Genetic analysis of cryIIIA gene expression in Bacillus thuringiensis

Sylvie Salamitou, Hervé Agaisse, Alejandra Bravo and Didier Lereclus

The Bacillus thuringiensis (Bt) cryIIIA gene is regulated by a different mechanism from that of most of the other cry genes. Its expression begins during late-exponential growth and not during sporulation as for the other classes of cry genes. Moreover, in Bacillus subtilis, cryIIIA expression is independent of the major sporulation-specific sigma factors and is increased in a spoOA genetic background. We used lacZ fusions and primer-extension analysis to follow the time-course of cryIIIA transcription in Bt wild-type and in various Spo- genetic backgrounds (spoOA, sigE and sigK). cryIIIA was activated from the end of vegetative growth to stage II of sporulation (t1) in the wild-type strain. Thereafter, transcription from the same promoter continued, at a decreasing rate, until the end of stage III. In the spoOA mutant strain, the same promoter was activated for at least 15 h during the stationary phase. cryIIIA activation in the sigK genetic background was similar to that in the wild-type but was extended in a sigE mutant strain. Thus cryIIIA expression in Bt is not directly dependent on the major sporulation-specific sigma factors. Furthermore, an event linked with the σE-dependent period of sporulation ends cryIIIA activation, although transcription of this gene does not switch off before the end of stage III.

Keywords: cryIIIA, Bacillus thuringiensis, gene expression, stationary phase, sporulation mutants

INTRODUCTION

The Gram-positive sporulating bacterium Bacillus thuringiensis (Bt) produces a variety of insecticidal proteins toxic to major crop pests and disease vectors. These proteins accumulate in the cytoplasm of the cells during the stationary phase or sporulation to form crystalline inclusions which can account for 25% of the dry weight of the cells. At the end of the sporulation process, crystals and spores are liberated concomitantly by lysis of the cells (for a review, see Agaisse & Lereclus, 1995; Baum & Malvar, 1995).

Sporulation in Bacillus subtilis is temporally and spatially regulated by a cascade of transcription factors in which six sigma factors are sequentially activated. They are σA, the primary vegetative-phase sigma factor, and five sporulation-specific factors, σH, σF and σG, active before septation, σK, active in the forespore compartment, and σE, active in the mother cell. σE is active from stage II (formation of the asymmetric septum in the mother cell) to stage IV (cortex formation), when σK becomes active (Errington, 1993). Ultrastructural studies showed that the various stages defined for B. subtilis sporulation can also be identified in Bt (Ribier & Lecadet, 1973; Bechtel & Bulla, 1976).

The various crystal proteins have been classified as CryI, CryII, CryIII and CryIV according to their activity spectra and sequence similarities (Höfte & Whiteley, 1989). Transcription of most of the cry genes is dependent on the sporulation-specific sigma factors σH, σK and σE, respectively (Brown & Whiteley, 1988, 1990; Adams et al., 1991). Promoters similar to those recognized by the sporulation sigma factors have been found upstream from many cry genes by sequence analysis (Ward & Ellar, 1986; Widner & Whiteley, 1989; Brizzard et al., 1991; Yoshisue et al., 1993, 1995).

The promoter region and the regulation of cryIIIA differ from those of the other cry genes (Agaisse & Lereclus, 1996).
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant genotype or plasmid characteristics*</th>
<th>Reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<td>407 Cry'</td>
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<td>Lereclus et al. (1989)</td>
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<td>407-SigE'</td>
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<td>Bravo et al. (1996)</td>
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<td>407-SigK'</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pH7304-18Z</td>
<td>Derived from pH7304; Em'</td>
<td>Agaisse &amp; Lereclus (1994b)</td>
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<tr>
<td>pHT7830</td>
<td>Derived from pH7304-18Z; cryIIIA-lacZ</td>
<td>Lereclus et al. (1994b)</td>
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<tr>
<td>pHTspoIID</td>
<td>Derived from pH7304-18Z; spoIID-lacZ</td>
<td>Bravo et al. (1996)</td>
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<tr>
<td>pHTcotA</td>
<td>Derived from pH7304-18Z;  cysA-lacZ</td>
<td>Bravo et al. (1996)</td>
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* kan, kanamycin; Em', erythromycin resistant.

1994b): cryIII A is expressed during vegetative growth and is activated at the onset of the stationary phase (Sekar, 1988; De-Souza et al., 1993; Agaisse & Lereclus, 1994a; Malvar et al., 1994). Moreover, experiments in B. subtilis sporulation mutants using lacZ fusions demonstrated that expression of cryIII A is not dependent on a sporulation-specific sigma factor and is increased in a spo0A genetic background (Agaisse & Lereclus, 1994a). This was confirmed by constructing a spo0A mutant of Bt and introducing the cryIII A gene in this strain: large amounts of CryIII A toxin were produced (Lereclus et al., 1995). Similarly, a spo0F mutant of Bt slightly overproduces CryIII A (Malvar & Baum, 1994).

CryIII A crystal synthesis and sporulation, which occur concomitantly in the mother cell, are regulated by different mechanisms. It is therefore of interest to investigate the interactions between the two processes. cryIII A activation during the transition phase between vegetative phase and sporulation has been thoroughly studied (De-Souza et al., 1993; Agaisse & Lereclus, 1994b; Malvar & Baum, 1994). In contrast, little information is available about the regulation of cryIII A expression at later stages of sporulation; in particular, when cryIII A transcription ends is unknown.

The cryIII A promoter region is located unusually far upstream from the translational start codon of the gene, i.e. between nucleotides -635 and -553 (Agaisse & Lereclus, 1994b). Thus, transcription of cryIII A leads to a large transcript with a 5' extremity at nucleotide position -588. However, this transcript is transformed into a stable form, the 5' extremity of which is nucleotide -129 (Agaisse & Lereclus, 1994b). Earlier work on the temporal regulation of cryIII A expression in the wild-type and in the spo0F Bt strains dealt essentially with the timing of appearance of the stable form of cryIII A mRNA (De-Souza et al., 1993; Malvar & Baum, 1994), and therefore does not necessarily represent the timing of cryIII A transcription.

The progress of sporulation in wild-type Bt was followed using sporulation reporter genes, and transcription of cryIII A was timed with respect to the different stages of the sporulation process using lacZ fusions and primer-extension analysis devised for detection of the transcription start site. The cryIII A transcription was shown to end during stage III of sporulation in wild-type Bt. The effect of various blocks in the sporulation process on cryIII A expression was then investigated using Bt mutant strains (spo0A, sigE and sigK disruptions). The results are consistent with the hypothesis that cryIII A is transcriptionally dependent on σ^8.

METHODS

Bacterial strains, plasmids and media. All strains and plasmids used are listed in Table 1. Bt strains were grown at 30 °C with shaking (150 r.p.m.) in Luria Broth (LB) or in nutrient broth sporulation medium (SP medium). SP medium contained 8 g nutrient broth (Difco) 1^-1, 1 mM MgSO_4, 13 mM KCl and 10 μM MnCl_2; after sterilization, 44 mg ferric ammonium citrate 1^-1 and 0.5 mM CaCl_2 were added.

Erythromycin was used at 5 μg ml^-1 and kanamycin was used at 200 μg ml^-1 for Bt. Bt was transformed as described previously by Lereclus et al. (1989).

To synchronize Bt cells grown in SP medium, cultures were started from frozen vegetative cells previously grown in selective LB medium. Samples from the cultures were centrifuged and stored frozen at -20 °C until used for β-galactosidase assays.

Synchronization of cell growth was checked by monitoring the development of sporulation as follows. Culture aliquots were heated at 80 °C for 20 min and plated on selective LB medium. By comparison with unheated samples similarly plated, the fraction of thermoresistant spores in the cell population was determined. Less than 1% of the cell population was thermoresistant at t₀ (t₀ meaning x hours after the beginning of the stationary phase), 27% was thermoresistant at t₁ and 100% at t₂. These results are in agreement with the results obtained using Bt cells grown in HCT medium by Ribier & Lecadet (1973). Due to the large size of Bt cells, it is possible to determine sporulation stage by phase-contrast microscopy. At
about \( t_0 \), the brown engulfed prespore (characteristic of stage III of sporulation) is clearly visible in the mother cell. At \( t_0 \), the prespores turn whiter (stage IV and V), reaching a maximum of whiteness at about \( t_1 \). At this time, thermoresistance is acquired, indicating that sporogenesis is complete (stage VI). At around \( t_2 \), spore liberation begins.

**β-Galactosidase assays.** Frozen cell pellets were thawed in Z buffer (Miller, 1972) and disrupted by sonication (2 × 30 s sonication at 4 °C with an intervening 30 s pause) using a Branson sonifier model 250. Cell debris was eliminated by centrifugation, and β-galactosidase activity was assayed as previously described by Miller (1972). Protein concentrations were determined using Bradford reagent with bovine serum albumin as standard. Specific activities are expressed in modified Miller units (Perego & Hoch, 1988). Results presented are means of at least two independent experiments.

RNA extraction and primer extension. Cells (5 ml) were pelleted by centrifugation (6000 r.p.m.; 10 min; 4 °C) and immediately stored frozen at −70 °C. Pellets were resuspended in 5 ml distilled water treated with diethyl pyrocarbonate. RNA was extracted as previously described by Glatron & Rapoport (1972). The cryIII-A transcription start site was determined by primer extension using as primer a 32-mer oligonucleotide (5'-CAAATCTCATTCGCTAAACCTCCAATTCAGGGCAGGC-3'), complementary to the DNA sequence from positions −452 to −420 with respect to the translation start site of cryIII-A (nucleotide position +1). The 5' end was labelled with \([\beta^{32P}]ATP\) (110 TBq mmol⁻¹) using T4 polynucleotide kinase.

Nucleotide sequences were determined using the dideoxy chain-termination method (Sanger et al., 1977) with double-stranded DNA as a template, a Sequenase version 2.0 kit (US Biochemical) and \([\alpha^{35S}]dATP\) (15 TBq; Amersham).

**RESULTS**

**Temporal regulation of cryIII-A expression in sporulating Bt cells**

The timing of cryIII-A expression in sporulating cells was followed with respect to that of σ^K and σ^K activity. In *B. subtilis*, the promoters of the spoIID and cotA genes are transcriptionally dependent on the σ/E and σ/K factors, respectively (Rong et al., 1986; Sandman et al., 1988). In *Bt*, the respective transcriptional dependency of these two promoters has been confirmed using σ^K and σ^K mutant strains (Bravo et al., 1996). σ^K activity, and thus the corresponding period of sporulation in *Bt*, were monitored using a transcriptional fusion of the spoIID promoter region to the lacZ reporter gene (plasmid pHsrolloID; Table 1). Similarly, σ^K activity was followed using a transcriptional fusion of the cotA promoter to the lacZ reporter gene (plasmid pHsrotocA; Table 1). cryIII-A expression was monitored using pH77830, which carries a transcriptional fusion of the complete promoter region of cryIII-A to the lacZ reporter gene (Table 1). All three plasmids were derived from pH304-182Z; the background β-galactosidase activity obtained with this plasmid is very low in *Bt* (< 10 U (mg protein)⁻¹) from \( t_2 \) to \( t_0 \) (Agaisse & Lereclus, 1994b).

The three plasmids were introduced into *Bt*, strain 407 Cry−. Cells were grown in SP medium and assayed for β-galactosidase production (Fig. 1). The β-galactosidase specific activity curve of the spoIID-lacZ fusion was bell-shaped and the maximum activity was at \( t_0 \) under the conditions of the experiment. A similar curve, displaying a maximum, has also been reported for *B. subtilis* (Rong et al., 1986). This indicates that lacZ was expressed from a transiently activated promoter and that β-galactosidase was not stable in the mother-cell compartment during sporulation. As assessed from the activity of the spoIID-lacZ fusion, σ^K was active from \( t_0 \) to \( t_1 / t_2 \). At that time, σ^K became active, as deduced from the β-galactosidase activity of the cotA-lacZ fusion. These data are in agreement with those deduced from microscopic examination, according to which \( t_0 \) corresponds to the end of stage III, and with the timing of activity of σ^K during stage II and III of sporulation in *B. subtilis* (Dricks & Losick, 1991).

In *Bt* cells harbouring pH77830, cryIII-A-mediated lacZ expression increased from late-exponential phase (\( t_2 \)) to \( t_0 \), remained constant for about 4 h, and declined thereafter. The activation of cryIII-A at the end of exponential growth to early sporulation is in close agreement with previous reports (De-Souza et al., 1993; Agaisse & Lereclus, 1994b; Malvar & Baum, 1994). The plateau in β-galactosidase specific activity beyond \( t_0 \) indicates that cryIII-A transcripts continued to be translated after \( t_0 \), such that β-galactosidase synthesis was equal to degradation. Indeed, if there was no synthesis, β-galactosidase specific activity would drop rapidly as in the case of the spoIID-lacZ fusion after \( t_0 \). These results suggest that there are two phases in cryIII-A expression in *Bt* cells: an activation period which ends concomitantly with the turning on of σ^K and a period during which cryIII-A expression is maintained (roughly until σ^K becomes functional in the mother-cell compartment, i.e. stage IV.

![Fig. 1. Timing of cryIII-A expression in wild-type Bt. Bt strain 407 Cry− harbouring pH77830 (cryIII-A-lacZ transcriptional fusion; [○]), pHsrolloID (σ^K-dependent lacZ fusion; [●]) or pHsrotocA (σ^K-dependent lacZ fusion; [□]) was grown in SP medium at 30 °C. Cells were assayed for β-galactosidase activity at various times. \( t_0 \) indicates the end of the exponential growth; \( t_0 \) is the number of hours before (−) or after (+) time zero.](image-url)
Fig. 2. Time-course of cryllA transcription in the wild-type Bt strain (407) and in the spo0A mutant strain (407-OA). (a) Equal amounts of total RNA (30 µg) were used for primer extension analysis for each time point. The same synthetic oligonucleotide (see Methods) was used to prime dideoxy sequencing reactions from a double-stranded pH7830 DNA template (lanes A, C, G, T). (b) The intensity of each band was determined using a Phosphorimager (Molecular Dynamics).


cryllA transcription is extended in a Bt spo0A genetic background

We investigated the transcription of cryllA by primer-extension analysis. The primer chosen was specific for the 5’ extremity at nucleotide −558 of the cryllA transcripts (see Methods). The activation of cryllA transcription at the onset of the stationary phase has already been studied in wild-type Bt strain (Agaisse & Lereclus, 1994b). We therefore looked at cryllA transcription from t3 to the end of sporulation, i.e. t15. As cryllA expression is increased in an spo0A genetic background of B. subtilis (Agaisse & Lereclus, 1994a), we also investigated the time-course of cryllA transcription in a spo0A mutant strain of Bt. mRNA was extracted from Bt cells sampled 3, 6, 9, 12 and 15 h after the onset of the stationary phase and the cryllA transcripts were characterized by primer-extension analysis in the wild-type strain (407) and in the spo0A mutant strain (407-OA) harbouring pH7830 (Fig. 2).

mRNA with the 5’ end at position −558 with reference to the translation start codon (Agaisse & Lereclus, 1994b) was detected from t3 to t6 during the sporulation process in the wild-type Bt strain (Fig. 2a). The primer-extension signals were quantified with a Phosphorimager (Fig. 2b). Transcription decreased from t6 onwards, and the signal could not be detected at t12 and t15. However, the signal intensity at t6 (about 50% of that at t3) and the β-galactosidase activity indicate that a large amount of cryllA transcript was still present in the cells at that time. These observations suggest that cryllA transcription continues during stage III of sporulation in Bt, even if it slows down after t5/t6.

The 5’ extremity corresponding to the cryllA promoter was also identified throughout the stationary phase in the spo0A mutant strain (Fig. 2a), demonstrating the ability of this promoter to function in Bt without any sporulation-dependent sigma factors. It is thus presumably responsible for the CryllA production previously observed in Spo~ mutant strains of Bt (Malvar & Baum, 1994; Malvar et al., 1994; Lereclus et al., 1995).

In the spo0A genetic background, the signal obtained by primer extension remained roughly constant from t3 to t15 (Fig. 2b). cryllA-directed β-galactosidase synthesis was
assessed hourly (Fig. 3). The accumulation of β-galactosidase up to $t_{10}$ correlated well with the constant quantity of cryIII-A transcripts. Addition of rifampicin to the cultures at $t_{10}$ led to a rapid ($< 5$ min) disappearance of the signal corresponding to the 5' end at nucleotide $-558$ of the cryIII-A-lacZ transcripts (data not shown). This demonstrated that these cryIII-A transcripts were not unusually stable, and that indeed the cryIII-A promoter was still functional at $t_{10}$ in the spo0A mutant background.

The rate of β-galactosidase production in the spo0A mutant strain was similar to that in the wild-type strain until $t_5$. After $t_5$, β-galactosidase specific activity reached a maximum in the wild-type strain whereas it continued to increase at the same rate in the spo0A mutant strain. This produced a much higher level of β-galactosidase synthesis in the spo0A mutant strain [about 55000 U (mg protein)$^{-1}$] than in the wild-type [10000 U (mg protein)$^{-1}$].

cryIII-A expression in sigE and sigK sporulation mutants of Bt

cryIII-A transcription is maintained in wild-type Bt roughly until $\sigma^K$ is turned on. To assess whether the absence of $\sigma^K$ prolonged cryIII-A expression, we introduced pHT7830 into the sigK strain of Bt, and cryIII-A-directed β-galactosidase synthesis in this genetic background was compared to that of the wild-type (Fig. 4). The patterns of cryIII-A-directed β-galactosidase synthesis in the two strains were not significantly different, suggesting that the turn off of cryIII-A transcription in wild-type Bt is not dependent on the turning on of $\sigma^K$.

The other prominent aspect of cryIII-A expression in wild-type Bt is that the activation of this gene ends concomitantly with the appearance of an active $\sigma^K$ in the cell. This effect disappears in a spo0A genetic background in which the whole sporulation transcription factor cascade is interrupted. We investigated the effect of a sigE disruption on cryIII-A expression in Bt. In this genetic background, cryIII-A-directed β-galactosidase synthesis increased from the late-exponential phase of growth to $t_5$, when it reached a maximum of about 27000 U (mg protein)$^{-1}$. From the end of vegetative growth to $t_6$, the rate of β-galactosidase accumulation in the sigE strain was similar to that in the wild-type strain, but it continued increasing in the mutant strain until $t_7$.

cryIII-A expression was not negatively affected in $\sigma^K$ or $\sigma^K$ mutant strains, indicating that cryIII-A is not significantly transcribed by these sporulation-specific sigma factors. Unlike the situation in B. subtilis, where cryIII-A expression was similar in the wild-type and in the sigE strains (Agaisse & Lereclus, 1994a), a block in sporulation at $\sigma^K$-dependent stages led to extended cryIII-A expression in Bt. This is consistent with the presence of a $\sigma^K$-dependent negative regulator in wild-type Bt which may repress cryIII-A expression at the transcriptional level. The prolonged transcription in the spo0A strain is also consistent with this hypothesis. However, the finding that the maximum β-galactosidase activity level is higher in the spo0A strain than in the sigE strain suggests that there is an additional effect in the spo0A strain to that mediated by $\sigma^K$. Indeed, as sigE activation is dependent on Sp0A (Satola et al., 1992; York et al., 1992), the pattern of cryIII-A expression in the spo0A and in the sigE mutant strains should be identical if the spo0A interruption effect was merely due to the absence of $\sigma^K$.

**DISCUSSION**

We show here that cryIII-A is transcribed from stage II to stage IV by the promoter previously identified between positions $-635$ and $-553$ (Agaisse & Lereclus, 1994b).
The cryIII A promoter has already been shown to be activated at the end of vegetative growth (De-Souza et al., 1993; Agaisse & Lereclus, 1994b; Malvar & Baum, 1994). Thus, two distinct periods can be distinguished in cryIII A expression: an activation which ends during stage II (concomitantly with the turning on of σE), and a period during which transcription is maintained, albeit at a slower rate, until stage IV of sporulation. The cryIII A promoter is thus functional in Bt from the end of the vegetative phase until the middle of sporulation.

Transcription by this promoter is not dependent on a sporulation-specific sigma factor, as previously reported for B. subtilis (Agaisse & Lereclus, 1994a). Progression of Bt cells beyond stage II of sporulation even had a negative effect on cryIII A transcription. Indeed, in the spo0A and sigE mutant strains, cryIII A expression undergoes the same activation as in the wild-type strain but this activation does not end at tso, and transcription remains high during the stationary phase in the spo0A genetic background. In contrast, the absence of σA has no significant effect on cryIII A expression. These results could indicate a shut off of cryIII A activation caused by a σE-dependent negative regulator. A similar transcriptional turn off, σE-dependent in this case, has been proposed for the amyE gene. In this case, it was observed that B. subtilis spo0A strains continued producing α-amylase after tso at the rate observed between tso and tso, in contrast with the wild-type strain (Weickert et al., 1990).

However, as the patterns of cryIII A expression in the spo0A and in the sigE strains are not alike, this explanation does not appear to be sufficient. Another hypothesis accounting for the different patterns of transcription of the cryIII A promoter, depending on the genetic backgrounds used, could be the regulation of the sigma factor which transcribes cryIII A. The putative -35 and -10 regions of the cryIII A promoter are similar to promoters recognized by the EσA form of RNA polymerase (Agaisse & Lereclus, 1994b). Our observations, in particular the prolonged transcription by the cryIII A promoter in a sporulation-deficient background, are in agreement with the expression of a σE-dependent gene. However, it cannot be ruled out that cryIII A is under the transcriptional dependency of an unknown sigma factor, active during the stationary phase. Were cryIII A σA-dependent, the appearance of an activator or disappearance of a repressor (such as the transition-state regulators in B. subtilis; Strauch & Hoch, 1993) would be responsible for the activation of cryIII A at the end of the exponential growth phase. Mutagenesis experiments are in progress to identify this hypothetical regulator.

No information relevant to the time-course of σA expression in Bt is available. In B. subtilis, the presence of an active σA at stages of sporulation later than the period during which σA is active, is controversial (Linn et al., 1973; Tjian & Losick, 1974; Carter et al., 1988; Qi & Døi, 1990; for a review, see Haldenwang, 1995). However, aprE mRNA, directing the synthesis of B. subtilis subtilisin, the transcription of which is dependent on σA, was reported to be present 5 h after the onset of the stationary phase (Park et al., 1989). These results suggest that a σA-dependent transcription could occur in cells at late stages of the sporulation process. In the case of cryIII A expression, the dependence of transcription on σA is yet to be confirmed.

ACKNOWLEDGEMENTS

We are grateful to Georges Rapoport in whose laboratory this work was conducted. We thank Evelyne Bégaud for her help with Bt culture and β-galactosidase assays, Alex Edelman for the English corrections, and Frank Kunst and Georges Rapoport for critical reading of the manuscript. 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 Agrevo. S. Salamitou and H. Agaisse were supported by a grant from Agrevo. A. Bravo was supported by a fellowship from CONACyT and DGAPA/UNAM.

REFERENCES


Glatron, M. F. & Rapoport, G. (1972). Biosynthesis of the parasporal inclusion of Bacillus thuringiensis: half-life of its corresponding messenger RNA. Biochimie 54, 1291-1301.


Received 13 February 1996; revised 3 April 1996; accepted 12 April 1996.