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 t_{4-6} was σ^E -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 '0A' 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 *Cyt1A*) has been characterized. The high toxicity of the inclusions is due in part to synergy between these components (Delécluse *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^A and σ^H . 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^{35} and σ^{28} factors [homologous to the mother-cell-specific σ^E 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^{35} -RNA polymerase; moreover, transcription of the *cyt1A* and *cry11A* genes continues into late-sporulation from promoters recognized by the σ^{28} -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 'OA' box and 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 1). *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 μ g ml⁻¹).

Escherichia coli K-12 strain TG1 [Δ (*lac-proAB*) *supE thi hds D5 F'*(*tra D36 proA⁺ proB⁺ lacI^q lacZ* Δ M15)] was used for plasmid construction. Transformants were selected on LB plates supplemented with ampicillin (100 μ g 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 μ g ml⁻¹), kanamycin (10 μ g ml⁻¹) or chloramphenicol (10 μ g 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) l⁻¹, 1 mM MgSO₄, 13 mM KCl and 10 μ M MnCl₂; after sterilization, 4.4 mg ferric ammonium citrate l⁻¹, and CaCl₂ 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

Strain	Genotype	Source or reference	β -Galactosidase activity*
168	<i>trpC2</i>	Laboratory stock	ND
JH12586	<i>trpC2 pheA1 abrB::cat</i>	Perego <i>et al.</i> (1988)	ND
IS233	<i>trpC2 pheA1 ΔsigH</i>	Weir <i>et al.</i> (1984)	ND
FBT11	<i>trpC2 amyE:: (cry11A'-lacZ cat)</i>	Dervyn <i>et al.</i> (1995)	28 (26-31)
FBT12	<i>trpC2 amyE:: (cry11A'-lacZ cat) spo0A::kan</i>	Dervyn <i>et al.</i> (1995)	5 (4-6)
FBT14	<i>trpC2 amyE:: (cry11A'-lacZ cat) ΔsigE</i>	Dervyn <i>et al.</i> (1995)	32 (29-35)
FBT17	<i>trpC2 pheA1 amyE:: (cry11A'-lacZ cat) ΔsigH</i>	This work	4 (3-6)
FBT20	<i>trpC2 amyE:: (cry11A'-lacZ kan)</i>	This work	ND
FBT21	<i>trpC2 pheA1 amyE:: (cry11A'-lacZ kan) abrB::cat</i>	This work	90 (85-95)

ND, Not determined.

* Values shown are the means of four to eight independent experiments and are expressed in Miller units (mg protein)⁻¹. Numbers in parentheses are 95% confidence limits as determined by probit analysis.

Fig. 1. Construction of plasmids for the analysis of *cry11A* expression. (a) Simplified restriction maps of the recombinant plasmids containing all or part of the *cry11A* operon from which the '0A' box has been deleted (pHT643 and pHT643Δ0A, respectively). The arrows indicate the position and direction of transcription of the *p19*, *cry11A* and *p20* genes. Asterisks indicate that restriction sites have been lost. (b) Nucleotide sequence of the promoter region from the *cry11A* operon. The ribosome-binding site (RBS) is underlined and -35 and -10 consensus boxes of the PI promoter are in bold letters and underlined. The transcriptional start point (+1) and putative '0A' box are also indicated. The sequence of the primer used for deletion of the '0A' box, creating a *Bam*HI site, is given above the DNA sequence.

1a). pHT643Δ0A was obtained as follows. A DNA fragment was amplified by PCR to create a *Bam*HI restriction site upstream from the -35 box of the *cry11A* gene PI transcriptional start point (corresponding primer 5' CCGAT-CCGATACATTTTGTGTTTATACATGC 3'; see Fig. 1b). The second primer (5' GAAATAACCCTTGGCTG 3') is complementary to nucleotides overlapping the *Sty*I site in the coding sequence of *cry11A*. pEG217 was used as the template (Donovan *et al.*, 1988). The 1191 bp PCR product was digested with *Bam*HI and *Sty*I, purified and ligated to pHT643 digested with *Bam*HI and *Sty*I. The result was pHT643Δ0A

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) (Delécluse *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 '0A' box and the *lacZ* gene, was obtained as follows. pHT643Δ0A was hydrolysed with *Sty*I, blunt-ended with Klenow fragment, then digested with *Hind*III. The resulting DNA fragment carrying the modified *cry11A* promoter region was inserted into pHT304-18Z (Agaisse & Lereclus, 1994) cut with *Bam*HI, blunt-ended with Klenow fragment and restricted with *Hind*III.

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 (Glatron & 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 - 32 S]dATPαS (15 TBq; Amersham).

The specific primer used to detect the *cry4A* transcript was 5' TGACCCTTATGCATTTATCCTCTCC 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' CATACAGTCTCTGGACAATTTACAG-AAATC 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'*-, Δ 0A*cry11A'*-, *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 '0A' boxes from *B. thuringiensis* with the consensus '0A' box from *B. subtilis* (a) and alignment of DNA sequences matching consensus promoters recognized by either σ^E or σ^H in *B. subtilis* (b). (a) Sequence comparison of putative Spo0A-binding boxes of *B. thuringiensis* subsp. *israelensis* *cry4A*, *cry4B* and *cry11A* toxin genes with the consensus *B. subtilis* '0A' 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 σ^E recognition site of *B. subtilis* are boxed. The underlined nucleotides correspond to those which match the consensus promoter recognized by σ^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} (σ^H -dependent, see Fig. 3a, c, lanes t_{-2}) and in mid-sporulation (σ^E -dependent, this work and data not shown; Yoshisue *et al.*, 1995; Dervyn *et al.*, 1995). For *cry4B*, the transcriptional start point detected in the 407-0A 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).

similarity was found upstream from the -35 and -10 promoter boxes of the three promoter regions. These sequences (Fig. 2a) show significant similarities with the consensus '0A' box of *B. subtilis* (Strauch *et al.*, 1990; for a review, see Errington, 1993) and with the putative Spo0A-binding site downstream from the σ^A -dependent promoter of the *B. thuringiensis spo0A* gene (Lereclus *et al.*, 1994). In particular, the highly conserved G and C residues were found (Baldus *et al.*, 1994). A sequence was also found upstream from the *cry4A*, *cry4B* and *cry11A* genes which matches both the -35 and -10 consensus promoter sites recognized by the RNA polymerase of *B. subtilis* associated with the σ^H factor (Fig. 2b).

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

The σ^H -associated RNA polymerase transcribes genes during the transition phase in *B. subtilis*; the regulatory protein Spo0A interacts with both σ^A - and σ^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). No *cry4B* transcript was detected at t_{-2} in wild-type cells (strain 4Q2-72). Primer extension experiments were performed on RNA extracted from 407-0A(pHT692) cells, containing a *cry4B'-lacZ* transcriptional fusion. The *cry4B'-lacZ* mRNA was detected in this *spo0A* background (Fig. 3b, lane t_{-2} ; the 5' end of this transcript corresponded to the 5' end of the *cry4B* transcript detected at t_0 in the wild-type strain 4Q2-72 (Fig. 3b, lane t_{+9}). 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 σ^H . By analogy with *B. subtilis*, σ^H may transcribe the *B. thuringiensis* subsp. *israelensis* *cry* genes, and Spo0A may repress *cry* gene transcription through interaction with the putative '0A' boxes. The '0A' box located upstream from the *cry11A* operon was therefore investigated. We tested the level of

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_{-2}) and at the beginning of sporulation (lane t_0) as previously described; (b) total RNA was extracted from strain 407-0A(pHT692) at t_{-2} and from strain 4Q2-72 at t_0 (lanes t_{-2} and t_{+9} , 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 '0A' 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 '0A' box located upstream from the *cry11A* gene promoter was tested by deletion analysis. Plasmid pHT643 Δ 0A is a derivative of pHT643 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_{-2} and t_0 (Fig. 4). The 5' end of the Δ 0A*cry11A* transcript was the same

as that identified for the *cry11A* operon (Fig. 3c, lane t_{-2}), although two lower bands were detected, which may correspond to additional promoters or to reverse transcriptase pause sites. Thus the putative '0A' 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(pHT643 Δ 0A), respectively] of the putative Spo0A box as assessed by SDS-PAGE (data not shown).

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

Plasmids pHT691 (*cry4A'*-*lacZ*, Fig. 5a), pHT692 (*cry4B'*-*lacZ*, Fig. 5b) and pHT693 (*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_4 to t_5 ; however, small amounts of β -galactosidase were also detected in early sporulation (t_{-1} to t_4) (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 *B. thuringiensis*. Cell extracts from various strains of *B. thuringiensis* harbouring either (a) pHT691 (*cry4A'*-*lacZ*); (b) pHT692 (*cry4B'*-*lacZ*); (c) pHT693 (*cry11A'*-*lacZ*) or (d) pHT694 (*cry11AΔ0A'*-*lacZ*) were used. β -Galactosidase specific activity [in Miller units (mg protein)⁻¹] was determined at various sporulation phases (t_0 corresponding to the entry to sporulation) for *B. thuringiensis* strain 4Q2-81 (*Spo*⁺ background, open circles), strain 407-sigE (triangles) and strain 407-0A (*spo0A* background, closed circles).

was dependent on σ^E . However, there was β -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 σ^E . β -Galactosidase expression from the *cry'*-*lacZ* transcriptional fusions in strain 407-0A (*spo0A*) 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 *B. thuringiensis* subsp. *israelensis* *cry* genes may also be controlled by a vegetative- or a transition-phase-specific sigma factor. Sequence analysis suggests the presence of σ^H -specific promoters overlapping the σ^E -specific promoters for the *cry4A*, *cry4B* and *cry11A* genes (Fig. 2b). Expression studies were performed in *B. subtilis* to demonstrate the σ^H -dependence of the *cry11A* gene.

Regulation of *cry11A* expression in *B. subtilis*

No *spo0H* *B. thuringiensis* strain is currently available. Therefore, to test for a σ^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 *B. subtilis* (Table 1). Recombinant cells were cultured in SP medium at 37 °C, and β -galactosidase activity was assayed at the $t_{-1.5}$ stage (Table 1). β -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)⁻¹; data not shown]. β -Galactosidase activities in strains FBT11 (wild-type background) and FBT14 (*sigE*) were equivalent (see Table 1), evidence that σ^E is not involved in the *cry11A* expression at this stage of sporulation in *B. subtilis* as in *B. thuringiensis*. 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 σ^H are involved in the positive regulation of *cry11A*. In *B. subtilis*, *spo0H* is negatively regulated by *AbrB* (Weir *et al.*, 1991). β -Galactosidase activity in strain FBT21 (Δ *abrB*) 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 σ^H -dependent. In *B. subtilis*, Spo0A positively regulates the expression of σ^H . Weir *et al.* (1991) showed that *spo0H* expression is five- to tenfold reduced in a *B. subtilis* *spo0A* mutant. This may explain why expression of *cry11A* in *B. subtilis* was lower in strain FBT12 (*spo0A* background, Table 1) than in the wild-type strain.

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

β -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 β -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), β -galactosidase synthesis started at t_3 , 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_1 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* *spo0A* strains is not equal (threefold 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 Spo0A induces the expression of σ^H in *B. thuringiensis* as in *B. subtilis*, the absence of Spo0A in *B. thuringiensis* 407-0A would lead to a lower expression of σ^H . In *B. thuringiensis* 407-0A, the absence of Spo0A may therefore indirectly down-regulate the σ^H -dependent transcription of *cry11A* during the transition phase. Indeed, no obvious induction of the *cry11A'*-*lacZ* fusion was observed in a *spo0A* background (Fig. 5c, closed circles). The Spo0A and σ^H regulatory factors are synthesized in the *sigE* background. In the presence of the '0A' box, Spo0A 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 (*cry11A* Δ 0A'-*lacZ*, Fig. 5d, triangles), the Spo0A regulatory protein would not interact with the *cry11A* promoter region and the RNA polymerase associated with σ^H would transcribe the fusion, leading to the synthesis of β -galactosidase after t_1 .

DISCUSSION

In *B. thuringiensis* subsp. *israelensis* *cry4A*, *cry4B* and *cry11A* are believed to be transcribed by the RNA polymerase associated with the sporulation-specific σ^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_5 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 σ^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 *spo0A* 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_{-2} and after t_5 but is undetectable between t_0 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 σ^H factor, from a promoter overlapping the σ^E -dependent promoter. Primer extension and expression analysis in a *spo0A* mutant strain of *B. thuringiensis* suggests that the early transcription of the *cry4* genes is also σ^H -dependent. Our results partially confirm those obtained by Yoshisue *et al.* (1995), who showed that the *cry4A* promoter region is recognized by the σ^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 Spo0A 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 σ^H -RNA polymerase during the transition phase and repressed on entry into the sporulation process by interaction of Spo0A with its DNA target. These genes are transcriptionally active in mid-sporulation, due to the σ^E -RNA polymerase. Yoshisue *et al.* (1995) showed that in *B. subtilis* *cry4A*, but not *cry4B*, is transcribed by the σ^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 *spo0A* 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 *cry11A* gene is different, although they

both harbour σ^E -dependent promoters (Bravo *et al.*, 1996). β -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_4 - t_5 . Moreover, we confirmed that in HCT medium a *spoIID'-lacZ* fusion, harbouring a σ^E -dependent promoter, is induced at t_2 in *B. thuringiensis* (data not shown). These results showed that under our experimental conditions (i) σ^E is present – and active – 2 h after the onset of sporulation, and (ii) the σ^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_5 , whereas after the deletion of the putative '0A' box, the *cry11A Δ 0A'-lacZ* is induced at t_3 . It is therefore tempting to suggest that interaction of the Spo0A regulatory protein with its DNA target is responsible for the delay of the σ^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* (Delécluse *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.

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REFERENCES

- Adams, L. F., Brown, K. L. & Whiteley, H. R. (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.
- Agaisse, H. & Lereclus, D. (1994). Expression of the *cryIIIA* toxin gene of *Bacillus thuringiensis* is not dependent on sporulation-specific sigma factor and is increased in a *spo0A* mutant of *B. subtilis*. *J Bacteriol* **6**, 4734–4741.
- Agaisse, H. & Lereclus, D. (1995). How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? *J Bacteriol* **177**, 6027–6032.
- Baldus, J. M., Green, B. D., Youngman, P. & Moran, C. P., Jr (1994). Phosphorylation of *Bacillus subtilis* transcription factor Spo0A stimulates transcription from the *spoIIIG* promoter by enhancing binding to weak 0A boxes. *J Bacteriol* **176**, 296–306.
- Baldus, J. M., Buckner, C. M. & Moran, C. P., Jr (1995). Evidence that the transcriptional activator Spo0A interacts with two sigma factors in *Bacillus subtilis*. *Mol Microbiol* **17**, 281–290.
- Baum, J. A. & Malvar, T. (1995). Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. *Mol Microbiol* **18**, 1–12.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513–1523.
- Bravo, A., Agaisse, H., Salamitou, S. & Lereclus, D. (1996). Analysis of *cryIAa* expression in *sigE* and *sigK* mutants of *Bacillus thuringiensis*. *Mol Gen Genet* **250**, 734–741.
- Brown, K. L. & Whiteley, H. R. (1988). Isolation of a *Bacillus thuringiensis* RNA polymerase capable of transcribing crystal protein genes. *Proc Natl Acad Sci USA* **85**, 4166–4170.
- Brown, K. L. & Whiteley, H. R. (1990). Isolation of the second *Bacillus thuringiensis* RNA polymerase that transcribes from a crystal protein gene promoter. *J Bacteriol* **172**, 6682–6688.
- Calogero, S., Gardan, R., Glaser, P., Schweizer, J., Rapoport, G. & Débarbouillé, M. (1994). RocR, a novel regulatory protein controlling arginine utilization in *Bacillus subtilis*, belongs to the NtrC/NifA family of transcriptional activators. *J. Bacteriol* **176**, 1234–1241.
- Delécluse, A., Poncet, S., Klier, A. & Rapoport, G. (1993). Expression of *cryIVA* and *cryIVB* genes, independently or in combination, in a crystal minus strain of *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol* **59**, 3922–3927.
- Dervyn, E., Poncet, S., Klier, A. & Rapoport, G. (1995). Transcriptional regulation of *Bacillus thuringiensis* subsp. *israelensis* *cryIVD* gene operon. *J Bacteriol* **177**, 2283–2291.
- Donovan, W. P., Dankocsik, C. & Gilbert, M. P. (1988). Molecular characterization of a gene encoding a 72-kilodalton mosquito-toxic crystal protein from *Bacillus thuringiensis* subsp. *israelensis*. *J Bacteriol* **170**, 4732–4738.
- Errington, J. (1993). *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. *Microbiol Rev* **57**, 1–33.
- 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.
- Haldenwang, W. G. (1995). The sigma factors of *Bacillus subtilis*. *Microbiol Rev* **59**, 1–30.
- Lecadet, M.-M., Blondel, M.-O. & Ribier, J. (1980). Generalized transduction in *Bacillus thuringiensis* var. *berliner* 1715 using bacteriophage CP54Ber. *J Gen Microbiol* **121**, 203–212.
- Lereclus, D., Arantes, O., Chaufaux, J. & Lecadet, M.-M. (1989). Transformation and expression of a cloned δ -endotoxin gene in *Bacillus thuringiensis*. *FEMS Microbiol Lett* **60**, 211–218.

- Lereclus, D., Agaisse, H., Gominet, M. & Chaufaux, J. (1994). Overproduction of encapsulated insecticidal crystal proteins in a *Bacillus thuringiensis* *spo0A* mutant. *Bio/Technology* 13, 67–71.
- Malvar, T. & Baum, J. A. (1994). Tn5401 disruption of the *spo0F* gene, identified by direct chromosomal sequencing, results in CryIIIA overproduction in *Bacillus thuringiensis*. *J Bacteriol* 176, 4750–4753.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Msadek, T., Kunst, F., Henner, D., Klier, A., Rapoport, G. & Dedonder, R. (1990). Signal transduction pathway controlling synthesis of a class of degradative enzymes in *Bacillus subtilis*: expression of the regulatory gene and analysis of mutations in *degS* and *degU*. *J Bacteriol* 172, 824–834.
- Perego, M., Spiegelman, G. B. & Hoch, J. A. (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.
- Poncet, S., Delécluse, A., Klier, A. & Rapoport, G. (1995a). Evaluation of synergistic interactions between the CryIVA, CryIVB and CryIVD toxic components of *Bacillus thuringiensis* subsp. *israelensis* crystals. *J Invertebr Pathol* 66, 131–135.
- Poncet, S., Dervyn, E., Klier, A. & Rapoport, G. (1995b). Regulation of the *cryIVD* operon in *Bacillus thuringiensis* subsp. *israelensis*. In *8th International Conference on Bacilli* (Abstracts), p. 58, Stanford, CA, USA.
- Porter, A. G., Davidson, E. W. & Liu, J.-W. (1993). Mosquitocidal toxins of *Bacilli* and their genetic manipulation for effective biological control of mosquitoes. *Microbiol Rev* 57, 838–861.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74, 5463–5467.
- Strauch, M., Webb, V., Spiegelman, G. & Hoch, J. A. (1990). The Spo0A protein of *Bacillus subtilis* is a repressor of the *abrB* gene. *Proc Natl Acad Sci USA* 87, 1801–1805.
- Waalwijk, C., Dullemans, A. M., vanWorkum, M. E. S. & Visser, B. (1985). Molecular cloning and the nucleotide sequence of the *M_r* 28000 crystal protein gene of *Bacillus thuringiensis* subsp. *israelensis*. *Nucleic Acids Res* 13, 8207–8217.
- Ward, E. S. & Ellar, D. J. (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.
- Weinrauch, Y., Msadek, T., Kunst, F. & Dubnau, D. (1991). Sequence and properties of *comQ*, a new competence regulatory gene of *Bacillus subtilis*. *J Bacteriol* 173, 5685–5693.
- Weir, J., Dubnau, E., Ramakrishna, N. & Smith, I. (1984). *Bacillus subtilis* *spo0H* gene. *J Bacteriol* 157, 405–412.
- Weir, J., Predich, M., Dubnau, E., Nair, G. & Smith, I. (1991). Regulation of *spo0H*, a gene coding for the *Bacillus subtilis* σ^H factor. *J Bacteriol* 173, 521–529.
- Wong, H. C., Schnepf, H. E. & Whiteley, H. R. (1983). Transcriptional and translational start sites for the *Bacillus thuringiensis* crystal protein gene. *J Biol Chem* 258, 1960–1967.
- Yoshisue, H., Fukada, T., Yoshida, K.-I., Sen, K., Kurosawa, S.-I., Sakai, H. & Komano, T. (1993). Transcriptional regulation of *Bacillus thuringiensis* subsp. *israelensis* mosquito larvicidal crystal protein gene *cryIVA*. *J Bacteriol* 175, 2750–2753.
- Yoshisue, H., Nishimoto, T., Sakai, H. & Komano, T. (1994). Identification of a promoter for the crystal protein-encoding gene *cryIVB* from *Bacillus thuringiensis* subsp. *israelensis*. *Gene* 137, 247–251.
- Yoshisue, H., Ihara, K., Nishimoto, T., Sakai, H. & Komano, T. (1995). Expression of the genes for insecticidal crystal proteins in *Bacillus thuringiensis*: *cryIVA*, not *cryIVB*, is transcribed by RNA polymerase containing σ^H and that containing σ^E . *FEMS Microbiol Lett* 127, 65–72.

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