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Analysis of *cryIAa* expression in *sigE* and *sigK* mutants of *Bacillus thuringiensis*

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Abstract The *sigE* and *sigK* genes, encoding the sporulation-specific sigma factors σ^{35} and σ^{28} of *Bacillus thuringiensis*, were each disrupted by inserting a gene conferring resistance to kanamycin into their coding sequence. The *B. thuringiensis* SigE$^-$ and SigK$^-$ mutant strains were blocked at different sporulation stages and were unable to sporulate. The SigE$^-$ strain was blocked at stage II of sporulation, whereas the SigK$^-$ strain was blocked at stage IV. The expression of a *cryIAa'-'lacZ* transcriptional fusion was analysed in these genetic backgrounds and it was found that both sigma factors are involved in the in vivo transcription of this gene. However, the SigK$^-$ strain harbouring the *cryIAa* gene produced amounts of toxin similar to those produced by the *B. thuringiensis* Spo$^+$ strain. The toxins accumulated in the mother cell compartment to form a crystal inclusion which remained encapsulated within the cell wall. Thus, transcription from the σ^E-dependent promoter alone (Bt I promoter) is sufficient to support high levels of toxin production in *B. thuringiensis*.

Key words *Bacillus thuringiensis* · *cry* gene expression · Encapsulation · Sigma factors · Sporulation

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Introduction

Bacillus thuringiensis (*Bt*) is a gram-positive bacterium that produces insecticidal proteins toxic to the larvae of various insects, many of which are disease vectors or major crop pests. These proteins, called δ-endotoxins, are produced abundantly during sporulation and accumulate as parasporal crystalline inclusions. They can account for up to 25% of the dry weight of the sporulated cells. Numerous δ-endotoxin genes have been isolated, sequenced and classified into five groups and several subgroups according to sequence homology and toxicity spectrum (Höfte and Whiteley 1989).

Bt formulations have been used as biopesticides for 30 years and a variety of commercial preparations are available. The use of *Bt* as a biological control agent has several advantages over chemical pesticides: it has a narrow and highly specific host range and it is harmless to non-target insects, to vertebrates and to the environment. However, the low persistence of the δ-endotoxins in the environment and the presence of spores in the formulations represent two disadvantages in the commercialisation of *Bt*-based products. These two problems could be overcome by encapsulation of the insecticidal toxins within a cell wall membrane, as suggested by the encapsulation of CryIAc toxin in *Pseudomonas fluorescens* cells (Gelernter 1990) and by expression of the CryIIIA toxin in a Spo$^-$ *Bt* mutant strain affected in the *spo0A* gene (Lereclus et al. 1995). The latter strategy was possible because, unlike other *cry* genes, *cryIIIA* expression is activated at the onset of the stationary phase from a promoter resembling those recognized by the primary sigma factor of vegetative cells (Agaisse and Lereclus 1994b). Thus, the activation of this gene is independent of the genes involved in the initiation of sporulation and of the sporulation-specific sigma factors (Agaisse and Lereclus 1994a; Malvar and Baum 1994). In contrast, the other *cry* genes are developmentally regulated and are

dependent on sporulation (see Agaisse and Lereclus 1995; Baum and Malvar 1995 for recent reviews on the regulation of *cry* gene expression).

The sporulation process is temporally regulated at the transcriptional level by the successive activation of six sigma factors. These factors are the primary sigma factor of vegetative cells, σ^A, and five factors that are activated during development and called σ^H, σ^F, σ^E, σ^G and σ^K, in order of their appearance during sporulation (Haldenwang 1995; Moran 1993). The σ^A and σ^H factors are active before the septum forms, σ^E and σ^K in the mother cell and σ^F and σ^G in the forespore.

In *Bt*, the *cryIAa* gene is sequentially transcribed during the sporulation from two overlapping promoters, Bt I and Bt II. Promoter Bt I is active between t_2 and t_6 of sporulation and Bt II from t_5 onwards (Wong et al. 1983). In vitro, transcription from each promoter is initiated by *Bt* RNA polymerase containing a different sigma factor. Transcription from Bt I is dependent on the σ^{35} factor (Brown and Whiteley 1988), and Bt II is recognized by the σ^{28} factor (Brown and Whiteley 1990). The σ^{35} factor has 88% identity with the *B. subtilis* σ^E factor and the σ^{28} factor has 85% identity with the corresponding *B. subtilis* σ^K factor (Adams et al. 1991). However, genetic studies on the transcription of *cry* genes have never been done in *Bt*, because characterized *Bt* mutants blocked at various stages of sporulation are not available. The participation of both sigma factors in *cry* gene expression in vivo has not been demonstrated in *B. thuringiensis*. However, this information could be important for the design of *Bt* Spo$^-$ strains that produce encapsulated crystals.

We report here the construction of *Bt* mutants affected in σ^{35} and σ^{28} structural genes. These mutants are blocked at early and late sporulation, respectively. Based on these results, and by comparison with the *B. subtilis* sigma factors (see above), the genes encoding σ^{35} and σ^{28} factors are designated throughout the text as *sigE* and *sigK*, respectively. Although no spore formation occurs in the *Bt sigK* mutant, the expression of the *cryIAa* gene was sufficient to produce insecticidal crystals which remained enclosed within the cell wall. This provides an alternative means to improve the stability of the insecticidal protein.

Materials and methods

Bacterial strains and media

Bt strain 407 (H1 serotype) and its acrystalliferous derivative (Cry$^-$) were isolated by O. Arantes as previously reported (Lereclus et al. 1989). *Escherichia coli* K-12 strain TG1 [Δ (*lac-proAB*) *supE thi hsdD5* (F′*traD36 pro*$^+$ *proB*$^+$ *lacI*^{q} *lacZ* ΔM15)] (Gibson 1984) was used for the cloning experiments. *Bt* strains were grown at 30° C in Luria Broth (LB), in HCT medium (Lecadet et al. 1980) or in nutrient broth sporulation medium (SP medium) (Lereclus et al. 1995). *E. coli* cells were grown at 37° C in LB medium. Antibiotic concentrations for bacterial selection were as follows: ampicillin, 100 μg/ml (for *E. coli*); erythromycin, 5 μg/ml (for *Bt*); kanamycin, 10 μg/ml for *E. coli* and 200 μg/ml for *Bt*.

Plasmids and DNA fragments

Plasmid pRN5101 was kindly provided by A. Gruss. This plasmid has a thermosensitive replication origin in gram-positive hosts; it was constructed by inserting pE194ts (Villafane et al. 1987) into the *Cla*I site of pBR322. Plasmid Bluescript (pBS KS$^-$) was from Stratagene (La Jolla, Calif.). Construction of plasmids pHT304-18Z and pHT410 has been described previously (Agaisse and Lereclus 1994b; Lereclus et al. 1989). The oligonucleotides CryIA-1 and CryIA-2 (Table 1) were used for PCR amplification of the 362-bp fragment containing the promoter region of *cryIAa* (Wong et al. 1983). Primer CryIA-1 had an extra 7 bp at the 5′ end containing a *Hin*dIII restriction site and primer CryIA-2 contained an extra 8 bp with the *Bam*HI restriction site. Both restriction sites were introduced to facilitate the cloning into pHT304-18Z. To disrupt *Bt sigE* and *sigK* genes, the 5′ and the 3′ regions of the corresponding genes were PCR-amplified by using oligonucleotides with appropriate restriction sites at the 5′ ends (Table 1), and subcloned separately into pBS KS$^-$. The 5′ region of the *sigE* and *sigK* genes consisted of *Bam*HI-*Xba*I restriction fragments of 857 and 611 bp, respectively. Their 3′

Table 1 Nucleotide sequences of the PCR primers used

Primer	Sequence	Position[a]	Restriction site at the 5′ end
cryIA-1	5′ CCCAAGCTTGCAGGTAAATGGTTCTAAC 3′	156-177*	*Hin*dIII
cryIA-2	5′ CGCGGATCCATCTCTTTTATTAAGATACC 3	495-518*	*Bam*HI
sigE-1	5′ CGGGATCCCGTTGAAAGCGTAGAGGTCAGAA 3′	16-38**	*Bam*HI
sigE-2	5′ GCTCTAGAGCCAACGCGATGCATATGTTGCTA 3′	834-855**	*Xba*I
sigE-3	5′ GGAATTCCATTGTCTGACGTGTTAGGTACA 3′	961-982**	*Eco*RI
sigE-4	5′ CGGGATCCCGATACGCAATATCTCGCAATGA 3′	1730-1751**	*Bam*HI
sigK-1	5′ CGGGATCCCGTCCAGTTATAATTTGAGCTCCAA 3′	31-53**	*Bam*HI
sigK-2	5′ GCTCTAGAGCCCCGATTGTACCAATTGAAAT 3′	603-623**	*Xba*I
sigK-3	5′ GGAATTCCATTAAAGCGATCGAGAGCTATT 3′	627-648**	*Eco*RI
sigK-4	5′ CGGGATCCCGGCACCTTCTAATATTACAGATAGAA 3′	1194-1217**	*Bam*HI
sigE-Ch	5′ TTTTCTAAAAAGCGTATTGAA 3′	1-22**	None
sigK-Ch	5′ GGAGAAACCATAGTTATGAA 3′	1-20**	None

[a] The positions of the oligonucleotides were determined from the published nucleotide sequences: *(Wong et al. 1983) and **(Adams et al. 1991)

regions consisted of *Eco*RI-*Bam*HI restriction fragments of 807 and 606 bp, respectively. The DNA fragments containing the 5′ and 3′ regions of each gene were purified, ligated with an 1.5 kb *Xba*I-*Eco*RI fragment carrying the *aphA3* gene of *Enterococcus faecalis* (Km^R cassette; Trieu-Cuot and Courvalin 1983), and inserted into the *Bam*HI restriction site of the pRN5101 plasmid. In resulting thermosensitive plasmids, pAB1 and pAB2, the *sigE* and *sigK* genes, respectively, are interrupted by the Km^R cassette.

Plasmids pDG675 and pDG676, carrying the promoter regions of the *B. subtilis spoIID* and *cotA* genes, respectively, were kindly provided by Dr. P. Stragier (Institut de Biologie Physico-Chimique, Paris, France). pHTspoIID was constructed by subcloning the 300 bp *Hin*dIII-*Bam*HI restriction fragment from pDG675 between the *Hin*dIII and *Bam*HI restriction sites of pHT304-18Z. pHTcotA was constructed as follows. The 400 bp *Eco*RI-*Bam*HI segment from pDG676 was first subcloned into pBS KS^-, giving pKScotA. The *Hin*dIII-*Bam*HI restriction fragment from pKScotA was then subcloned between the *Hin*dIII and *Bam*HI restriction sites of pHT304-18Z. The resulting plasmid was designated pHTcotA.

DNA manipulations and transformation

Plasmid DNA was extracted from *E. coli* by the standard alkaline lysis procedure. Chromosomal DNA was extracted from *Bt* as previously described (Msadek et al. 1990). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, Mass.). DNA fragments were purified from agarose gels using a Prep-A-Gene kit (Bio-Rad Laboratories, Richmond, Calif.). Oligonucleotide primers were synthesized by Genset (Paris, France) and PCR amplifications were performed using a thermal cycler GeneAmp PCR System 2400 (Perkin-Elmer, Foster City, Calif.). The template DNA used in the PCR amplification reactions was either the *cryIAa* gene previously cloned from the *Bt* strain 407 (Lereclus et al. 1989) or the chromosomal DNA from the 407 Cry^- strain. Reaction conditions were as follows: one incubation of 5 min at 95° C, followed by 30 cycles of 1 min at 57° C for annealing, 1 min at 72° C for extension and 1 min at 92° C for denaturation; at the end an extra incubation at 72° C for 10 min was included. *Taq* polymerase was purchased from USB Laboratories (Cleveland, Ohio). Standard procedures were used for *E. coli* transformation. *Bt* cells were transformed by electroporation as previously described (Lereclus et al. 1989).

β-Galactosidase assay

Bt strains containing *lacZ* transcriptional fusions were cultured in SP medium at 30° C with shaking at 150 rpm. Samples of 1 ml were taken at the end of log phase (t_0) and at 1 h intervals thereafter until late sporulation (t_{14}). Sporulation was monitored by phase-contrast microscopy. The cells were harvested by centrifugation and dried pellets were immediately frozen at −20° C. The samples were thawed, disrupted by sonication (Branson Sonifier Model 250) and assayed for β-galactosidase activity (Msadek et al. 1990). The specific activities (β-galactosidase units/mg protein) are expressed in Miller (1992) units; they are the average of at least two independent experiments.

Protein analysis

Bt stains were grown in LB or HCT medium with shaking at 30° C for 48 to 72 h. Aliquots (10 ml) of spore-crystal or cell-crystal suspensions were centrifuged 10 min at 8000 rpm, washed twice with 10 ml H_2O and finally suspended in 1 ml H_2O, 0.1 mM phenylmethylsulfonylfluoride. These preparations were sonicated twice for 1 min and analysed by 0.1% SDS-12% PAGE.

Bioassays of insecticidal activity

The protein concentration in the samples was estimated by Bradford assay (Bradford 1976) using bovine serum albumin as standard. The toxicity of the preparations was estimated using second-instar *Plutella xylostella* larvae and free ingestion techniques as previously described (Sanchis et al. 1988).

Electron microscopy examination

Cells grown in HCT medium for 48 and 72 h at 30° C were centrifuged for 1 min at 3000 rpm. Samples were fixed in Karnowsky's fixative (4% paraformaldehyde and 5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4) and post-fixed in 1% OsO_4. Samples were sequentially dehydrated in ascending solutions of alcohol (30 to 100%) and embedded in Spurr resin (Ladd Research Industries, Burlington, Vt.). Thin sections were cut on a LKB Nova ultramicrotome, collected on Formvar-coated nickel grids and examined with a Philips CM12 electron microscope.

Results

Construction of $SigE^-$ and $SigK^-$ *Bt* mutant strains

The thermosensitive pAB1 and pAB2 plasmids, containing Km^R-disrupted copies of the *sigE* and *sigK* genes, respectively (see Materials and methods for their construction), were introduced into *Bt* strain 407 Cry^- by electroporation. Replacement of the *sigE* and *sigK* genes by the disrupted copies *sigE*::*Km* and *sigK*::*Km* was obtained by culture of the transformants in the presence of kanamycin at a nonpermissive temperature (40° C) (see Fig. 1). The Spo^- mutants (designated 407-$SigE^-$ and 407-$SigK^-$) were resistant to kanamycin and sensitive to erythromycin. The replacement of the *Bt sigE* and *sigK* genes by the corresponding disrupted copy was verified by PCR analysis: the chromosomal DNAs from the selected $SigE^-$ and $SigK^-$ mutant strains were used as templates, and oligonucleotides complementary to flanking sequences of each gene (sigE-Ch and sigK-Ch, see Table 1), were used as primers in association with the oligonucleotides sigE-4 and sigK-4, respectively. The sizes of the PCR products were as expected from the Km^R-disruptants (data not shown).

Phenotype of *B. thuringiensis* strains mutant for sporulation-specific sigma factors

The *Bt* $SigE^-$ and $SigK^-$ mutant strains were both unable to sporulate: no thermoresistant spores were produced after 72 h of growth at 30° C in HCT or SP medium. Under similar growth conditions at least 90% of the cells of the wild type strain had sporulated after 24 to 48 h. Examination of the cells by phase-contrast microscopy indicated that the $SigE^-$ strain was blocked early in sporulation (stage II), after the formation of the asymmetric septum dividing the mother cell

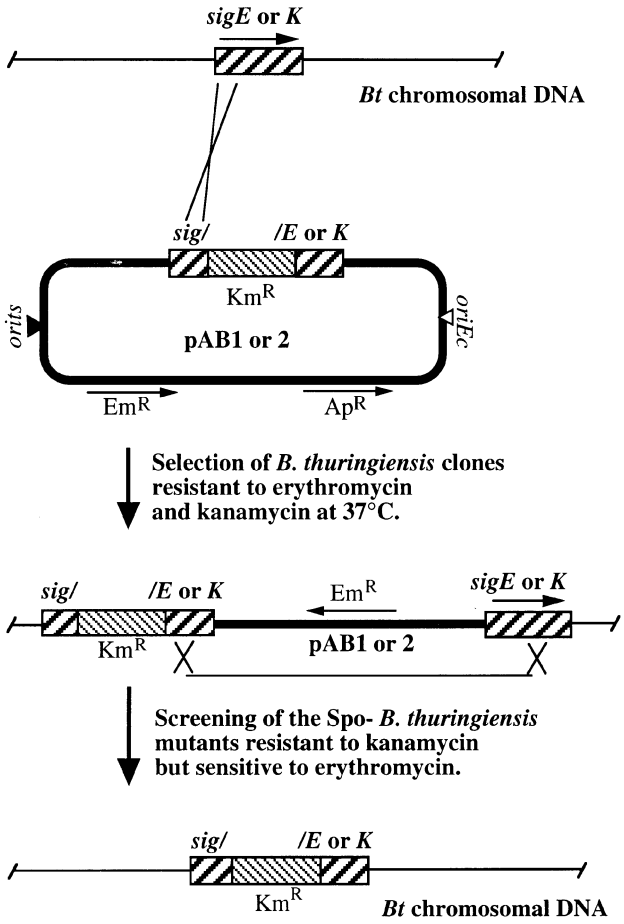

Fig. 1 Disruption of the chromosomal *sigE* and *sigK* genes in *Bacillus thuringiensis* (*Bt*). Plasmids pAB1 and pAB2 were integrated into the *Bt* chromosome by homologous recombination. A second homologous recombination event resulted in the loss of all pRN5101 vector sequences (solid line). *Arrows* indicate the direction of transcription of the genes. Ap^R, Em^R and Km^R indicate the genes conferring resistance to ampicillin, erythromycin and kanamycin, respectively. The *open triangle* represents the replication origin of pBR322 (*oriEc*) and the *solid triangle* represents the replication origin of pE194ts (*orits*)

and the forespore compartments. Disporic cells were observed, as previously reported for a $SigE^-$ mutant of *B. subtilis* (Illing and Errington 1991). The $SigK^-$ mutant strain was blocked late in sporulation (stage IV). A phase-gray body located near one pole of the cell was observed within the cells.

Plasmids pHTspoIID and pHTcotA (Fig. 2), carrying the promoter region of the *B. subtilis spoIID* and *cotA* genes, respectively, fused to *lacZ*, were constructed to follow the appearance and disappearance of the σ^E or σ^K factors during sporulation of *Bt*. The *spoIID* gene is transcribed by an RNA polymerase that contains the sigma factor σ^E (Lopez-Diaz et al. 1986; Rong et al. 1986); this gene is involved in the morphological development of the spore at stage II (Young and Mandelstam 1979). The *cotA* gene encodes a spore coat protein (Donovan et al. 1987) and its transcription is dependent on σ^K (Sandman et al. 1988). The plasmids

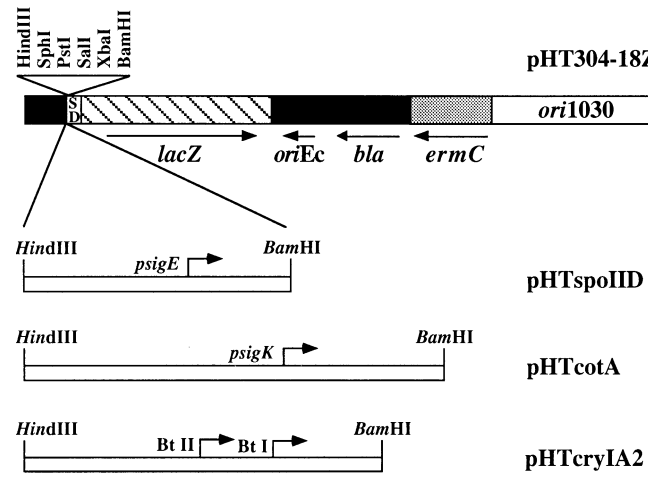

Fig. 2 Construction of plasmids for transcriptional analysis in *B. thuringiensis*. pHT304-18Z was previously described (Agaisse and Lereclus 1994b): *arrows* indicate the direction of transcription of *ermC*, *bla* and *lacZ* genes and the direction of replication functional in *E. coli* (*ori*Ec). *ori*1030 is the replication region of the *Bt* plasmid pHT1030 (Lereclus and Arantes 1992). *Broken arrows* indicate the direction of transcription initiated from promoters *psigE*, *psigK*, Bt I and Bt II as previously reported (Rong et al. 1986; Sandman et al. 1988; Wong et al. 1983). The *Hin*dIII-*Bam*HI fragments carrying the *spoIID*, *cotA* and *cryIAa* promoter regions were prepared and subcloned into pHT304-18Z as described in Materials and methods. They are not represented to scale

pHTspoIID and pHTcotA were introduced into *Bt* 407 Cry^- Spo^+, 407-$SigE^-$ and 407-$SigK^-$ strains by electroporation and the β-galactosidase synthesis was monitored during growth in SP medium (Fig. 3A, B). In the Spo^+ strain, *spoIID*-directed β-galactosidase synthesis started at t_2, reached a maximum of about 10000 Miller units at t_5, and decreased thereafter. In this strain, the *cotA*-directed β-galactosidase expression was detected only at t_6 and rose to about 4000 Miller units at t_{11}. There was no detectable *lacZ* expression (<10 Miller units) from either the *spoIID'* or *cotA'-'lacZ* transcriptional fusions in the 407-$SigE^-$ mutant. Because *sigK* transcription depends on σ^E (Sandman et al. 1988), there is no production of σ^K factor in this mutant strain. There was no detectable *lacZ* expression from the *cotA* promoter in the 407-$SigK^-$ mutant strain and expression from the *spoIID* promoter reached a maximum at about t_6, as in the wild-type strain.

Expression of a *cryIAa'-'lacZ* transcriptional fusion in $SigE^-$ and $SigK^-$ *Bt* strains

To determine the temporal pattern of regulation of the *cryIAa* promoters in *Bt* wild-type and Spo^- mutant strains, a plasmid containing the *cryIAa'-'lacZ* promoter fusion was constructed. A region containing the promoter region of *cryIAa* was amplified by PCR as described in Materials and methods, and inserted upstream of the *lacZ* reporter gene in pHT304-18Z. The

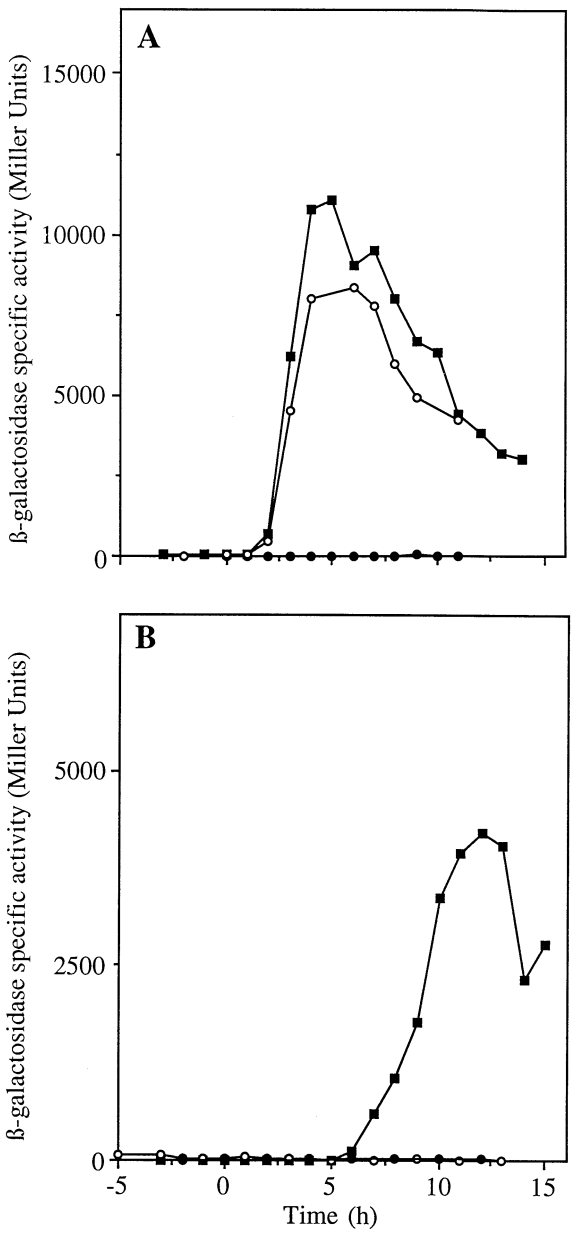

Fig. 3A, B *spoIID*- and *cotA*-directed *β*-galactosidase expression in *Bt*. Cells were grown in SP medium at 30° C. Time zero indicates the end of the exponential phase. t_n is the number of hours before (−) or after time zero. **A** *β*-Galactosidase activity profiles of *Bt* strains habouring pHTspoIID. **B** *β*-galactosidase activity profiles of *Bt* strains habouring pHTcotA. The specific activity of *β*-galactosidase was determined at the indicated times in strains Spo$^+$ 407 (■), 407-SigE$^-$ (●) and 407-SigK$^-$ (○)

resulting plasmid, designated pHTcryIA2 (Fig. 2), was introduced into *Bt* 407 Cry$^-$ Spo$^+$, 407-SigE$^-$ and 407-SigK$^-$ strains by electroporation. The production of *β*-galactosidase in the Spo$^+$ 407 Cry$^-$ strain started at t_2 and showed two peaks, the first at t_7 and the second at t_{11} (Fig. 4). As indicated by the *spoIID'-'lacZ* and *cotA'-'lacZ* fusions (Fig. 3A, B), t_7 and t_{11} corresponded with the period of maximal expression of σ^E- and σ^K-specific promoters, respectively. The expression

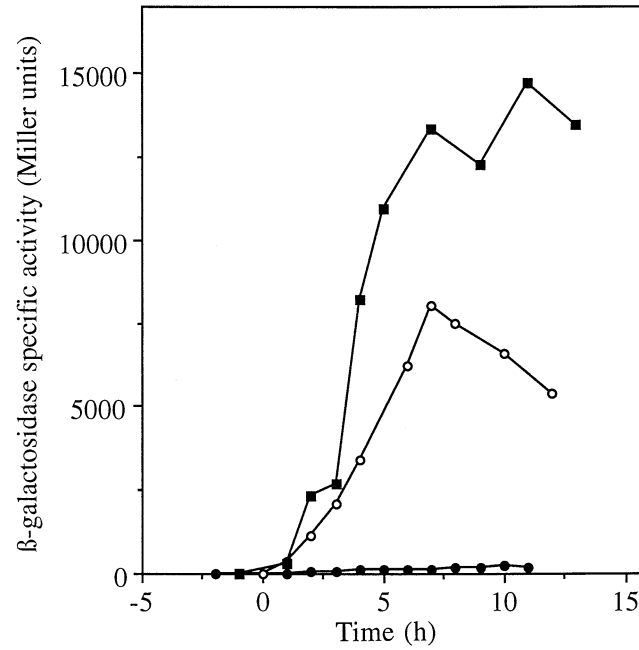

Fig. 4 *cryIAa*-directed *β*-galactosidase expression in *Bt* strains harbouring pHTcryIA2. Cells were grown in SP medium at 30° C and *β*-galactosidase specific activity was determined at the indicated times in strains Spo$^+$ 407 (■), 407-SigE$^-$ (●) and 407-SigK$^-$ (○)

of *cryIAa*-directed *β*-galactosidase synthesis was severely reduced in the 407-SigE$^-$ mutant strain (Fig. 4). However, low *β*-galactosidase activity was detected at t_2, reaching a maximum of 200 Miller units at t_{10} (because of the scale used, this enhancement is not apparent in Fig. 4). The *cryIAa*-directed *β*-galactosidase synthesis started at t_2 and reached a maximum of 9000 Miller units at t_7 in the 407-SigK$^-$ mutant (Fig. 4). The second peak of expression during late sporulation observed in the Spo$^+$ strain was not apparent in the SigK$^-$ mutant, suggesting an important role for the σ^K factor in the transcription of this gene during late sporulation.

Production of CryIAa toxin in the *sigE* and *sigK Bt* strains

Plasmid pHT410, carrying the *cryIAa* gene of the *Bt* 407 wild-type strain (Lereclus et al. 1989), was introduced into *Bt* 407 Cry$^-$ Spo$^+$, 407-SigE$^-$ and 407-SigK$^-$ strains by electroporation. Transformants were grown in HCT and SP medium at 30° C and production of crystal inclusions was examined by phase-contrast microscopy and electron microscopy (Fig. 5). After 48 h of growth in HCT medium, large bipyramidal crystals were observed in the 407-Spo$^+$ and 407-SigK$^-$ transformants. However, the crystals were released from the Spo$^+$ strain, whereas those from the SigK$^-$ mutant strain remained encapsulated within the cell wall. Even after 72 h of growth in HCT medium, there was no

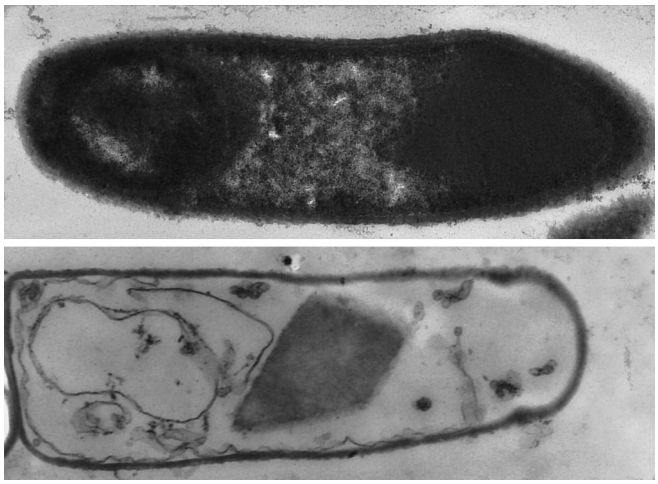

Fig. 5 Electron micrographs of cells of *Bt* strain 407-SigK$^-$ carrying plasmid pHT410. *Bt* cells were grown in HCT medium at 30° C for 48 h (top panel) or for 72 h (bottom panel)

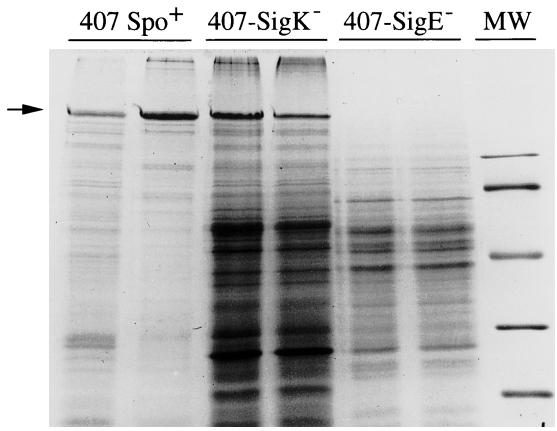

Fig. 6 Analysis of crystal proteins produced by strain 407-SigK$^-$ harbouring plasmid pHT410. Strains (indicated in the Figure) carrying pHT410 were grown at 30° C in HCT medium. Spore-crystal and cell-crystal preparations were harvested from 2- to 3-day-old cultures. Duplicate samples (5 µl) of these preparations were analysed by 0.1% SDS-12% PAGE and Coomassie Blue staining. The *arrow* indicates the 130 kDa crystal component. Lane MW contains molecular weight markers (from top to bottom: 97, 66, 43, 30 and 20 kDa)

release of crystal inclusions from the SigK$^-$ mutant strain. Typical examples of cells recovered after 48 and 72 h were selected from a set of electron micrographs (Fig. 5). No crystals were observed in the 407-SigE$^-$ strain harbouring pHT410 (result not shown).

SDS-PAGE analysis of the proteins contained in the cell-crystal or spore-crystal preparations from cells grown in HCT medium, showed that strain 407-SigE$^-$ harbouring pHT410 did not produce the 130 kDa CryIAa polypeptide (Fig. 6). In contrast, strain 407-SigK$^-$ harbouring pHT410 produced amounts of CryIAa toxin similar to those obtained from the strain 407 Cry$^-$ Spo$^+$ containing the same plasmid (Fig. 6).

Table 2 Insecticidal activity of the *Bt* strains

Strain	LD^a_{50}
407 Spo$^+$ (pHT410)	14.7 (7.9–21.8)
407-SigK$^-$(pHT410)	124.6 (68.6–1358.7)
407-SigK$^-$ (pHT410) sonicated for 1 min[b]	35.6 (15.2–71.2)
407-SigK$^-$ (pHT410) sonicated for 5 min[c]	9.5 (6.2–12.6)

[a] The LD_{50} is the volume (in µl) of spore-crystal or cell-crystal solution required to kill 50% of insect larvae. Confidence intervals are indicated in parentheses

[b] Cells were partially broken by sonication for 1 min. Examination of the preparation by phase-contrast microscopy showed that the crystal inclusions remained inside the cells

[c] Cells were totally broken by sonication for 5 min. Microscopic examination showed that at least 95% of the crystals were liberated

The insecticidal activities of spore-crystal and cell-crystal preparations were analysed using second-instar larvae of the lepidopteran *P. xylostella* (Table 2). Due to the presence of many proteins other than CryIAa in the 407-SigK$^-$ mutant, it was not possible to determine the precise concentration of toxin in the cell-crystal preparation from this strain. Therefore, the LD_{50} was defined in terms of volume of culture to estimate the insecticidal activity of the products. Bioassays indicated that the CryIAa toxin produced in the 407-SigK$^-$ strain was highly toxic to the *P. xylostella* larvae. However, the insecticidal activity of the products was significantly improved by sonication.

Discussion

We describe the construction of two *Bt* mutants deficient in the sporulation-specific sigma factors σ^E (σ^{35}) and σ^K (σ^{28}). The SigE$^-$ and SigK$^-$ *Bt* mutant strains are blocked at different sporulation stages and are unable to sporulate. As reported for the σ^E- and σ^K-deficient mutants in *B. subtilis* (Illing and Errington 1991; Piggot and Coote 1976), the *Bt* SigE$^-$ strain is blocked at stage II, whereas the *Bt* SigK$^-$ strain is blocked at stage IV. These two *Bt* mutant strains were used to analyse the in vivo expression of a *cryIAa′-′lacZ* transcriptional fusion during sporulation of *Bt*.

Two promoters (Bt I and Bt II) involved in *cryIAa* transcription have previously been characterized (Wong et al. 1983). Promoter Bt I is recognized by the σ^{35} factor, and Bt II is recognized by the σ^{28} factor (Brown and Whiteley 1988, 1990). Genetic studies to assess the contribution of σ^{35} (σ^E)- and σ^{28} (σ^K)-dependent promoters to the expression of *cryIAa* were previously carried out in *B. subtilis* (Whiteley et al. 1990). The Bt I promoter was not used in a *spoIIAC* mutant affected in σ^F, which is required for σ^E processing, but was used in a *spoIIIC* mutant, which is deficient in the

C-terminal half of σ^K. However, in a *B. subtilis* Spo^+ strain expression from Bt I was detected at t_3, increased markedly at t_7 and strong expression from this promoter continued to late sporulation stages (t_{13}) (Whiteley et al. 1990). In contrast, there was only a weak utilization of the Bt II promoter. The data on Bt I and Bt II promoter utilization in *B. subtilis* are confusing, because the level of σ^E in *B. subtilis*, as detected by reaction with a monoclonal antibody, was reported to decline after t_5 (Trempy et al. 1985). Whiteley et al. (1990) suggested that some other regulatory mechanisms could account for the continued expression from the Bt I promoter in *B. subtilis*. Therefore we have quantified the contributions of Bt I and Bt II promoters to *cryIAa* expression, using $SigE^-$ and $SigK^-$ mutants of *Bt*. Our results showed that the previous genetic study of these promoters, using *B. subtilis* as a model, underestimated the role of the Bt II promoter in the expression of the *cryIAa* gene.

The *spoIID* and *cotA* promoters, which are controlled by σ^E and σ^K factors, respectively, have been used as control promoters to determine the timing of σ^E- and σ^K-dependent promoter expression. The σ^E-dependent promoter shows maximal induction at about t_6, whereas the σ^K-dependent promoter is maximally induced at about t_{11} (Fig. 3A, B). The β-galactosidase expression directed from the *cryIAa* promoter region in the wild-type strain shows two peaks of maximal induction, one at t_7 and the other at t_{11}, which are correlated with the time of maximal induction of σ^E- and σ^K-specific promoters (Fig. 4). These results are in agreement with the data based on S1 mapping showing that both promoters were used sequentially during sporulation in *Bt* (Wong et al. 1983).

In the *Bt* 407-$SigE^-$ mutant strain, expression from the *cryIAa′-′lacZ* transcriptional fusion was severely reduced but not completely abolished (75-fold less β-galactosidase specific activity than the wild-type strain). This expression started at t_3 and reached a maximum (200 Miller units) at t_9 . Recently, it was proposed that the *cryIVA* gene may also be transcribed by a RNA polymerase containing σ^H (Yoshisue et al. 1995). To test the putative involvement of σ^H in *cryIAa* expression, the *cryIAa′-′lacZ* transcriptional fusion was introduced into the previously constructed *Bt spo0A* mutant (Lereclus et al. 1995). Spo0A is a transcriptional regulator essential for the initiation of sporulation and is required for the transcription of the *spo0H* gene that encodes σ^H (Dubnau et al. 1988). A low transcriptional activity (200 Miller units) was also observed in the *Bt spo0A* mutant (data not shown), thus suggesting that the low level of β-galactosidase activity observed in the *Bt* 407-$SigE^-$ mutant strain was not due to σ^H.

In the *Bt* 407-$SigK^-$ mutant strain, the transcription from the *cryIAa* promoter started at t_2 and reached a maximum at t_7. Transcription from this promoter continued at a moderate level until late sporulation (6000 Miller units versus 15000 Miller units in the wild type at t_{10}). The second induction with a maximum at t_{11}, observed in the wild-type strain, was not detectable in the *Bt* 407-$SigK^-$ mutant strain. Thus, σ^K may have an important role in *cryIAa* gene transcription during late sporulation stages and may be responsible for a large part of toxin production in the wild-type *Bt* strains. However, the level of production of CryIAa protein in the $SigK^-$ strain was as high as that in the Spo^+ strain (Figs. 5 and 6, and Table 2). This apparent discrepancy between β-galactosidase assays and toxin production may result from the following two points: (i) liberation of spores and crystals occurs at t_{15} in the wild-type strain and hence the crystal protein production ends at this time; (ii) cell lysis did not occur in the $SigK^-$ mutant strain and expression from the Bt I promoter probably continues for a longer time allowing accumulation of crystal protein within the cells.

The bioassays with the *P. xylostella* larvae demonstrated that the crystal protein enclosed within the cells (*Bt* 407-$SigK^-$ strain) was highly active. However, maximal insecticidal activity, similar to that obtained with the Spo^+ strain, was found only when the crystals were liberated from the cells by sonication. Thus, encapsulation of the crystals in a $SigK^-$ Bt mutant may reduce the rate of release and, hence, the solubilization of the crystals in the insect gut environment. Possibly, this apparent disavantage can be avoided by modifying the growth conditions to facilitate cell lysis. The production of encapsulated *Bt* crystal proteins has two principal advantages: first, release of spores into the environment can be avoided and secondly, the crystal protein may persist longer in the environment than conventional *Bt* products. However, the persistence and activity of encapsulated crystal protein within the 407-$SigK^-$ cells need to be assayed in field trials in comparison with commercial *Bt* products.

Most of the *cry* genes (*cryI*, *cryII*, *cryIV* and *cyt*) are expressed from a Bt I type promoter (Brown and Whiteley 1988; Dervyn et al. 1995; Ward and Ellar 1986; Widner and Whiteley 1989; Yoshisue et al. 1993a, b). Therefore, these genes could be introduced into a $SigK^-$ mutant to obtain strains that produce different encapsulated crystal proteins. Also, commercial strains like *B. thuringiensis* subsp. *kurstaki* HD-1 Dipel with a mutation in the *sigK* gene can be isolated and thus provide a new generation of *Bt*-based biopesticides.

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