. subtilis* 168 carrying the *cryIIIA'*-*lacZ* fusion with the *spc* gene as selectable marker (strain HC3) and mutant strains (*spo0A::kan* strain HC30A; *abrB::cat* strain HC3AabrB; *spo0A::kan abrB::cat* strain HC30AabrB).

AbrB in *cryIIIA* expression (Fig. 4C). Strains HC3 and HC3AabrB were cultured in parallel on the same day in the same preparation of SP medium to minimize day-to-day variations.

Since Spo0A~P negatively regulates expression of *abrB* (31), it was therefore hypothesized that the increased expression of *cryIIIA* in a *spo0A* mutant was due to the increased level of AbrB in this genetic background. To test this hypothesis, we constructed a double *spo0A abrB* mutant. The disrupted *spo0A::kan* gene was introduced into strain HC3AabrB, giving strain HC3AabrB0A. *cryIIIA*-directed β -galactosidase production in a *spo0A abrB* double mutant (strain HC3AabrB0A) was not higher than that in a single *abrB* mutant (strain HC3AabrB) but did not decrease after t_2 . However, β -galactosidase production in a *spo0A abrB* double mutant (strain HC3AabrB0A) was strongly reduced compared with the production in a *spo0A* mutant (strain HC30A), confirming the positive role played by AbrB in *cryIIIA* expression.

CryIIIA production in *B. subtilis* wild-type and *spo0A* mutant strains. *B. thuringiensis* HD1 CryB[–] harboring the plasmid pHT305P carrying the *cryIIIA* gene produces 73- and 67-kDa *Coleoptera*-specific toxins (10); the 67-kDa polypeptide results from the processing of the 73-kDa CryIIIA protein.

To confirm the genetic analysis of the regulation of *cryIIIA* expression in *B. subtilis*, we tested whether it was possible to produce CryIIIA crystal protein in wild-type *B. subtilis* and in the *spo0A* mutant. pHT305P was introduced into *B. subtilis* 168, giving strain 168P, and the corresponding *spo0A* mutant was constructed by introducing the disrupted *spo0A::kan* gene into strain 168P, giving the strain 168P0A. Both strains 168P and 168P0A were allowed to sporulate on SP plates and produced large amounts of the flat rhomboidal crystals that are characteristic of *B. thuringiensis* strains active against coleopteran larvae. Spore-crystal preparations were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 5). Both strains produced large amounts of CryIIIA; strain 168P contained more of the 67-kDa (processed) form, whereas strain 168P0A contained mainly the 73-kDa (unprocessed) form.

DISCUSSION

In this study, we show that the regulation of *cryIIIA* expression in *B. subtilis* is similar to that in *B. thuringiensis*. As monitored by *cryIIIA'*-*lacZ* fusions, *cryIIIA* gene expression is appropriately temporally regulated in *B. subtilis* in the following order: relatively weak expression during vegetative growth which is followed by activation at the onset of the stationary phase in HC medium, with continued expression at least until t_6 . As demonstrated by the deletion of the 1-kb H2-H3 fragment and primer extension analysis (mapping of a 5' end at nucleotide position –558), the essential promoter involved in the full expression of *cryIIIA* is likely to be the same in *B.*

FIG. 5. Protein analysis of *B. subtilis* transformants expressing the *cryIIIA* gene. Spore-crystal preparations were analyzed by 0.1% SDS-10.5% PAGE gel and Coomassie blue staining as previously described (22). Lanes: 1, molecular weight markers (from top to bottom, 97, 66, 60, 43, and 30 kDa); 2, *B. subtilis* 168 carrying the plasmid vector pHT304; 3, *B. subtilis* *spo0A::kan* strain carrying plasmid pHT305P; 4, *B. subtilis* 168 carrying plasmid pHT305P. Identical volumes of spore-crystal preparation (20 μ l) were loaded in wells 2, 3, and 4. Lane 5, 3 μ g of CryIIIA protein purified from the *B. thuringiensis* strain HD1 CryB[–] harboring pHT305P (10). Arrowheads indicate the 73- and 67-kDa crystal components.

subtilis and in *B. thuringiensis*. Moreover, primer extension analysis shows that the same major form of cryIII α mRNA with a 5' end at nucleotide position -129 is found in both species. We have recently shown that this major form probably results from the processing of the cryIII α mRNA initiated at nucleotide position -558, generating a stable transcript with a 5' end at nucleotide position -129 (2). Thus, it is likely that this posttranscriptional event occurs both in *B. subtilis* and in *B. thuringiensis*. All of these data suggest that the expression of cryIII α is similarly regulated in *B. thuringiensis* and in *B. subtilis*.

To identify the sigma factor implicated in the regulation of cryIII α transcription, we introduced the cryIII α '-lacZ promoter fusion into various mutants deficient for sigma factors in *B. subtilis*. The activation of the cryIII α '-lacZ fusion was not dependent in any of the sigma factor-deficient strains tested (σ^E , σ^H , σ^B , σ^D , and σ^L). This was surprising, in view of the σ^E -like and/or σ^K -like dependent transcription of other cry genes. However, the putative -10 and -35 sequences of the essential promoter identified in the DNA region involved in the full expression of cryIII α are not similar to the consensus sequences recognized by the *B. thuringiensis* E σ^{35} or E σ^{28} form of RNA polymerase but instead resemble those recognized by the E σ^A form of RNA polymerase (2).

The activation observed at the onset of the stationary phase may be due to the appearance of a stationary-phase-specific activator. Because Spo0A~P is a key regulator at the onset of stationary phase and activates the expression of various sporulation-stage-specific genes transcribed by E σ^A (36, 48), we tested the expression of cryIII α in a spo0A mutant. We have shown that the activation of cryIII α expression is not dependent on Spo0A. Indeed, in a spo0A mutant, β -galactosidase activity was elevated and remained high after t_2 in SP medium. This could be explained by the absence of degradation of β -galactosidase in the spo0A mutant, which produces low levels of proteases during stationary phase. The absence of the 67-kDa processed form of CryIII α protein in the spo0A mutant strain harboring the pHT305P (cryIII α gene) is consistent with this. However, although the kinA mutant overproduces proteases (30), cryIII α expression did not decrease in this genetic background. It is thus likely that repression of the cryIII α promoter activity after t_2 in SP medium is mediated by the phosphorylated form of Spo0A produced by the activity of KinA. The absence of shutoff in the σ^H mutant could be therefore explained by a defect in Spo0A synthesis, since E σ^H stimulates the transcription of spo0A during sporulation (32). The prevalent role of the phosphorelay (via Spo0A~P) in the shutoff of cryIII α expression (both in *B. subtilis* and in *B. thuringiensis*) is strengthened by the result presented in the second accompanying paper (24); a *B. thuringiensis* strain defective in the phosphorylation of Spo0A (spo0F mutant) overproduces CryIII α during stationary phase. Moreover, in the first accompanying paper (25), the authors show that another Spo $^-$ *B. thuringiensis* strain overproducing CryIII α is complemented for the Spo $^+$ phenotype by a kinase protein.

It is likely that the initiation of, or an early event in, sporulation turns off cryIII α expression. It is noteworthy that the temporal pattern of cryIII α expression is dissimilar in HC medium and in sporulation medium (compare Fig. 2 and 4). The event responsible for the shutoff of cryIII α expression observed at t_2 in SP medium might be absent or delayed in HC medium. This type of shutoff depending on spo0A has been described for at least two other genes expressed early during sporulation: *gsiA* (29) and *phoA* (17). Expression of these genes is increased in a spo0A mutant, and a second mutation in *abrB* does not suppress the enhancing effect of the spo0A

mutation. However, unlike *gsiA* and *phoA*, expression of cryIII α in a spo0A *abrB* double mutant is no longer increased. These data and the results obtained with an *abrB* single mutant suggest that AbrB acts as a positive regulator of cryIII α expression. AbrB is well known as a member of the so-called transition state regulator family (42). During vegetative growth, these regulators act as repressors to prevent expression of genes implicated in the production of secondary metabolites such as degradative enzymes and antibiotics (12, 26). However, AbrB can also regulate the expression of regulators or components in alternative metabolic pathways as an activator. A positive effect of AbrB has been described for the expression of at least two genes: *hpr* (42) and *hut* (13). Whether the positive role of AbrB in the expression of *hpr* is direct is unclear. However, it has recently been shown that AbrB positively regulates the expression of *hut* by competing with the *hut* catabolite repressor for binding to the *hutO*_{CR2} operator (13). Regarding cryIII α expression, it is not known whether the effect of AbrB is direct.

The respective roles of Spo0A and AbrB in cryIII α expression are thus still unclear. However, it is noteworthy that cryIII α -directed β -galactosidase synthesis does not decrease after t_2 both in a spo0A single mutant and in a spo0A *abrB* double mutant, compared with those in the parental wild-type and *abrB* single mutant strains, respectively. Thus, the shutoff of cryIII α depends on spo0A in both the wild-type and the *abrB* genetic background. This suggests that Spo0A~P acts in two ways concerning cryIII α expression: (i) Spo0A~P negatively modulates the expression of cryIII α by repressing *abrB*, whose product is apparently positively involved in cryIII α expression; and (ii) Spo0A~P is involved in the shutoff of cryIII α after t_2 in SP medium in an *abrB*-independent fashion.

In conclusion, activation of cryIII α expression appears to be independent of the key genes involved in the initiation of sporulation (key regulators or sigma factors). It differs in this respect from other cry genes. The utilization of *B. thuringiensis* as a biopesticide entails the dissemination of large amounts of spores into the environment. An obvious implication of our study is that it should be possible to produce high levels of CryIII α toxin in a Spo $^-$ genetic background. It is also possible to express other cry genes using the cryIII α promoter region in a Spo $^-$ *B. thuringiensis* genetic background. Nonsporulating and consequently nonpersisting strains could thereby be constructed for release into the environment.

ACKNOWLEDGMENTS

We are grateful to Georges Rapoport, in whose laboratory this work was conducted, and to Tarek Msadek and Frank Kunst for many helpful discussions on the stationary phase of *B. subtilis*. We thank D. Dubnau, A. Grossman, J. Hoch, C. Karmazyn-Campelli, I. Smith, and P. Stragier for the generous gift of *B. subtilis* mutant strains. We thank M. Steinmetz for the generous gift of plasmid pCm::Sp. We gratefully acknowledge Myriam Gomet for her assistance with β -galactosidase assays and James Baum and Thomas Malvar for providing results before publication.

This work was supported by research funds from the Institut Pasteur, the Institut National de la Recherche Agronomique, the Centre National de la Recherche Scientifique, and Roussel Uclaf. H. Agaisse was supported by a fellowship from the Ministère de l'Éducation Nationale, and D. Lereclus was supported by a grant from INRA.

REFERENCES

- Adams, L. F., K. L. Brown, and H. R. Whiteley. 1991. Molecular cloning and characterization of two genes encoding sigma factors that direct transcription from a *Bacillus thuringiensis* crystal protein gene promoter. *J. Bacteriol.* 173:3846-3854.

2. Agaisse, H., and D. Lereclus. 1994. Structural and functional analysis of the promoter region involved in the full expression of the *cryIIIA* toxin gene of *Bacillus thuringiensis*. Mol. Microbiol., in press.
3. Anagnostopoulos, C., and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. 81:741-746.
4. Antoniewski, C., B. Savelli, and P. Stragier. 1990. The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. J. Bacteriol. 172:86-93.
5. Arantes, O., and D. Lereclus. 1991. Construction of cloning vectors for *Bacillus thuringiensis*. Gene 108:115-119.
6. Brizzard, B. L., H. E. Schnepf, and J. W. Kronstad. 1991. Expression of the *cryIB* crystal protein gene of *Bacillus thuringiensis*. Mol. Gen. Genet. 231:59-64.
7. Brown, K. L., and H. R. Whiteley. 1988. Isolation of a *Bacillus thuringiensis* RNA polymerase capable of transcribing crystal protein genes. Proc. Natl. Acad. Sci. USA 85:4166-4170.
8. Brown, K. L., and H. R. Whiteley. 1990. Isolation at the second *Bacillus thuringiensis*, RNA polymerase that transcribes from a crystal protein gene promoter. J. Bacteriol. 172:6682-6688.
9. Burbulys, D., K. A. Trach, and J. A. Hoch. 1991. Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell 64:545-552.
10. De-Souza, M. T., M.-M. Lecadet, and D. Lereclus. 1993. Full expression of the *cryIIIA* toxin gene of *Bacillus thuringiensis* requires a distant upstream DNA sequence affecting transcription. J. Bacteriol. 175:2952-2960.
11. Donovan, W. P., M. J. Rugar, A. C. Slaney, T. Malvar, M. C. Gawron-Burke, and T. B. Johnson. 1992. Characterization of two genes encoding *Bacillus thuringiensis* insecticidal crystal proteins toxic to *Coleoptera* species. Appl. Environ. Microbiol. 58:3921-3927.
12. Ferrari, E., A. S. Jarnagin, and B. F. Schmidt. 1993. Commercial production of extracellular enzymes, p. 917-937. In A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
13. Fisher, S. H., M. A. Strauch, M. R. Atkinson, and J. L. W. Wray. 1994. Modulation of *Bacillus subtilis* catabolite repression by transition state regulatory protein AbrB. J. Bacteriol. 176:1903-1912.
14. Fouet, A., and A. L. Sonenshein. 1990. A target for carbon source-dependent negative regulation of the *citB* promoter of *Bacillus subtilis*. J. Bacteriol. 172:835-844.
15. Gonzy-Tréboul, G., C. Karmazyn-Campelli, and P. Stragier. 1992. Developmental regulation of transcription of the *Bacillus subtilis* *ftsAZ* operon. J. Mol. Biol. 224:967-979.
16. Höfte, H., and H. R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53:242-255.
17. Jensen, K. K., E. Sharkova, M. F. Duggan, Y. Qi, A. Koide, J. A. Hoch, and F. M. Hulett. 1993. *Bacillus subtilis* transcription regulator, *spo0A*, decreases alkaline phosphatase levels induced by phosphate starvation. J. Bacteriol. 175:3749-3756.
18. Klier, A., F. Fargette, J. Ribier, and G. Rapoport. 1982. Cloning and expression of the crystal protein genes from *Bacillus thuringiensis* strain *berliner* 1715. EMBO J. 1:791-799.
19. Kunst, F., T. Msadek, and G. Rapoport. 1994. Signal transduction network controlling degradative enzyme synthesis and competence in *Bacillus subtilis*, p. 1-19. In P. Piggot (ed.), Regulation of bacterial differentiation. American Society for Microbiology, Washington, D.C.
20. Lecadet, M.-M., J. Chauvaux, J. Ribier, and D. Lereclus. 1992. Construction of novel *Bacillus thuringiensis* strains with different insecticidal specificities by transduction and by transformation. Appl. Environ. Microbiol. 58:840-849.
21. Lederberg, E. M., and S. N. Cohen. 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. 119:1072-1074.
22. Lereclus, D., O. Arantes, J. Chauvaux, and M.-M. Lecadet. 1989. Transformation and expression of a cloned δ -endotoxin gene in *Bacillus thuringiensis*. FEMS Microbiol. Lett. 60:211-218.
23. Lereclus, D., A. Delécluse, and M.-M. Lecadet. 1993. Diversity of *Bacillus thuringiensis* toxins and genes, p. 37-69. In P. Entwistle, J. S. Cory, M. J. Bailey, and S. Higgs (ed.), *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. John Wiley & Sons, Ltd., New York.
24. Malvar, T., and J. A. Baum. 1994. Tn5401 disruption of the *spo0F* gene, identified by direct chromosomal sequencing, results in *CryIIIA* overproduction in *Bacillus thuringiensis*. J. Bacteriol. 176:4750-4753.
25. Malvar, T., C. Gawron-Burke, and J. A. Baum. Overexpression of *Bacillus thuringiensis* HknA, a Histidine Protein Kinase Homolog, bypasses early *Spo*⁻ mutations that result in *CryIIIA* overproduction. J. Bacteriol. 176:4742-4749.
26. Marahiel, M. A., M. M. Nakano, and P. Zuber. 1993. Regulation of peptide antibiotic production in *Bacillus*. Mol. Microbiol. 7:631-636.
27. Meissner, P. S., W. P. Sisk, and M. L. Berman. 1987. Bacteriophage λ cloning system for the construction of directional cDNA libraries. Proc. Natl. Acad. Sci. USA 84:4171-4175.
28. Msadek, T., F. Kunst, D. Henner, A. Klier, G. Rapoport, and R. Dedonder. 1990. Signal transduction pathway controlling synthesis of a class of degradative enzymes in *Bacillus subtilis*: expression of the regulatory genes and analysis of mutations in *degS* and *degU*. J. Bacteriol. 172:824-834.
29. Mueller, J. P., and A. L. Sonenshein. 1992. Role of the *Bacillus subtilis* *gsiA* gene in regulation of early sporulation gene expression. J. Bacteriol. 174:4374-4383.
30. Perego, M., S. P. Cole, D. Burbulys, K. Trach, and J. A. Hoch. 1989. Characterization of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins *Spo0A* and *Spo0F* of *Bacillus subtilis*. J. Bacteriol. 171:6187-6196.
31. Perego, M., G. B. Spiegelman, and J. A. Hoch. 1988. Structure of the gene for the transition state regulator, *abrB*: regulator synthesis is controlled by the *spo0A* sporulation gene in *Bacillus subtilis*. Mol. Microbiol. 2:689-699.
32. Predich, M., G. Nair, and I. Smith. 1992. *Bacillus subtilis* early sporulation genes *kinA*, *spo0F* and *spo0A* are transcribed by the RNA polymerase containing σ^H . J. Bacteriol. 174:2771-2778.
33. Ribier, J., and M.-M. Lecadet. 1973. Etude ultrastructurale et cinétique de la sporulation de *Bacillus thuringiensis* var. *berliner* 1715. Remarques sur la formation de l'inclusion parasporale. Ann. Microbiol. (Inst. Pasteur) 124A:311-344.
34. Ribier, J., and M.-M. Lecadet. 1981. *Bacillus thuringiensis* var. *Berliner* 1715. Isolement et caractérisation de mutants de sporulation. C.R. Acad. Sci. Paris (Série III) 292:803-808.
35. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
36. Satola, S. W., J. M. Baldus, and C. P. Moran. 1992. Binding of *Spo0A* stimulates *spoIIIG* promoter activity in *Bacillus subtilis*. J. Bacteriol. 174:1448-1453.
37. Sekar, V. 1988. The insecticidal crystal protein gene is expressed in vegetative cells of *Bacillus thuringiensis* var. *tenebrionis*. Curr. Microbiol. 17:347-349.
38. Sekar, V., D. V. Thompson, M. J. Maroney, R. G. Bookland, and M. J. Adang. 1987. Molecular cloning and characterization of the insecticidal crystal protein gene of *Bacillus thuringiensis* var. *tenebrionis*. Proc. Natl. Acad. Sci. USA 84:7036-7040.
39. Steinmetz, M., and R. Richter. 1994. Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through *in vivo* recombination. Gene 142:79-83.
40. Stragier, P., C. Bonamy, and C. Karmazyn-Campelli. 1988. Processing of a sporulation sigma factor in *Bacillus subtilis*: how morphological structure could control gene expression. Cell 52:697-704.
41. Stragier, P., and R. Losick. 1990. Cascades of sigma factors revisited. Mol. Microbiol. 4:1801-1806.
42. Strauch, M. A., and J. A. Hoch. 1993. Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. Mol. Microbiol. 7:337-342.
43. Trach, K. A., and J. A. Hoch. 1993. Multisensory activation of the phosphorelay initiating sporulation in *Bacillus subtilis*: identification and sequence of the protein kinase of the alternate pathway. Mol. Microbiol. 8:69-79.
44. Ward, E. S., and D. J. Ellar. 1986. *Bacillus thuringiensis* var. *israelensis* δ -endotoxin. Nucleotide sequence and characterization

- of the transcripts in *Bacillus thuringiensis* and *Escherichia coli*. J. Mol. Biol. **191**:1–11.
45. Weir, J., E. Dubnau, N. Ramakrishna, and I. Smith. 1984. *Bacillus subtilis* *spo0H* gene. J. Bacteriol. **157**:405–412.
46. Whiteley, H. R., H. E. Schnepf, K. L. Brown, and W. R. Widner. 1990. Regulation of *Bacillus thuringiensis* crystal protein gene promoters in sporulating *Bacillus subtilis*, p. 201–210. In M. M. Zukowski, A. T. Ganesan, and J. A. Hoch (ed.), Genetics and biotechnology of bacilli, vol. 3. Academic Press, San Diego, Calif.
47. Widner, W. R., and H. R. Whiteley. 1989. Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* possess different host range specificities. J. Bacteriol. **171**:965–974.
48. York, K., T. J. Kenney, S. Satola, C. P. Moran, H. Poth, and P. Youngman. 1992. Spo0A controls the sigma A-dependent activation of *Bacillus subtilis* sporulation-specific transcription unit *spoIIIE*. J. Bacteriol. **174**:2648–2658.
49. Yoshisue, H., T. Fukada, K.-I. Yoshida, K. Sen, S.-I. Kurosawa, H. Sakai, and T. Komano. 1993. Transcriptional regulation of *Bacillus thuringiensis* subsp. *israelensis* mosquito larvicidal crystal protein gene *cryIV_A*. J. Bacteriol. **175**:2750–2753.