Antonie van Leeuwenhoek (2014) 105:1007–1015
DOI 10.1007/s10482-014-0160-1

ORIGINAL PAPER

Early transcription of *Bacillus thuringiensis cry* genes in strains active on Lepidopteran species and the role of gene content on their expression

Manuel Porcar · Armelle Déleclusse · Jorge E. Ibarra · Víctor Juárez-Pérez

Received: 22 November 2013 / Accepted: 22 March 2014 / Published online: 2 April 2014

Abstract Six strains of *Bacillus thuringiensis* previously selected as highly toxic against *Manduca sexta* and *Plutella xylostella* were analyzed by PCR screening in order to identify the *cry* genes active on Lepidoptera. According to their gene content and insecticidal potency, these strains were cultured and aliquots taken at different pre- and post-sporulation times. Total RNA was extracted and used as template in RT-PCR analyses directed to identify mRNAs of the previously identified *cry* genes. Results showed transcription of genes *cry1A*, *cry1E*, *cry1I*, and *cry2* even before the onset of sporulation. However, this early transcription did not lead to an appreciable parasporal protein synthesis until t_5–t_9, as deduced from SDS-PAGE profiles. As for *cry1I* gene, the corresponding protein was not detected, as expected, but *cry1I* mRNAs were present at least until t_5. Interestingly, strains expressing four *cry* genes from the end of the log phase onwards exhibited kinetics characterized by a very long transition phase, whereas the strain expressing only one *cry* gene showed a very short transition phase. Strains expressing three genes showed an intermediate profile. These results indicate that the transcription of *B. thuringiensis cry1* and *cry2* genes in natural strains can start several hours before massive crystal synthesis occurs and that this translation is probably competing with transcriptional regulators required for the sporulation onset.

Keywords Sporulation · *Cry* genes · Sporulation time courses

Electronic supplementary material The online version of this article (doi:10.1007/s10482-014-0160-1) contains supplementary material, which is available to authorized users.

M. Porcar · A. Déleclusse · V. Juárez-Pérez
Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France

M. Porcar
Fundació General de la Universitat de València, Cavanilles Institute of Biodiversity and Evolutive Biology, 46020 Valencia, Spain

J. E. Ibarra (✉)
Departamento de Biotecnología y Bioquímica, Centro de Investigación y Estudios Avanzados (CINVESTAV-I.P.N.), Apartado Postal 629, 36500 Irapuato, GTO, México
e-mail: jibarra@ira.cinvestav.mx

V. Juárez-Pérez
Stragen France, 60 Av. Rockefeller, 69008 Lyon, France

Introduction

Bacillus thuringiensis (Bt) is a spore-forming bacterium that has been used worldwide as bio-insecticide for more than five decades. The main toxic factors are proteins present in its parasporal crystal (Sanchis 2011). Crystal proteins are produced *de novo* during sporulation and may represent up to 30 % of the total dry weight of the culture (Schnepf 2012). The two main crystal components are the Cry and the Cyt proteins. Cry proteins are

the most frequent and diverse and display a wide insecticidal spectrum (Crickmore et al. 1998; de Maagd et al. 2003). Due to their insecticidal nature and their high level of production, basic research on the regulation mechanisms of expression of these proteins has kept great interest, mostly to improve the toxin production and thus the profitability of Bt-based products (Federici 2005).

The high expression level of those proteins is the result of a series of finely regulated pre- and post- transcriptional and translational events (Gong et al. 2012). First, the RNA transcripts of the *cry* genes have a longer life (10 min) than the rest of the other cell transcripts. The presence of stem or stem-loop structures located at the 3′-end of several *cry* genes transcripts may prevent their degradation by RNAses which could explain their long life (Agaisse and Lereclus 1995). Also, to prevent transcript degradation in *cry3* genes, a Shine-Dalgarno motif called STAB-SD, has been found upstream from the initiation codon, which stabilizes even heterologous transcripts (García-Gómez et al. 2013) and therefore increases the expression of the protein (Park et al. 1998). Additionally, it has been reported that some *cry1* genes are positively regulated by the 2E subunit of the pyruvate dehydrogenase, as a binding site of this co-factor, located upstream the promoter region (Walter and Aronson 1999).

Studies on *cry* gene regulation, based on fusions to the *lacZ* reporter gene, have shown that most of the *cry* genes are sporulation-dependent. These results demonstrate that *cry* genes are regulated by sigma factors homologous to σ^H, σ^E and/or σ^K factors of *B. subtilis*. Analyses of β-galactosidase kinetics showed that two overlapped promoters sequentially regulate *cry1* genes by the σ^E and the σ^K factors during the sporulation phase (Lereclus et al. 2000). However, the same genes may be regulated by σ^H-like promoters, upstream the coding sequences (Pérez-García et al. 2010). Some other genes, such as *cry4* and *cry10* seem to be transcribed earlier during the transition phase also by the σ^H factor (Poncet et al. 1997). Sequence analyses of promoter regions from other *cry* and *cyt* genes suggest a similar temporal regulatory course. Interestingly, the expression of *cry3* genes is regulated by a promoter that is recognized during the vegetative stage, whose sequence is highly similar to those recognized by the σ^A factor. Accordingly, the expression of the *cry3A* gene in a Bt *spo0A* mutant, unable to initiate sporulation, induces an overproduction of the Cry3A protein (Agaisse and Lereclus 1994), opposed to what occurs in the rest of sporulation-dependent *cry* genes, such as *cry1* (Yang et al. 2012).

Table 1 Insecticidal activity of selected *B. thuringiensis* strains against *Manduca sexta* and *Plutella xylostella*; and their Lepidopteran-active *cry* gene content as determined by PCR

Strain	Serovariety	LC_{50}	*cry* genes
Manduca sexta (ng/cm^2)			
LBIT-7	*kenyae*	94.85	*1A, 1E, 1I, 2*
LBIT-404	*kenyae*	37.47	*1A, 1E, 1I, 2*
LBIT-418	*kenyae*	14.97	*1A, 1E, 1I, 2*
Plutella xylostella (ng/ml)			
LBIT-127	*kurstaki*	192.4	*1A, 1I, 2*
LBIT-287	*kurstaki*	100.3	*1A, 1I, 2*
LBIT-290	*kurstaki*	102.9	*1A*

Besides Cry protein expression strategies (Gong et al. 2012), stability of the high levels of Bt toxin production is achieved by their crystallization, as proteins are protected from degradation by endo-proteases. It is generally accepted that large Cry proteins (120–140 kDa) crystallize spontaneously due to the presence of a conserved cystein-rich C-terminus moiety, called the "structural domain" (De Maagd et al. 2001, García-Gómez et al. 2013). To overcome the absence of the structural domain of some Cry proteins, Bt has developed different post-translational solutions: a) crystallization is directed either by the presence of helper proteins, found in the vicinity of some *cry* or *cyt* genes, or b) by chaperon proteins (Wu and Federici 1993). Exceptionally, Cry1I proteins do not form crystals and are secreted to the medium, although the mechanism involved in the secretion is unknown (Ruiz de Escudero et al. 2006).

Most of the information concerning the *cry* and *cyt* expression has been obtained from transcriptional fusions of the promoter regions or mutation of the putative motifs involved in their expression. However, few works has been done with strains in their natural genetic background. This report correlates the kinetics of Cry protein production by SDS-PAGE with the detection, by RT-PCR, of early transcripts of the *cry* genes detected in six strains highly toxic to lepidopteran pests.

Materials and methods

Bacterial strains

A set of six *B. thuringiensis* strains (Table 1), native to Mexico and originally isolated form dust from grain storage bins (5) and dead insect (1), were used in this study. These strains were previously selected by their high insecticidal activity towards *Manduca sexta* and *Plutella xylostella*, out of more than 400 native isolates, by using single-dose bioassays. These strains were serologically identified by the International Entomopathogenic Bacillus Center (IEBC) at the Institut Pasteur (Table 1), and subjected to further characterization, as described below.

Bioassays

Bioassays on *M. sexta* and *P. xylostella* larvae used freeze-dried powders of spore-crystal complexes from each strain, suspended in 0.02 % Tween 80. Bioassays on first instar *M. sexta* larvae were prepared by spreading 200 µl of specific concentrations of the spore-crystal complexes on artificial diet plates previously poured into Petri dishes. Once the suspension on the diet was air-dried, ten larvae were transferred to each plate, using two plates per concentration. Mortality was assessed after five days of incubation at 28 ± 2 °C, 70 ± 5 % relative humidity, and 16:8 h light/dark photoperiod. Bioassays on *P. xylostella* used a leaf-dip technique. Disks of ca. 25 cm^2 were cut from fully expanded leaves of greenhouse-grown broccoli and dipped for 1 min under vacuum conditions in 20 ml of spore-crystal complex suspensions. Disks were air-dried at room temperature and placed in Petri dishes. Ten first-instar larvae were placed on each disk, using two disks per concentration, and incubated for 5 days as described above before they were checked for mortality. In both bioassays a total of 6 concentrations per bioassay were used, plus the control (only Tween solution). Mean LC_{50} values were estimated from a minimum of three replicates and all the statistical requirements listed earlier (Ibarra and Federici 1987) were fulfilled.

PCR amplification

A loopfull of cells from an overnight Luria–Bertani (LB) agar plate was re-suspended in 100 µl sterile water and boiled for 10 min. 6 µl of this suspension were added as template to 44 µl of PCR mix containing primers (1 µM each), dNTPs (200 µM each), $MgCl_2$ (3 mM), and 2.5 units of Taq DNA Polymerase (Invitrogen). Table 2 shows the oligonucleotides used for the specific amplification of *cry* genes known to code for Lepidoptera-active δ-endotoxins: *cry1A, cry1B, cry1C, cry1D, cry1E, cry1F, cry1G, cry1H, cry1I, cry1J, cry1K, cry2A* and *cry9* (Porcar and Juárez-Pérez 2003). Novel oligonucleotides were designed from a sequence alignment of consensus *cry1*, *cry2* and *cry9* sequences. Forward primers specifically recognizing *cry1A-cry1H* were used in combination with the general I(-) reverse primer previously described (Juárez-Pérez et al. 1997), whereas for *cry1I, cry1 J, cry1 K, cry2A* and *cry9,* specific forward and reverse primers were designed. The PCR cycling conditions were set as follows: a 3-min initial step at 95 °C, and 30 cycles of amplification (1 min at 95 °C, 1 min 45 s at 48 °C and 1 min 45 s at 72 °C) followed by a final step of 72 °C during 10 min. All PCR were repeated twice with independent PCR kits, primer dilutions and bacterial cultures.

Culture conditions

Kinetics, SDS-PAGE and RT-PCR analyses were performed on the same culture, in all cases. Synchronous cultures were achieved by preparing precultures of 50 ml LB broth inoculated with a single colony from an LB plate and grown overnight at 180 rpm and 30 °C. A second preculture was inoculated with an aliquot from the first one at its log phase. Then two-liter flasks containing 500 ml LB broth were inoculated with this last preculture, at the middle of its log phase, diluted to a DO_{600} of 0.5 and grown with shaking (180 rpm) at 30 °C during 16 h. Aliquots (1 ml) were taken each hour and the DO_{600} measured in order to determine the time-growth curve.

SDS-PAGE analysis

1 ml of each culture was taken from t_{-1} onwards every two hours, washed once with NaCl 1 M and twice with sterile bi-distilled water. Pellets were finally re-suspended in 250 µl bi-distilled water and kept frozen (−20 °C) until required. 10 % SDS-PAGE gels were prepared and run as previously described (Laemli 1971).

Table 2 Primers used for the PCR-identification of Lepidoptera-active *cry* genes and RT-PCR analyses

Gene recognized	Primer (5′–3′ sequence)	Expected amplicon (bp)	Source
cry1A	A-FW (CAATAGTCGTTATAATGATT)	1720[a]	Juárez-Pérez et al. 1997
	A-RT (GTTAATTGGGAAACTGTTC)	224[b]	This work
cry1B	B-FW (GGCTACCAATACTTCTATTA)	1321[a]	Juárez-Pérez et al. 1997
cry1C	C-FW (ATTTAATTTACGTGGTGTTG)	1174[a]	Juárez-Pérez et al. 1997
cry1D	D-FW (CAGGCCTTGACAATTCAAAT)	1136[a]	Juárez-Pérez et al. 1997
cry1E	E-FW (TAGGGATAAATGTAGTACAG)	1133[a]	Juárez-Pérez et al. 1997
	E-RT (TGACTATATCCAACTAATGA)	135[b]	This work
cry1F	F-FW (GATTTCAGGAAGTGATTCAT)	967[a]	Juárez-Pérez et al. 1997
cry1G	G-FW (GCTTCTCTCCAAACAACG)	521[a]	This work
cry1H	H-FW (ACTCTTTTCACACCAATAAC)	567[a]	This work
cry1I	I-FW (ACAATTTACAGCTTATTAAG)	1133	This work
	I-RV (CTACATGTTACGCTCAATAT)		This work
	I-RT (ACTCCATTAACAGGTTGAG)	226–233[b]	This work
cry1 J	J-FW (GCGCTTAATAATATTTCACC)	1089[a]	This work
cry1 K	K-FW (TGATATGATATTTCGTAACC)	1132[a]	This work
cry1 s	I(-) (MDATYTCTAKRTCTTGACTA)		Juárez-Pérez et al. 1997
cry2A	2-FW (CGATATGTTAGAATTTAGAAC)	984	This work
	2-RV (TACCGTTTATAGTAACTCG)		This work
	2-RT (GAATTAACTTGGAAAAGAG)	194[b]	This work
cry9	9-FW (CAAAAAGATGTTATTGGAAG)	925	This work
	9-RV (GATACGATGCTTGTTGTAAG)		This work

[a] Amplification fragments when primers are used in combination with I(−)

[b] Amplification fragments when used in combination with the specific forward primer of the corresponding gene (i.e. A-FW and A-RT)

RNA extraction

Two polypropylene Falcon tubes were filled with 10 ml aliquots of the growing cultures taken at t_{-2}, t_5 and t_{11}. Each 10-ml culture was pelleted and then re-suspended in 3.6 ml of RLT buffer (Qiagen). Bacterial suspensions were transferred into 2 ml RNase-free tubes (600 µl in each tube) containing 0.25 g of glass beads (100 µm diameter). Cells were lysed in a FastPrep instrument (Bio101 Savant, Eppendorf) during 45 s at 4 °C. Tubes were then centrifuged (10,000 rpm, 20 min) and 500 µl of the supernatant were recovered and mixed with 500 µl of 70 % ethanol. Mixtures were decanted into an RNeasy mini column and processed as described at the manufacturer's protocol (Qiagen), including a digestion step with 10 µl of Dnase I (Amersham-Pharmacia Biotech) per column. Total RNA were eluted from the columns with 50 µl of RNAse-free water, pooled and subjected to a second Dnase I treatment during 15 min at 37 °C. The enzyme was inactivated at 70 °C for 10 min. Then, 1 ml Trizol (GibcoBRL) was added, samples were incubated at room temperature for 5 min and 200 µl of chloroform were added. After some vigorous inversions, samples were incubated 2 min at room temperature and then centrifuged at 12,000 × g for 15 min at 4 °C. The aqueous phase was transferred to a fresh RNase-free tube. RNA was precipitated by the addition of 0.5 ml of isopropyl alcohol, kept at room temperature during 30 min and recovered by centrifugation at 12,000 × g for 15 min at 4 °C. Pellets were washed with 70 % ethanol, re-suspended in 50 µl of RNase-free water and frozen immediately at −20 °C. To verify the integrity of the RNA, aliquots were visualized in RNase-free 2 % agarose TAE gels and quantified with an Eppendorf Biophotometer.

RT-PCR amplifications

Reverse transcriptase PCR from total RNA isolated from *B. thuringiensis* cultures was performed with Superscript II RNase H- Reverse Transcriptase (Invitrogen) following the instructions of the manufacturer. 250 ng of total RNA were used as template for each RT-PCR reaction carried out with specific reverse oligonucleotides ("RT" primers in Table 2). Polymerization was performed at 42 °C for 50 min and the enzyme was heat-inactivated at 70 °C for 15 min. In order to discard the presence of contaminating DNA in the purified RNA samples, 2 µl of the RT-PCR reaction were used as template for a standard PCR analysis, and control reactions were set with 25 ng of RNA. The same forward primers used for the standard PCR described above were used, combined with the reverse primers designed for RT-PCR (Table 2). PCRs were carried out with the following program: a first step at 95 °C for 2 min and 30 cycles of amplification (1 min at 95 °C, 1 min at 48 °C and 30 s at 72 °C) followed by a final 5 min step at 72 °C. Aliquots (10 µl) of each reaction were run in a 1.7 % TAE agarose gels.

Results

Strain diversity

Out of more than 400 native isolates used to select highly toxic strains of *B. thuringiensis*, the three selected strains toxic to *M. sexta* showed LC_{50} values ranging from 14.97 to 94.85 ng/cm^2, while the three strains active on *P. xylostella* exhibited LC_{50} values between 100.3 and 192.4 ng/ml (Table 1). In both cases, these values showed that all the strains showed high levels of toxicity. Additionally, two different serovars were represented among all the strains analyzed, belonging to serovar *kenyae* the strains toxic to *M. sexta*, and to *kurstaki* the strains toxic to *P. xylostella*.

PCR-based identification of Lepidoptera-active *cry* genes yielded three different profiles (Table 1). The combination *cry1A-cry1E-cry1I-cry2* was detected in the three strains toxic to *M. sexta*, and the profiles *cry1A-cry1I-cry2* and *cry1A* were detected in the strains toxic to *P. xylostella*. Strains LBIT-7, LBIT-404 and LBIT-418 shared the gene profile *cry1A-cry1E-cry1I-cry2* in spite of showing significant differences in their toxicity levels on *M. sexta* (Table 1). Likewise, strains LBIT-127 and LBIT-287 showed the gene profile *cry1A-cry1I-cry2* in spite of being the former almost half as toxic as the latter on *P. xylostella*. Strain LBIT-290 showed only one

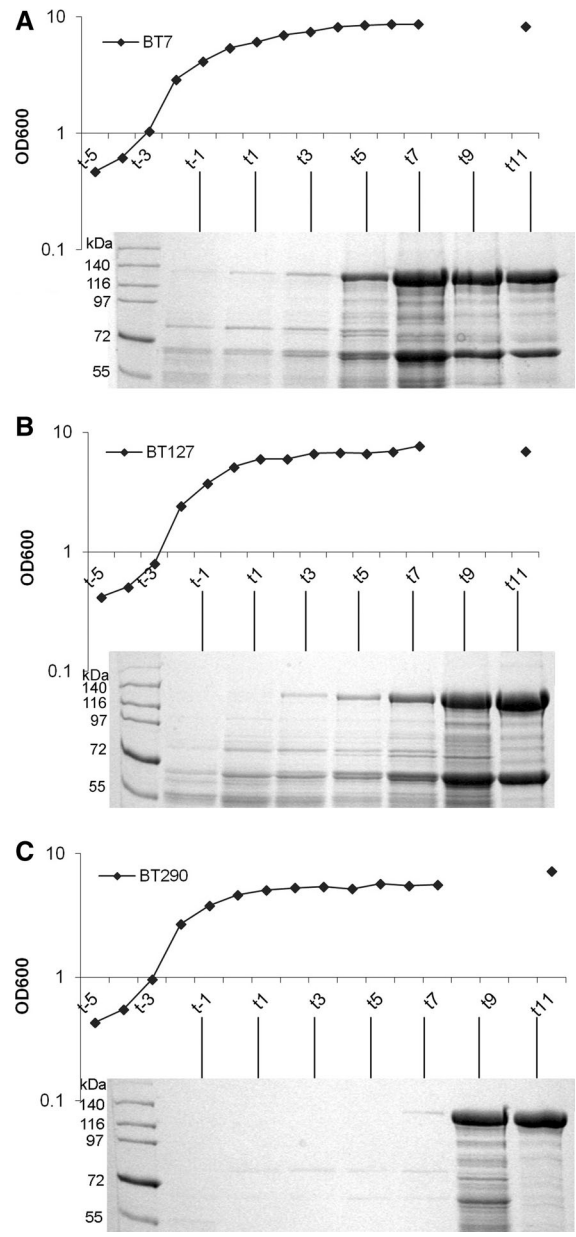

Fig. 1 Time courses (OD_{600} readings) and SDS-PAGE profiles of strains LBIT-7 (**a**), LBIT-127 (**b**) and LBIT-290 (**c**). Protein contents of cell extracts at the analyzed times are given below the corresponding points of the kinetics

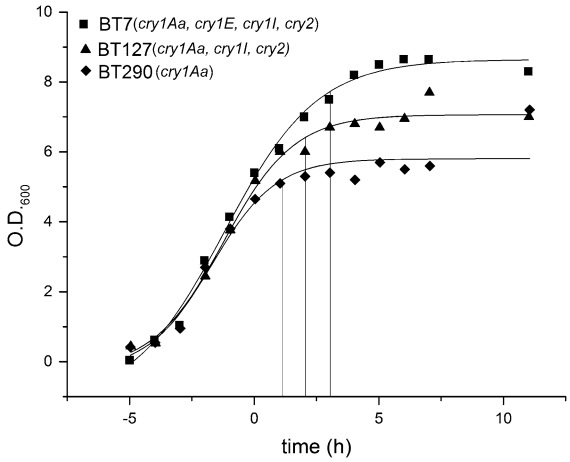

Fig. 2 Comparison of growth curves from strains LBIT-7, LBIT-127 and LBIT-290. Lines are defined by the best quadratic fit (logistic curve) of data. Vertical lines indicate the onset of the transition phase for each strain, which indicates that the higher the number of *cry* genes in the strain the longest it takes to the onset point

Lepidoptera-active gene type (*cry1A*), and still showed the same high toxicity as LBIT-287.

Sporulation kinetics and protein profiling of selected strains

Kinetics of strains LBIT-7, LBIT-404 and LBIT-418 were very similar and characterized by a long transition phase between the exponential growth phase and the stationary phase (see Supplementary Fig. 1). Cry protein synthesis, as revealed by two main bands of about 130 and 60 kDa in SDS-PAGE analysis, faintly appeared at t_1, and dramatically increased from t_5–t_7 onwards (Fig. 1a).

Strains LBIT-127 and LBIT-287 both exhibited similar kinetics (see Supplementary Fig. 2). They had a long transition phase, but to a lesser extent than that of *M. sexta*-active strains. Protein profiles of these strains consisted of two main peptides of about 130 and 60 kDa (Fig. 1b). Cry protein expression was slightly delayed but not as much as those shown by *M. sexta*-active strains, and the maximum Cry protein production in LBIT-127 and LBIT-287 growing cultures was achieved at t_9 and t_{11} (as compared to t_7 for LBIT-7, LBIT-404 and LBIT-418).

Strain LBIT-290 (Fig. 1c and Supplementary Fig. 2) showed a distinctive growth curve and protein profiles. The growing culture rapidly reached its maximum OD_{600}, within a very short transition phase. Cry protein synthesis (a main band of about 130 kDa) was not detectable till t_9 and t_{11}, when a major band of ca. 130 kDa. Suddenly appeared in the SDS-PAGE profile.

A comparison of the kinetics of one strain from each group (LBIT-7, LBIT-127 and LBIT-290) shows how the transition phase, indicated by its onset using the second derivate of the growth curve, takes more time as the number of *cry* genes is higher (Fig. 2). This observation is also related to the timing of highest Cry protein expression, by showing it earlier as the number of *cry* genes is higher (see above).

Transcription of *cry1* and *cry2* genes

Total RNA from the six selected strains was subjected to RT-PCR analyses directed to the identification of mRNAs corresponding to genes *cry1A, cry1E, cry1I*, and *cry2* at t_{-2}, t_5, and t_{11}. RT-PCR of strains LBIT-7, LBIT-404 and LBIT-418 yielded amplicons indicating the transcription of all four genes. While in LBIT-7 and LBIT-404 the mRNAs of all these genes were detected at all the tested times (see Supplementary Fig. 3), LBIT-418 lacked detectable amounts of *cry1I* mRNA at t_{11} (Table 3). Within the *P. xylostella*-active strains, LBIT-127 and LBIT-287 showed amplification of the expected size for of *cry1A, cry1I*, and *cry2*

Table 3 Detection of mRNAs corresponding to *cry1A, cry1E, cry1I,* and *cry2* genes by amplification in RT-PCR analyses performed at three times in the culture kinetics

Strain	*cry*-gen content	mRNA detected		
		t_{-2}	t_5	t_{11}
LBIT-7	1A, 1E, 1I, 2	*1A, 1E, 1I, 2*	*1A, 1E, 1I*[a], *2*	*1A, 1E, 1I, 2*
LBIT-404	*1A, 1E, 1I, 2*	*1A*[a], *1E, 1I*[a], *2*	*1A, 1E, 1I, 2*	*1A, 1E, 1I, 2*
LBIT-418	*1A, 1E, 1I, 2*	*1A, 1E, 1I*[a], *2*	*1A, 1E, 1I, 2*	*1A, 1E, 2*
LBIT-127	*1A, 1I, 2*	*1A, 1I, 2*	*1A, 1I, 2*	*1A, 2*
LBIT-287	*1A, 1I, 2*	*1A, 1I, 2*	*1A, 1I, 2*	*1A, 1I, 2*
LBIT-290	*1A*	*1A*	*1A*	*1A*

[a] Only a faint band was observed

at the three times of observation, except for *cry1I* in LBIT-127, which lacked detectable amounts of its mRNA at t_{11} (Table 3). Similar to these two strains, where all the mRNAs were detected as early as t_{-2}, the mRNA for *cry1A* was also detected in LBIT-290 at t_{-2}, t_5, and t_{11} (Table 3). Under the tested conditions, the negative control (standard PCR lacking the RT step but using the same amount (250 ng) of total RNA) always failed to produce amplification, indicating a lack of detectable DNA contamination.

Discussion

In a nation-wide isolation program of *B. thuringiensis* strains native to Mexico, a selection of highly toxic Lepidopteran-active isolates was carried out. The 6 isolates studied in this work were selected out of more than 400 native strains, based on their high toxicity to *M. sexta* and *P. xyllostella*, as well as on their *cry* gene content. Interestingly, the three strains active against *M. sexta* showed the same *cry*-gene profile, in spite of the significant difference among their toxicity, somewhat similar to the observations made on the three strains toxic to *P. xyllostella*. The lack of a direct correlation between *cry* gene content and the relative proportion of Cry proteins forming the *B. thuringiensis* parasporal crystal is the main limitation of PCR, to accurately predict the insecticidal activity of a given strain. Thus, determining which of the *cry* genes present are expressed and at what extent is critical to overcome the predicting abilities of the PCR technique.

For this purpose, we used RT-PCR to detect transcripts corresponding to genes *cry1A*, *cry1E*, *cry1I*, and *cry2*, which were previously identified by standard PCR. Transcripts from each gene present in the six strains were detected, at least in two of the three different times checked in the culture kinetics. Transcription of *cry1A*, *cry1E*, and *cry2* genes in Lepidopteran-active strains is not surprising, as the corresponding proteins are active on many Lepidopteran species. However, the presence of *cry1I* transcripts in almost all of the analyzed strains is somehow unexpected. In fact, *cry1I* genes were supposed to be cryptic due to the lack of a known promoter sequence (Gleave et al. 1993) until the Cry1I protein was reported to be present in the supernatant of cultures as a soluble protein secreted at the beginning of the sporulation phase (Kostichka et al. 1996). That should be the reason why peptide bands corresponding to Cry1I proteins were not detected in SDS-PAGE performed from spore-crystal preparations. This may also indicate that these proteins lack the crystalogenic fraction, in accordance with Kostichka et al. (1996), but it may also be due to a very low transcription and/or translation level. Also, in accordance to the same report, our results indicate that *cry1I* genes have a functional promoter that can be active as late as t_{11}.

More interesting is that transcription of *cry* genes, as revealed by RT-PCR, was detected at very early stages of sporulation, even before the sporulation onset. This early detection of transcripts is in contrast with the widely accepted fact that *cry* genes are transcribed after the sporulation onset (Lereclus et al. 2000). In fact, regulation of gene expression in post-exponential phase is a complex process involving many control mechanisms (for a revision see de Hoon et al. 2010). In *B. thuringiensis*, two different mechanisms regulate the expression of *cry* genes. The first one is sporulation-dependent and involves transcription by sporulation-specific sigma factors σ^E and σ^K from t_0 onwards. The second mechanism is independent of sporulation, such as the coleopteran-active *cry3A* gene, which is expressed during the stationary phase from t_0 to t_{10}, but also, to a lesser extent, during the vegetative growth (Agaisse and Lereclus 1994).

Weak transcription during the late exponential phase of several sporulation-dependent *cry* genes has been reported earlier (Poncet et al. 1997). The early transcription of Lepidopteran-active protein we report in this work is in accordance with these previous reports and suggests that some transcription of so called sporulation-dependent *cry* genes at the end of the exponential phase is a general phenomenon among *B. thuringiensis* strains. Since the early transcription does not correlate with important protein production as deduced by SDS-PAGE profiles we obtained, it is likely that a limited transcription occurs during the transitional phase, regulated by factors such as σ^H (Lereclus et al. 2000; Pérez-García et al. 2010). Also, it must be noticed that reliable synchrony of the cultures was achieved by the serial pre-cultures used to inoculate the final cultures used for analysis.

Another interesting observation made in this work was a correlation between gene content and protein profile found in the six analyzed strains. The crystal protein synthesis of the three strains sharing the same four *cry* genes was detected rather soon after the

sporulation onset and peaked at t_7. The strains with three *cry* genes showed peptide bands corresponding to Cry proteins starting at t_3–t_5 and peaking at t_9–t_{11}; while the strain with only one cry gene showed a distinctive profile, as no detectable protein was observed but until t_9, when an important protein concentration was found. Interestingly, *cry* gene content not only correlated with SDS-PAGE profiles, but also with time courses. A very long transition phase was found in strains expressing four genes, as opposed to a very short one in the strain with only one *cry* gene. Similar results were observed in a *B. thuringiensis* strain containing five *cry* genes (Wang et al. 2012).

These results suggest a competence between the expression of the *cry* genes and the sporulation-related genes. The transcription of *cry* genes is mediated by RNA polymerases associated to sigma factors. Some of these sigma factors, such as σ^H, are active early in the sporulation process and allow the transcription of *cry* genes but also of other genes, such as the sporulation regulator *spo0A* (Lereclus et al. 2000; Pérez-García et al. 2010; Yang et al. 2012). It is well known that the concentration of this regulator determines whether the cell sporulates or not, and at what pace. Accordingly, it may be suggested that in strains bearing several *cry* genes, the titration of the sigma factors would result in a low Spo0A concentration and hence in a long transition phase, causing a delay in the sporulation completion. On the other hand, in *B. thuringiensis* strain expressing a single *cry* gene, the production of Spo0A might be significantly greater. These strains could rapidly reach the critical Spo0A threshold and thus exhibit short transition phases. Since Spo0A is known to repress the transcription of the *cry* genes (Yang et al. 2012), this is probably playing a role in the late protein production of strain LBIT-290, containing only one *cry* gene.

The competence mechanism we propose and the repressing effect of Spo0A can explain the correlation between *cry* genes array, time courses and protein profiles. However, the explosive crystal protein expression typical of *B. thuringiensis* was produced too late, especially in strain LBIT-290, which may be the consequence of other mechanisms involved, as indicated by a proteomics study of the late stationary phase (Gong et al. 2012). The time required for the translation of *cry* transcripts may partially explain this phenomenon. But it is likely that other repressing factors, such as AbrB (Lereclus et al. 2000), take the relay of Spo0A to control Cry proteins expression during the late stationary phase.

References

Agaisse H, Lereclus D (1994) Expression in *Bacillus thuringiensis* cryIIIA toxin gene is not dependent on a sporulation-specific sigma factor and is increased in a spoOA mutant. J Bacteriol 176:4734–4741

Agaisse H, Lereclus D (1995) How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? J Bacteriol 177:6027–6032

Crickmore N, Zeigler DR, Feitelson J, Schnepf HE, Van Rie J, Lereclus D, Baum J, Dean DH (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol Mol Biol Rev 62:807–813

de Hoon MJL, Eichenberger P, Vitkup D (2010) Hierarchical evolution of the bacterial sporulation network. Curr Biol 20:R735–R745

De Maagd RA, Bravo A, Crickmore N (2001) How *Bacillus thuringiensis* has evoled specific toxins to colonize the insect world. Trends Genet 17:193–199

De Maagd RA, Bravo A, Berry C, Crickmore N, Schnepf HE (2003) Structure, diversity and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annu Rev Genet 37:409–433

Federici BA (2005) Insecticidal bacteria. An overwhelming success for invertebrate pathology. J Invertebr Pathol 89:30–38

García-Gómez BI, Sánchez J, Martínez de Castro DL, Ibarra JE, Bravo A, Soberón M (2013) Efficient production of *Bacillus thuringiensis* Cry1AMod toxins under regulation of *cry3Aa* promoter and single cysteine mutations in the protoxin region. Appl Environ Microbiol 79:6969–6973

Gleave AP, Williams R, Hedges RJ (1993) Screening by polymerase chain reaction of *Bacillus thuringiensis* serotypes for the presence of *cryV*-like insecticidal protein genes and characterization of a *cryV* gene cloned from *B. thuringiensis* subsp. *kurstaki*. Appl Environ Microbiol 59:1683–1687

Gong Y, Li M, Xu D, Wang H, He J, Wu D, Chen D, Qiu N, Bao Q, Sun M, Yu Z (2012) Comparative proteomic analysis revealed metabolic changes and the translational regulation of Cry protein synthesis in *Bacillus thuringiensis*. J Proteom 75:1235–1246

Ibarra JE, Federici BA (1987) An alternative bioassay employing neonate larvae for determining the toxicity of suspended particles to mosquitoes. J Am Mosq Control Assoc 3:187–192

Juárez-Pérez VM, Ferrandis MD, Frutos R (1997) PCR-based approach for the detection of novel *Bacillus thuringiensis* genes. Appl Environ Microbiol 63:2997–3002

Kostichka K, Warren GW, Mullins M, Mullins AD, Palekar NV, Craig JA, Koziel MG, Estruch JJ (1996) Cloning of a *cryV*-type insecticidal protein gene from *Bacillus thuringiensis*: the *cryV*-encoded protein is expressed early in stationary phase. J Bacteriol 178:2141–2144

Lereclus D, Agaisse H, Grandvalet C, Salamitou S, Gominet M (2000) Regulation of toxin and virulence gene transcription in *Bacillus thuringiensis*. Int J Med Microbiol 290:295–299

Park H-W, Ge B, Bauer LS, Federici BA (1998) Optimization of Cry3A Yields in *Bacillus thuringiensis* by use of sporulation-dependent promoters in combination with the STAB-SD mRNA sequence. Appl Environ Microbiol 64:3932–3938

Pérez-García G, Basurto-Ríos R, Ibarra JE (2010) Potential effect of a putative σ^H-driven promoter on the over expression of the Cry1Ac toxin of *Bacillus thuringiensis*. J Invertebr Pathol 104:140–146

Poncet S, Dervyn E, Klier A, Rapoport G (1997) SpoOA represses transcription of the *cry* toxin genes in *Bacillus thuringiensis*. Microbiology 143:2743–2751

Porcar M, Juárez-Pérez VM (2003) PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. FEMS Microbiol Rev 26:419–432

Ruiz de Escudero I, Estela A, Porcar M, Martínez C, Oguiza JA, Escriche B, Ferré J, Caballero P (2006) Molecular and insecticidal characterization of a Cry1I protein toxic to insects of the families Noctuidae, Tortricidae, Plutellidae, and Chrysomelidae. Appl Environ Microbiol 72:4796–4804

Sanchis V (2011) From microbial sprays to insect-resistant transgenic plants: history of the biospesticide *Bacillus thuringiensis*. A review. Agron Sustain Dev 31:217–231

Schnepf HE (2012) *Bacillus thuringiensis* Recombinant Insecticidal Protein Production. In: Sansinenea E (ed) *Bacillus thuringiensis* Biotechnology. Springer, Heidelberg, pp 259–281

Walter T, Aronson A (1999) Specific binding of the E2 subunit of pyruvate dehydrogenase to the upstream region of *Bacillus thuringiensis* protoxin genes. J Biol Chem 274:7901–7906

Wang J, Mei H, Qian H, Tang Q, Liu X, Yu Z, He J (2012) Expression profile and regulation of spore and parasporal crystal formation-associated genes in *Bacillus thuringiensis*. J Proteom Res 12:5487–5501

Wu D, Federici BA (1993) A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. J Bacteriol 175:5276–5280

Yang H, Wang P, Peng Q, Rong R, Liu C, Lereclus D, Zhang J, Song F, Huang D (2012) Weak transcription of the *cry1Ac* gene in nonsporulating *Bacillus thuringiensis* cells. Appl Environ Microbiol 78:6466–6474