Spo0A represses transcription of the cry toxin genes in *Bacillus thuringiensis*

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The DNA regions upstream from the genes encoding polypeptides of *Bacillus thuringiensis* subsp. *israelensis* larvicidal crystals (cry4A, cry4B, cry11A) contain sequences with similarities to the spo0A box of *Bacillus subtilis* (or '0A' box) and the promoter recognized by the σ^H^-associated RNA polymerase of *B. subtilis*. Expression of cry–lacZ transcriptional fusions was analysed in various *B. thuringiensis* genetic backgrounds. The early transcription of the toxin genes was not sporulation-dependent, whereas the late-stage expression at t₅₄ was σ^H^-dependent. Primer extension analysis confirmed that the cry4- and cry11-type toxin genes were weakly transcribed during the transition phase; expression analysis of a cry11A'–lacZ transcriptional fusion in *B. subtilis* sporulation mutants confirmed the involvement of the σ^H^-RNA polymerase. Primer extension analysis showed that in *B. thuringiensis* subsp. *israelensis*, the cry4A and cry11A gene transcription observed at the end of the growth stage was turned off at the beginning of the sporulation phase. The DNA region located upstream from the cry11A gene promoter including the putative 'OA' box was deleted. This led to a derepression of the expression of the cry11A operon. These results suggest that the cry4A, cry4B and cry11A toxin genes of *B. thuringiensis* subsp. *israelensis* are transcribed during the transition phase by the RNA polymerase associated with the σ^H^-factor and are subject to Spo0A repression.

**Keywords:** *Bacillus thuringiensis*, cry toxin genes, Spo0A, transcription

**INTRODUCTION**

*Bacillus thuringiensis* subsp. *israelensis* produces complex composite inclusions highly toxic to mosquito and blackfly larvae. The larvicidal specificity of each of the four major crystal components (Cry4A, Cry4B, Cry11A and Cry11A) has been characterized. The high toxicity of the inclusions is due in part to synergy between these components (Delcluse et al., 1993; Poncet et al., 1995a). The corresponding genes have been cloned and sequenced from a 72 MDa resident plasmid. Each of these four genes is expressed during the sporulation phase (for a review, see Porter et al., 1993).

In *Bacillus subtilis*, two transcriptional regulators, Spo0H (or σ^H^-factor) and Spo0A, are required for the onset of sporulation. σ^H^-is involved in the initiation of the sporulation process in *B. subtilis*, but its precise role has not been yet defined. However, it is known to be involved in post-exponential phase gene transcription (for a review, see Haldenwang, 1995). The spo0A gene is transcribed from two promoters recognized by the RNA polymerase associated with σ^H^- and σ^K^- factors. The regulatory protein Spo0A plays a key role in the initiation of sporulation (Baldus et al., 1994). The phosphorylated form interacts with its DNA target (5' TGTTCGAA 3') and thereby represses one set of genes and activates other sporulation-specific genes. After the start of sporulation, the activation of a series of sigma factors, which bind to the core RNA polymerase, allows a time- and compartment-specific regulation of sporulation genes (for reviews, see Haldenwang, 1995; Baldus et al., 1994; Errington, 1993).

The temporal and compartmental gene regulation during the sporulation process in *B. thuringiensis* is very similar to that in *B. subtilis*, and the Spo0A proteins of *B. thuringiensis* and *B. subtilis* are homologous (Lereclus et al., 1994). Moreover, genes for two sporulation sigma factors have been isolated and sequenced from *B. thuringiensis*: the σ^K^- and σ^K^- factors [homologous to the mother-cell-specific σ^K^- and σ^K^- factors of *B. subtilis*].
respectively (Adams et al., 1991). More recently, a gene encoding a Spo0F-like protein has been characterized in B. thuringiensis (Malvar & Baum, 1994).

Most known crystal protein genes of B. thuringiensis are under the control of sporulation-specific sigma factors. In B. thuringiensis, the cry1Aa gene is transcribed during the sporulation phase from two sporulation-specific promoters (Wong et al., 1983; Brown & Whiteley, 1988, 1990). In B. thuringiensis subsp. israelensis, the cry4A, cry4B, cry11A and cyt1A genes are transcribed during mid-sporulation from promoters recognized by the σ^H–RNA polymerase; moreover, transcription of the cyt1A and cry11A genes continues into late-sporulation from promoters recognized by the σ^B–RNA polymerase (Waalwijk et al., 1985; Ward & Ellar, 1986; Yoshisue et al., 1993, 1994; Dervyn et al., 1995).

We compared DNA regions located upstream from each of the toxin genes to identify putative conserved sequences possibly involved in the regulation of the four toxin genes in B. thuringiensis subsp. israelensis. We found sequences similar to the consensus -10 and -35 boxes recognized by the σ^H–RNA polymerase in B. subtilis. Thus, B. thuringiensis subsp. israelensis toxin genes may also be transcribed early during sporulation. Expression studies and deletion analysis were performed to identify the role of these sequences in toxin gene regulation. cry4A, cry4B and cry11A were weakly transcribed during the transition phase, probably under the control of the RNA polymerase associated with a σ^H-like factor, and the cry11A operon was negatively regulated by Spo0A.

**METHODS**

**Bacterial strains and media.** Total RNA was extracted from B. thuringiensis subsp. israelensis strain 4Q2-72, which harbours the 72 MDA resident plasmid encoding all the crystal proteins. The acrystalliferous isogenic strain 4Q2-81, B. thuringiensis strains 407-0A (spo0A::kan) (Lereclus et al., 1994) and B. thuringiensis 407-sigE (sigE::kan) (Bravo et al., 1996) were used as recipient strains for transformation experiments; they are derived from strain 407 (serotype I). B. thuringiensis subsp. israelensis strains 4Q2-72 and 4Q2-81 were kindly provided by D. H. Dean, Ohio State University, Columbus, OH, USA. B. thuringiensis strains were transformed by electroporation (Lereclus et al., 1989) and transformants selected on Luria Broth (LB) plates containing erythromycin (10 μg ml^-1^).

**Escherichia coli** K-12 strain TG1 (Δ(lac–proAB) supE thi hsd D5 F^tra D36 proA^ proB^ lacP lacZ ΔM15) was used for plasmid construction. Transformants were selected on LB plates supplemented with ampicillin (100 μg ml^-1^).

**B. subtilis** strains FBT17, FBT20 and FBT21 were constructed to investigate the role of σ^H^ and AbrB in the regulation of the cry11A operon (Table 1). The spo0H strain IS233 (Weir et al., 1984) was transformed with chromosomal DNA from strain FBT11, giving strain FBT17. As the *abrB* strain JH12586 (Perego et al., 1988) is chloramphenicol-resistant, chromosomal cry11A–lacZ cat DNA could not be used for studying the role of AbrB. Plasmid pBT11 was therefore constructed as follows. Plasmid pBT10 (Dervyn et al., 1995) was digested with EcoRI and SacI. The resulting DNA fragment was inserted between the EcoRI and SacI sites in pAC7 (Weinrauch et al., 1991) giving pBT11, which carries a cry11A–lacZ kan DNA fragment. pBT11 was used to transform B. subtilis strain 168, and strain FBT20 was obtained through integration of the cry11A–lacZ kan DNA fragment at the *amyE* locus by homologous recombination. Chromosomal DNA from strain FBT20 was used to transform strain JH12586, giving strain FBT21 (Table 1). Recombinants were selected on LB plates supplemented with erythromycin (0.3 μg ml^-1^), kanamycin (10 μg ml^-1^) or chloramphenicol (10 μg ml^-1^).

**B. thuringiensis** strains were grown at 37 °C in LB medium or at 30 °C in HCT medium (Lecadet et al., 1980). *E. coli* was grown at 37 °C in LB medium. **B. subtilis** was grown at 37 °C in nutrient broth sporulation medium (SP medium) containing 8 g nutrient broth (Difco) 1\(^{-1}\), 1 mM MgSO\(_4\), 13 mM KCl and 10 μM MnCl\(_2\); after sterilization, 4.4 mg ferric ammonium citrate 1\(^{-1}\), and CaCl\(_2\) to 0.5 mM were added.

**Plasmids.** pHT643, which carries the *p19*, cry11A and p20 genes, has already been described (Dervyn et al., 1995; see Fig.

### Table 1. B. subtilis strains used in this study and expression of cry11A–lacZ transcriptional fusion at t^-15^

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
<th>β-Galactosidase activity*</th>
</tr>
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<tbody>
<tr>
<td>168</td>
<td><em>trpC2</em></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>JH12586</td>
<td><em>trpC2 pheA1 aabrB::cat</em></td>
<td>Laboratory stock</td>
<td>ND</td>
</tr>
<tr>
<td>IS233</td>
<td><em>trpC2 pheA1 sigH</em></td>
<td>Perego et al. (1988)</td>
<td>ND</td>
</tr>
<tr>
<td>FBT11</td>
<td><em>trpC2 amyE::(cry11A–lacZ cat)</em></td>
<td>Weir et al. (1984)</td>
<td>ND</td>
</tr>
<tr>
<td>FBT20</td>
<td><em>trpC2 amyE::(cry11A–lacZ kan)</em></td>
<td>This work</td>
<td>4 (3–6)</td>
</tr>
<tr>
<td>FBT21</td>
<td><em>trpC2 pheA1 amyE::(cry11A–lacZ kan) abrB::cat</em></td>
<td>This work</td>
<td>90 (85–95)</td>
</tr>
</tbody>
</table>

ND, Not determined.

* Values shown are the means of four to eight independent experiments and are expressed in Miller units (mg protein)^-1\(^{-1}\). Numbers in parentheses are 95% confidence limits as determined by probit analysis.
which carries a 184 bp deletion in front of the PI promoter -35 box (see Fig. 1a).

pHT691 (containing the cry4A'–lacZ fusion), pHT692 (containing the cry4B'–lacZ fusion) (Delecse et al., 1993) and pHT693 (containing the cry11A'–lacZ fusion) (Dervyn et al., 1995) have already been described.

pHT694, carrying a transcriptional fusion between the cry11A operon promoter deleted for the putative 'OA' box and the lacZ gene, was obtained as follows. pHT643AOA was hydrolysed with Syl, blunt-ended with Klenow fragment, then digested with HindIII. The resulting DNA fragment carrying the modified cry11A promoter region was inserted into pHT304-182 (Agaissé & Lereclus, 1994) cut with BamHI, blunt-ended with Klenow fragment and restricted with HindIII.

**DNA manipulation.** Plasmid DNA was extracted from *E. coli* by the standard alkaline lysis procedure (Birnboim & Doly, 1979). Restriction enzymes and Klenow fragment were used as recommended by the manufacturers (Amersham and Boehringer Mannheim). Chromosomal DNA was extracted from *B. subtilis* as previously described (Msadek et al., 1990).

PCRs were carried out by using the thermostable *Thermus aquaticus* DNA polymerase as recommended by the supplier (Amersham), using oligonucleotides synthesized by Genset.

The GCG program (Genetics Computer Group, University of Wisconsin, Madison, WI, USA) was used for computer analysis.

**RNA extraction and primer extension.** Total RNA was extracted from *B. thuringiensis* cells grown in HCT medium as previously described (Glaton & Rapoport, 1972). Elongation of the radioactive DNA primers and analysis of the products were performed as previously described (Calogero et al., 1994). DNA sequences were determined by the dideoxy chain-termination method (Sanger et al., 1977) with a Sequenase version 2.0 kit (US Biochemical) and [α-35S]dATPαS (15 TBq; Amersham).

The specific primer used to detect the cry4A transcript was 5'-TGACCCCTTATGATTTACCTCTCC 3' (complementary to nucleotides +56 to +80 of the cry4A transcript (Yoshisue et al., 1993); the primer used to detect the cry4B transcript was 5'-CATACGTCTCGAAGATTACAG-3' (corresponding to primer 2, Yoshisue et al., 1994); the probe used to detect the cry11A-specific transcript has been described previously (Dervyn et al., 1995).

**β-Galactosidase assays.** *B. subtilis* strains containing the cry11A'–lacZ fusion and *B. thuringiensis* strains containing either the cry11A', Δ0cry11A'-, cry4A' or cry4B'–lacZ fusions were grown in SP or HCT medium, respectively, supplemented with appropriate antibiotics. β-Galactosidase was assayed as follows. *B. thuringiensis* cultures were concentrated twofold in Z buffer (Miller, 1972) and disrupted by ultrasonic disintegration (for 1 min with a Branson sonifier at 30% duty cycle). Cell debris was eliminated by centrifugation at 7000 g for 10 min, and the β-galactosidase activity in the supernatant was determined as described by Miller (1972). *B. subtilis* cultures were tested for β-galactosidase activity as previously described (Msadek et al., 1990).

**RESULTS**

**DNA sequence comparison**

The DNA sequences of the cry4A, cry4B and cry11A promoter regions were compared. No extensive similarities were found. However, local DNA sequence
Fig. 2. Sequence comparison of putative ‘OA’ boxes from B. thuringiensis with the consensus ‘OA’ box from B. subtilis (a) and alignment of DNA sequences matching consensus promoters recognized by either $\sigma^A$ or $\sigma^H$ in B. subtilis (b). (a) Sequence comparison of putative SpoOA-binding boxes of B. thuringiensis subsp. israelensis cry4A, cry4B and cry11A toxin genes with the consensus B. subtilis ‘OA’ box. Identical nucleotides are boxed. The position of each box is given with respect to the nearest corresponding start point. (b) Alignment of nucleotide sequences from promoter regions of the cry4-type and cry11A genes from B. thuringiensis subsp. israelensis. The nucleotides which match the consensus sequence of the $\sigma^A$ recognition site of B. subtilis are boxed. The underlined nucleotides correspond to those which match the promoter consensus recognized by $\sigma^H$ in B. subtilis. The asterisks designate the nucleotides corresponding to the 5' end of the mRNA. For cry4A and cry11A, the same transcriptional start points were detected at $t_2$ ($\sigma^A$-dependent, see Fig. 3a, c, lanes $t_2$) and in mid-sporulation ($\sigma^A$-dependent, this work and data not shown; Yoshisue et al., 1995; Dervyn et al., 1995). For cry4B, the transcriptional start point detected in the 407-OA and the 4Q2-72 strains (this work, bold letter with arrow) was five nucleotides upstream from that determined by Yoshisue et al. (1994) in sporulating cells (asterisk).

Negative regulation of the toxin genes at the onset of the sporulation process

The $\sigma^H$-associated RNA polymerase transcribes genes during the transition phase in B. subtilis; the regulatory protein SpoOA interacts with both $\sigma^A$- and $\sigma^H$-dependent promoters, acting as either a negative or a positive effector of gene expression (Baldus et al., 1994, 1995). Primer extension experiments were performed using total RNA extracted from B. thuringiensis with primers specific for cry4A or cry4B and a probe specific for cry11A, respectively. The cry4A and cry11A genes were transcribed at the end of the exponential phase (Fig. 3a, c, lanes $t_2$, where $t_n$ denotes $n$ hours after the start of sporulation). No vegetative transcription of the cry4A and cry11A genes was detected at the beginning of the sporulation (Fig. 3a, c, lanes $t_0$) or in mid-sporulation ($\sigma^A$-dependent, see Fig. 3a, c, lanes $t_2$). No cry4B transcript was detected at $t_2$ in wild-type cells (strain 4Q2-72). Primer extension experiments were performed on RNA extracted from 407-OA(pHT692) cells, containing a cry4B′- lacZ transcriptional fusion. The cry4B′-lacZ mRNA was detected in this spoOA background (Fig. 3b, lane $t_2$; the 5' end of this transcript corresponded to the 5' end of the cry4B′ transcript detected at $t_2$ in the wild-type strain 4Q2-72 (Fig. 3b, lane $t_2$). The failure to detect the cry4B transcript in the wild-type background may therefore be due to the sensitivity level rather than to the absence of the corresponding transcript in strain 4Q2-72. Yoshisue et al. (1995) previously showed that in B. subtilis cry4A is recognized by $\sigma^H$. By analogy with B. subtilis, $\sigma^H$ may transcribe the B. thuringiensis subsp. israelensis cry genes, and SpoOA may repress cry gene transcription through interaction with the putative ‘OA’ boxes. The ‘OA’ box located upstream from the cry11A operon was therefore investigated. We tested the level of
Repression of cry11A by Spo0A in B. thuringiensis

**Fig. 3.** Reverse transcriptase mapping of the transcriptional start sites of the cry4A (a), cry4B (b) and cry11A (c) genes. (a, c) Total RNA was extracted from B. thuringiensis subsp. israelensis strain 4Q2-72 (harbouring the 72 MDa plasmid encoding all the crystal protein genes). Cells were grown in HCT medium and harvested 2 h before the beginning of the sporulation phase (lane t-) and at the beginning of sporulation (lane t0) as previously described; (b) total RNA was extracted from strain 407-0A(pHT692) at t- and from strain 4Q2-72 at t0 (lanes t- and t0, respectively). Extension products are designated by arrows. Lengths of the cry4A and cry11A extension products were determined by comparison with the sequence obtained by sequencing the M13mp18 ssDNA; the position of the cry4B mRNA 5' end is given with respect to the sequence of the corresponding region from pHT692 (containing a cry4B'-lacZ fusion).

**Fig. 4.** Mapping of the cry11A gene start site after deletion of the upstream region, including the putative ‘OA’ box. Primer extension experiments were performed as described in Fig. 3. The extension product corresponding to the 5' end of the cry11A transcript shown in Fig. 3(c) is shown by an arrow.

expression of transcriptional cry'-lacZ fusions in sporulation mutants of B. thuringiensis to characterize the temporal regulation of the toxin genes.

**Effect of deletion of the putative Spo0A box on cry11A transcription**

The role of the putative ‘OA’ box located upstream from the cry11A gene promoter was tested by deletion analysis. Plasmid pH643Δ0A is a derivative of pH643 with a 184 bp deletion upstream from the -35 box of the cry11A PI promoter (Fig. 1). It was introduced by electroporation into the B. thuringiensis subsp. israelensis acrystalliferous strain 4Q2-81. Its transcriptional start site PI was active at both t- and t0 (Fig. 4). The 5' end of the Δ0Acry11A transcript was the same as that identified for the cry11A operon (Fig. 3c, lane t0), although two lower bands were detected, which may correspond to additional promoters or to reverse transcriptase pause sites. Thus the putative ‘OA’ box or a sequence in the 184 bp deleted segment is responsible for the cry11A transcriptional repression at the onset of the sporulation process. Similar amounts of Cry11A inclusion were produced in the presence or in the absence [strains 4Q2-81(pHT643) and 4Q2-81(pHT643Δ0A), respectively] of the putative Spo0A box as assessed by SDS-PAGE (data not shown).

**Time course of β-galactosidase expression in B. thuringiensis**

Plasmids pH691 (cry4A'-lacZ, Fig. 5a), pH692 (cry4B'-lacZ, Fig. 5b) and pH693 (cry11A'-lacZ, Fig. 5c) were introduced into B. thuringiensis strains 4Q2-81, 407-0A and 407-sigE. The strains were cultured in HCT medium at 30 °C; aliquots were harvested at different stages of sporulation and β-galactosidase activity assayed. No significant β-galactosidase activity was detected in extracts from strains carrying the plasmid vector alone (pHT304-18Z) (less than 7 Miller units (mg protein)⁻¹; data not shown). In the Spo+ background (strain 4Q2-81), the cry-type promoters drove synthesis of β-galactosidase from t- to t0; however, small amounts of β-galactosidase were also detected in early sporulation (t- to t0) (Fig. 5a, b, c, open circles). To test whether the early transcription was dependent on the sporulation-specific sigma factors, strains 407-sigE(pHT691), 407-sigE(pHT692) and 407-sigE(pHT693) were assayed for β-galactosidase activity. Late expression was abolished in a sigE background for all the fusions (Fig. 5a, b, c, triangles) but not in a sigK strain (data not shown). Thus, late cry4A, cry4B and cry11A gene transcription...
Fig. 5. Time course of cry gene expression in \textit{B. thuringiensis}. Cell extracts from various strains of \textit{B. thuringiensis} harbouring either (a) pHT691 (cry4A'-lacZ); (b) pHT692 (cry4B'-lacZ); (c) pHT693 (cry11A'-lacZ) or (d) pHT694 (cry11A0A'-lacZ) were used. \(\beta\)-Galactosidase specific activity [in Miller units (mg protein)] was determined at various sporulation phases (time corresponding to the entry to sporulation) for \textit{B. thuringiensis} strain 442-81 (Spo+ background, open circles), strain 407-sigE (triangles) and strain 407-OA (spoOA background, closed circles).

was dependent on \(\sigma^E\). However, there was \(\beta\)-galactosidase synthesis, albeit low, from \(t_1\) to \(t_4\) in sigE strains (Fig. 5a, b, c, triangles), suggesting that the transcriptional activity of the cry4A, cry4B and cry11A promoters from \(t_1\) to \(t_4\) was not dependent on \(\sigma^E\). \(\beta\)-Galactosidase expression from the cry'-lacZ transcriptional fusions in strain 407-0A (spoOA) between \(t_1\) and \(t_4\) (Fig. 5a, b, c, closed circles) was about threefold higher than in the wild-type strain (Fig. 5a, b, c, open circles). These results suggest that the \textit{B. thuringiensis} subsp. \textit{israelensis} cry genes may also be controlled by a vegetative- or a transition-phase-specific sigma factor. Sequence analysis suggests the presence of \(\sigma^H\)-specific promoters overlapping the \(\sigma^E\)-specific promoters for the cry4A, cry4B and cry11A genes (Fig. 2b). Expression studies were performed in \textit{B. subtilis} to demonstrate the \(\sigma^H\)-dependence of the cry11A gene.

\textbf{Regulation of cry11A expression in \textit{B. subtilis}}

No spoOH \textit{B. thuringiensis} strain is currently available. Therefore, to test for a \(\sigma^H\)-dependent promoter of the cry11A operon, the cry11A'-lacZ transcriptional fusion was introduced as a single copy at the amyE locus in the chromosome of various sporulation mutants of \textit{B. subtilis} (Table 1). Recombinant cells were cultured in SP medium at 37 °C, and \(\beta\)-galactosidase activity was assayed at the \(t_{1.5}\) stage (Table 1). \(\beta\)-Galactosidase activity in strains bearing the lacZ gene without a promoter at the amyE locus was low [less than 2-3 Miller units (mg protein)]; data not shown]. \(\beta\)-Galactosidase activities in strains FBT11 (wild-type background) and FBT14 (sigE) were equivalent (see Table 1), evidence that \(\sigma^E\) is not involved in the cry11A expression at this stage of sporulation in \textit{B. subtilis}. In contrast, lacZ expression in strains FBT12 (spo0A background) and FBT17 (sigH) corresponded to the level of activity in control strains bearing the lacZ gene without the cry promoter. These results showed that both Spo0A and \(\sigma^H\) are involved in the positive regulation of cry11A. In \textit{B. subtilis}, spoOH is negatively regulated by AbrB (Weir et al., 1991). \(\beta\)-Galactosidase activity in strain FBT21 (\textit{aabrB}) was therefore tested; it was threefold higher than that of the
wild-type strain. It seems therefore that in *B. subtilis* cry11A expression during the transition state is $\sigma^H$-dependent. In *B. subtilis*, SpoOA positively regulates the expression of $\sigma^H$. Weir et al. (1991) showed that spoOH expression is five- to tenfold reduced in a *B. subtilis* spoOA mutant. This may explain why expression of cry11A in *B. subtilis* was lower in strain FBT12 (spoOA background, Table 1) than in the wild-type strain.

**Role of the putative '0A' box in *B. thuringiensis***

$\beta$-Galactosidase synthesis directed by the cry11A promoter in the presence (pHT693, Fig. 5c) or absence (pHT694, Fig. 5d) of the putative '0A' box was analysed in various *B. thuringiensis* sporulation mutants. The time course of expression of $\beta$-galactosidase in strains 4Q2-81 and 407-0A, harbouring either pHT693 or pHT694, respectively (Fig. 5c, d) was roughly comparable (open and closed circles, respectively); however, in the Spo+ strain, in the absence of the '0A' box (Fig. 5d), $\beta$-galactosidase synthesis started at $t_4$, rather than $t_5$ for the wild-type promoter (Fig. 5c). In addition, deletion of the putative '0A' box led to a significant derepression at $t_4$ in the sigE strain (Fig. 5d, triangles) compared to the wild-type promoter (Fig. 5c, triangles). These results suggest that the '0A' box, or a sequence in the deleted DNA region, is involved in the transcriptional repression of the cry11A operon during entry into sporulation. As cry11A expression in *B. thuringiensis* and *B. subtilis* spoOA strains is not equal (threelfold higher and sixfold lower than in the corresponding Spo+ strains, respectively), we cannot exclude the possibility that regulation of the cry11A operon is different in the two bacteria. If SpoOA induces the expression of $\sigma^H$ in *B. thuringiensis* as in *B. subtilis*, the absence of SpoOA in *B. thuringiensis* 407-0A would lead to a lower expression of $\sigma^H$. In *B. thuringiensis* 407-0A, the absence of SpoOA may therefore indirectly down-regulate the $\sigma^H$-dependent transcription of cry11A during the transition phase. Indeed, no obvious induction of the cry11A′-lacZ fusion was observed in a spoOA background (Fig. 5c, closed circles). The SpoOA and $\sigma^H$ regulatory factors are synthesized in the sigE background. In the presence of the '0A' box, SpoOA interacts with its target and may thereby repress the expression of the cry11A′-lacZ fusion (Fig. 5c, triangles). By contrast, in the absence of the '0A' box (cry11AΔ0A′-lacZ, Fig. 5d, triangles), the SpoOA regulatory protein would not interact with the cry11A promoter region and the RNA polymerase associated with $\sigma^H$ would transcribe the fusion, leading to the synthesis of $\beta$-galactosidase after $t_4$.

**DISCUSSION**

In *B. thuringiensis* subsp. *israelensis* cry4A, cry4B and cry11A are believed to be transcribed by the RNA polymerase associated with the sporulation-specific $\sigma^E$ factor at the mid-sporulation stage (for reviews, see Agaisse & Lereclus, 1995; Baum & Malvar, 1995). Our findings support this view, since cry4′-lacZ and cry11A′-lacZ transcriptional fusions were not expressed after $t_4$ in a sigE strain of *B. thuringiensis*. However, primer extension analysis and expression studies in sporulation mutants of *B. thuringiensis* showed that cry4A, cry4B and cry11A were weakly transcribed during the late exponential phase from a promoter overlapping the $\sigma^E$ consensus sequences. The transcriptional start sites of the cry4A and cry11A genes have already been determined in sporulating *B. thuringiensis* cells (Yoshisue et al., 1993; Dervyn et al., 1995). The 5′ end of the cry4B′-lacZ mRNA in both spoOA and wild-type backgrounds is located five nucleotides upstream from the cry4B mRNA 5′ end reported by Yoshisue et al. (1994). The cry11A transcript is present in *B. thuringiensis* cells grown in a sporulation medium at $t_{-4}$ and after $t_4$ but is undetectable between $t_4$ and $t_5$ (Poncet et al., 1995b). Here we report evidence that cry11A is transcribed during the transition phase by the RNA polymerase associated with the $\sigma^H$ factor, from a promoter overlapping the $\sigma^H$-dependent promoter. Primer extension and expression analysis in a spoOA mutant strain of *B. thuringiensis* suggests that the early transcription of the cry4A and cry11A genes is also $\sigma^H$-dependent. Our results partially confirm those obtained by Yoshisue et al. (1995), who showed that the cry4A promoter region is recognized by the $\sigma^H$-RNA polymerase during the transition phase. In contrast, no cry4B mRNA was detected by these authors during the late-exponential growth stage (Yoshisue et al., 1994, 1995).

Primer extension analysis also indicates that cry4A and cry11A expression is turned off on entry into sporulation in *B. thuringiensis*. Deletion analysis of the cry11A promoter region implicates the regulatory protein SpoOA in the negative regulation of the cry11A operon, probably through interaction with a '0A' box. Nevertheless, it seems that this box has no essential role in cry11A synthesis; also, we cannot exclude the possibility that other regulatory factors are involved in the accumulation of cry11A inclusions in *B. thuringiensis*. Our data suggest that the cry4A, cry4B and cry11A genes are all subject to the same early transcriptional regulation, involving a dual regulation system: their promoters are controlled by the $\sigma^H$-RNA polymerase during the transition phase and repressed on entry into the sporulation process by interaction of SpoOA with its DNA target. These genes are transcriptionally active in mid-sporulation, due to the $\sigma^E$-RNA polymerase. Yoshisue et al. (1995) showed that in *B. subtilis* cry4A, but not cry4B, is transcribed by the $\sigma^H$-RNA polymerase. However, they did not observe cry4A repression on entry into sporulation. This difference may be due to different regulation in *B. subtilis* and in *B. thuringiensis* and/or to poor cell synchronization. In addition, we detected cry4B mRNA by primer extension experiments using total RNA extracted from a spoOA strain [407-0A(pHT692)], see Results, in which the cry4B transcriptional activity was threefold higher than in a wild-type background.

The temporal pattern of expression of the cry4-type genes and the cry11Aa gene is different, although they
both harbour $\sigma^E$-dependent promoters (Bravo et al., 1996). $\beta$-Galactosidase synthesis mediated by a transcriptional cry1Aa'-lacZ fusion in B. thuringiensis cells grown in HCT medium is induced 2 h after the entry to sporulation, whereas we showed in the present study that the transcription mediated by the cry4A, cry4B and cry11A sigma-dependent promoters started at $t_q-2h$. Moreover, we confirmed that in HCT medium a spoIIIG-lacZ fusion, harbouring a $\sigma^E$-dependent promoter, is induced at $t_q$ in B. thuringiensis (data not shown). These results showed that under our experimental conditions (i) $\sigma^E$ is present – and active – 2 h after the onset of sporulation, and (ii) the $\sigma^E$-dependent induction of the cry4 and cry11A genes is delayed in B. thuringiensis. In B. subtilis, Spo0A is detected within the sporulating cell at least 4 h after the beginning of sporulation (Baldus et al., 1994). Moreover, as shown in Fig. 5, the cry11A-lacZ fusion is induced at $t_q$ whereas the deletion of the putative ‘0A’ box, the cry11AA0A-lacZ is induced at $t_q$. It is therefore tempting to suggest that interaction of the Spo0A regulatory protein with its DNA target is responsible for the delay of the $\sigma^E$-dependent transcription of the cry genes in B. thuringiensis.

Transcription of the cry4 and cry11A genes on entry into sporulation may contribute to invasion of insect larvae following spore-crystal ingestion. However, it appears from recent studies that the regulation of toxin genes in B. thuringiensis is more complex than previously believed (for a review, see Agaisse & Lereclus, 1995; Baum & Malvar, 1995). Spo0A repression may provide a stringent control of cry gene expression from B. thuringiensis subsp. israelensis and avoid the titration of regulatory elements at the beginning of the sporulation process. During the transition phase, expression from the cry4A promoter is higher than that from the cry4B or cry11A promoters. Unlike Cry4B and Cry11A, Cry4A does not crystallize when produced alone in a crystal-minus strain of B. thuringiensis (Delcluse et al., 1993). Early transcriptional regulation of cry gene expression is therefore not sufficient to explain the accumulation of the toxins as parasporal inclusions in the mother-cell compartment. Further work is needed to determine the role of sporulation-specific regulators on cry gene expression.

ACKNOWLEDGEMENTS

We are grateful to Alex Edelman for revising the English manuscript. This work was supported by grants from the Institut Pasteur, the Centre National de la Recherche Scientifique and AgrEvo. S. Poncet was supported by a grant from Université Paris 7 and E. Dervyn by a grant from the Institut National de la Recherche Agronomique.

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Received 2 April 1997; accepted 7 April 1997.

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