Note

EXPRESSION OF THE SIGMA^{35} AND *CRY2AB* GENES INVOLVED IN *Bacillus thuringiensis* VIRULENCE

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ABSTRACT: There are several genes involved in *Bacillus thuringiensis* sporulation. The regulation and expression of these genes results in an upregulation in Cry protein production, and this is responsible for the death of insect larvae infected by *Bacillus thuringiensis*. Gene expression was monitored in *Bacillus thuringiensis* during three developmental phases. DNA *macroarrays* were constructed for selected genes whose sequences are available in the GenBank database. These genes were hybridized to cDNA sequences from *B. thuringiensis* var. *kurstaki* HD-1. cDNA probes were synthesized by reverse transcription from *B. thuringiensis* RNA templates extracted during the exponential (log) growth, stationary and sporulation phases, and labeled with ^{33}PadCTP. Two genes were differentially expressed levels during the different developmental phases. One of these genes is related to sigma factor (sigma^{35}), and the other is a *cry* gene (*cry2Ab*). There were differences between the differential levels of expression of various genes and among the expression detected for different combinations of the sigma factor and *cry2Ab* genes. The maximum difference in expression was observed for the gene encoding sigma^{35} factor in the log phase, which was also expressed at a high level during the sporulation phase. The *cry2Ab* gene was only expressed at a high level in the log phase, but at very low levels in the other phases when compared to the sigma^{35}.
Key words: *cry* genes, gene expression, sigma factor

EXPRESSÃO DOS GENES SIGMA^{35} E *CRY2AB* ENVOLVIDOS NA VIRULÊNCIA DE *Bacillus thuringiensis*

RESUMO: Muitos genes estão envolvidos nos mecanismos de esporulação da bactéria *Bacillus thuringiensis.* A regulação e expressão desses genes resultam em uma produção massiva da proteína Cry, responsável pela morte das larvas de muitos insetos. Neste trabalho monitorou-se a expressão de genes de *Bacillus thuringiensis*, ao longo de três fases de seu desenvolvimento. Foram construídos ma*croarrays* de DNA dos genes selecionados, cujas seqüências estão disponibilizadas no GenBank. Estes genes foram hibridizados com cDNAs obtidos de *B. thuringiensis kurstaki* HD-1. As sondas de cDNA foram sintetizadas a partir da transcrição reversa do RNA da bactéria, extraído durante as fases de crescimento logarítmico, estacionária e esporulativa, marcadas com ^{33}PadCTP. A expressão diferencial encontrada foi significativa para dois genes de *B.thuringiensis*, um relacionado aos fatores sigma (sigma^{35}) e outro ao gene *cry* (*cry2Ab*). Detectaram-se diferenças entre as médias de expressão do fator sigma e do gene *cry2Ab*. Os valores máximos de expressão diferencial foram obtidos para o gene codificador do fator sigma^{35} na fase log e na fase esporulativa. Na análise de médias observou-se expressão do gene *cry2Ab* apenas na fase log; no entanto, de forma bem mais baixa quando comparado com a expressão de sigma^{35}, nas três fases.
Palavras-chave: genes *cry*, expressão gênica, fator sigma

INTRODUCTION

Many genes are involved in sporulation mechanisms. It is estimated that about 800 genes are active in this phase in *Bacillus subtilis*, and this is in addition to the pleiotropic effects of many genes and other cell functions that determine the production of enzymes and antimicrobial substances. Sporulation is the result of the successive actions of several genes that act in cascade (Balassa, 1969).

During sporulation, the regulation of *cry* genes results in upregulation of the Cry proteins, which kill insect larvae (Lereclus et al., 2000). These proteins also allow the germination of bacterial spores and multiplication of bacterial cells in larval intestines. The production of extracellular factors in response to nutrient deficiency allow bacterial cells to wound and invade host tissues, accessing alternative nutrient sources and providing favorable conditions for a new multiplication or exponential growth cycle.

The expression of *cry* genes is regulated by two mechanisms. One of them, which regulates most of the *cry* genes, depends on specific sigma factors of the sporulation phase. The other mechanism does not depend on sporulation, and regulates factors that are typical of the stationary phase, e.g., *cry* 3 (Valadares-Inglis et al*.,* 1998).

The selection of *B. thuringiensis* isolates with high virulence is a very important step for the development of biopesticides (Polanczyk & Alves, 2005). There have been some reports that characterized *B. thuringiensis* according to *cry* gene content (Bravo et al., 1998; Ferrandis et al., 1999; Uribe et al., 2003, Vilas-Bôas & Lemos, 2004), but the expression level of these genes, or even their product precursors, have still not been completely elucidated. Therefore, the search for the phases in which the *cry* genes and their precursors are expressed, as well as their expression levels in a standard strain such as HD-1, becomes of fundamental importance for the selection of other *B. thuringiensis* strains that may be used for insect control.

In this study, the differential expression of genes from *B. thuringiensis kurstaki* HD-1 related to virulence, sporulation and crystal protein formation was monitored in order to determine the genes expressed at the logarithmic, stationary and sporulation phases of the development cycle of this bacterium.

MATERIAL AND METHODS

Bacterial strain - The *B. thuringiensis* var. *kurstaki* HD-1 strain used was obtained from the '*Bacillus* Genetic Stock Center' and is kept at the Laboratory of Bacterial Genetics and Applied Biotechnology, FCAV - UNESP/ Jaboticabal, São Paulo, Brazil.

Gene selection for DNA array production - For DNA array production, *B. thuringiensis* genes were selected, cloned, sequenced and annotated based on information from the GenBank database. The genes selected (Table 1) are related to virulence factors (expressed during the stationary phase), sporulation mechanisms and formation of protein crystals (expressed during the stationary and sporulation phases). DNA arrays were produced at the 'Brazilian Clone Collection Center' (BCCCenter).

Extraction and quantification of genomic DNA - *B. thuringiensis* var. *kurstaki* (HD1) cells were grown in a semi-solid medium (Nutrient Agar) (Gordon et al*.,* 1973), from 'BIOBRÁS' and incubated at 30ºC for approximately 18 h. Colonies isolated from each line were inoculated in 50 mL of Brain Heart Infusion medium, also from 'BIOBRÁS' and were grown with agitation at 200 rpm at 30ºC for 4 h and 30 min.

Genomic DNA was extracted as described by Marmur (1961), and after ethanol precipitation, the DNA samples were resuspended in TE buffer (10 mM Tris; EDTA 1 mM, pH 8.0) and stored at –20ºC. DNA was quantified from samples of each strain using a spectrophotometer (BECKMAN, model DU-640B), and DNA quality was determined by electrophoresis in a 0.8% agarose gel with TBE buffer (89 mM Tris; 89 mM boric acid and 2.5 mM EDTA, pH 8.2), at 90 V for 90 min. The DNA samples were diluted to 10 ng μL^{-1} for analysis.

Design of oligonucleotide primers - Pairs of oligonucleotide primers were designed using the *Gene Runner* 3.0 software (Hastings Software, Inc.) and sequences obtained from GenBank (Table 1). The selected genes were amplified by PCR (DeRisi et al., 1997) using genomic DNA as a template, and the PCR products were designed to be used in macroarrays for the evaluation of gene expression at different developmental stages of *B. thuringiensis* var. *kurstaki* (HD1). Oligonucleotides were synthesized by *MWG* (The Genomic Company) and primers were used at a concentration of 1 pmol mL^{-1} in the PCRs.

B. thuringiensis var. *kurstaki* HD-1 DNA was amplified using the specific primers. PCR amplification reactions were carried out in a final volume of 20 µL in aPTC – 100™ thermal cycler (MJ Research). Amplifications with the primers for *cry1Ac*, spo0I, ori44, sigma 28 (K), slp, *cry11A* and chi were performed with 0.6 mM $MgCl_2$ (Invitrogen), 250 µM dNTPs, 1 pmol primers, 1X PCR buffer (Invitrogen), 1U Taq polymerase (Invitrogen) and 30 ng genomic DNA. Amplifications with the primers *cry1Ab*, *cry1-2*, *cry1Ia*, *pew3*, *gyrB*, *cry3Ca*, *cry1Aa*, *cry218*, *crybns3*, *Hkna*, *npra*, *ori43*, *cry2Ab*, sigma35 (K) and (I), *mob1*, *cry10Aa*, *cyt2Ba*, sigma 28(I), *cry4B*, Bt8, *spoIIID* and *cry4Aa* were performed using the same conditions, except that the $MgCl_2$ concentration was increased to 0.8 mM.

Table 1 - Oligonucleotide primers used in this study.

Primers	Sequences	Base pairs amplified
Cry1Aa	5' ATATTTCCTTGTCGCTAACGC 3'	850
D17518	5' CTGTTATTTGATGCCCTGACC 3'	
Cry1Ab	5' CGGGATTAGAGCGTGTATG 3'	450
M12661	5' CATCCAGCGAATCTACCG 3'	
Cry1Ac	5' ATCGCTCGTCTATCGGCATTG 3'	400
U87793	5' AGCCAGCCCTCACGTTCTTC 3'	
Cry218	5' ACAGTGCCCTTACAACCG 3'	403
AJ002514	5' CCATAAGCAAATTCTGTCCC 3'	
Cry1B	5' TACAGATACCCTTGCTCGTG 3'	798
M23723	5' GGTGTTGATAAAGGAGGGAG 3'	
Cry1-2	5' GCTCAGGGCATAGAAGGAAG 3'	600
D00117	5' TTCTGTGGCGGTATTTCATC 3'	
CryV465	5'CTATGGCAGAGAACGAAG 3'	650
U07642	5'CAGACTTGAGAGGATTAGG 3'	
Crybns3	5' TGGTCAGGGCATCAAATAAC 3'	401
Y09663	5' GCTCTCAAGGTGTAAACTGC 3'	
Hkna	5' ATGCTACCCGCCACTTCAG 3'	422
U03552	5' CCCTCCATTTCCATACGAC 3'	
Npra	5' ATCTAACTGAAGCAACTGGC 3'	495
L77763	5' CTTTACCGCTGTCTGCCTC 3'	
Ori43	5' CCCGCATCATCCATCATAG 3'	455
M60513	5' TTGTCCCGTAGAAAGTTGC 3'	
Ori44	5' TCGTAAAGCTGGAAAGAAAGG 3'	402
M60465	5' AAATACCAAGCATCTCTGTCG 3'	
Pew3	5' GCTCAGGGCATAGAAAGAAG 3'	400
E01234	5' TCTGTGGCGGTATTTCATC 3'	
Spo0A	5' GAATGTGTTGCGAAAGGG 3'	600
X80639	5' TCCATCTACTGTTGTTGCTG 3'	
Slp	5' ACTTGCTCGTTAGGTTGCTC 3'	543
X62090	5' AGCAAGGAAGAGAAGTAGTCG 3'	
Cry4B	5' ATCTCGTGTAAGTGCTGCCTC 3'	541
X07423	5' AAGAGAGCCTAATAACCAGTCC 3'	
Cry11A	5'GGATGGATAGGAAACGGAAG 3'	593
M31737	5' ATACTGCCGTCTGTTGCTTG 3'	
MOB1	5' CACAATGATGAGGCAAGTC 3'	404
U67921	5'TTGGGCTTTACCTCCGTC 3'	
*Bt*8	5' TCCGTTAGCGAATGACTTAC 3'	477
X07423	5' AAATAAACTGCGGTCTCTCG 3'	
Cry10Aa	5' CATCCTGCTACCGAAACG 3'	400
M12662	5' ATCAAATCGCCTCCTATCC 3'	
SpoIIID	5' GACAAGAAAGACAGTGCGTG 3'	538
D28169	5' CCCACCATACTTCTCGCTTC 3'	
Cry4Aa	5' GTGTCCAATCTGAACTTACTCG 3'	501
Y00423	5' GGTTTCCTTGATTCTCTCTTCG 3´	
Cry2Ab	5' ACGCCATTTGTTAGGAGTTG 3'	512
X55416	5' TTTCCATTCCCTCTAAGCG 3'	
Cyt2Ba	5' CAAATGGTCTTCCTAATGCAG 3'	498
U52043	5' TATGATTTGGACGATGTAAGC 3'	
Gyrb	5' TGGTGGGAAGTTTGACGG 3'	654
AF136390	5' TCACGAACATCCTCACCAG 3'	
Rep63	5' GCGTAAGCCCATTCTCTATTC 3'	749
AJ011655	5' CAGGTGTTGTGTTGGATGAAAG 3'	
Sigma 28	5' CGCAGCCGTTATCATCAG 3'	606
X56696	5' CCCTTACAAACTCGTGGAAC 3'	
Sigma 35	5' TGGGATGGGAATGAACTG 3'	451
X56697	5' TCTCGTCAAAGTGTTCCCTC 3'	
Cry3Ca	5' TGACAAGAAAGGGAGGAAG 3'	501
X59797	5' TTCGTCCATCTCGTAAAGTC 3'	
chi	5' GCAGATTCACCAAAGCAAAG 3'	533
AF526379	5' TCCCAGTCTAAATCTACGCC 3'	

Genomic DNA amplification and analysis of amplified products - After sample preparation, thermocycling was carried out using the following conditions: one initial denaturing step of 2 min at 94ºC, 30 cycles (1 min at 94ºC; 1 min at 48ºC; 1 min at 72ºC) and one final extension step at 72ºC for 10 min, with the primers mob1, *cry10Aa*, *cyt2Ba*, sigma 28(I), sigma35 (K) and (I), *cry1Aa*, *cry218*, *crybns3*, *Hkna*, *npra*, *ori43* and *cry2Ab*. A similar program was used for the other primer sets, but the annealing temperature was changed to 50ºC.

Amplified products were analyzed by EBAGE (ethidium bromide gel electrophoresis) using a 1.5% gel (Sambrook & Russel, 2001) and TBE buffer (70 V for 2.5 h). DNA fragments were observed under UV light and gels were visualized using the Gel Documentation System (BIO-RAD).

The amplification products were then resuspended in 50% DMSO at concentrations of 2.5 μg μL^{-1} and 100 μg μL^{-1}, and transferred to a 384-well plate, then applied to nylon membranes at the Brazilian Clone Collection Center (BCCCenter).

Bacterial culture for total RNA extraction - Samples were extracted for RNA preparation at regular intervals during bacterial culture (Gordon et al., 1973). *B. thuringiensis* var. *kurstaki* HD-1 RNA at vegetative growth, stationary and sporulation phases was extracted (Ribier & Lecadet, 1973; Bulla Jr, et al., 1980).

Growth phases were defined through successive microscopic analyses of the cultures and by spectrophotometer readings at 600 nm.

The strain was cultured in Nutrient Agar (BIOBRÁS) (Gordon et al., 1973) and incubated at 30ºC for approximately 18 h. Colonies isolated from the strains were inoculated in 50 mL of nutritious broth medium (BIOBRÁS) and cultured with shaking at 200 rpm at 30ºC for 12 h (DO 0.4) to prepare the pre-inoculum. Inocula for the three growth phases were obtained by culturing 1 mL of the pre-inoculum in 50 mL of medium. Cells were cultured for 4h30 min (DO 0.1), 6h30 min (DO 0.2) and 9 h (DO 0.3) to obtain log phase, stationary phase and sporulation phase samples, respectively.

Samples were collected at each of the three phases of development and total RNA was immediately extracted.

Total RNA extraction - Bacterial cultures (50 mL) were centrifuged at 4500 g for 10 min at 4ºC, and total RNA extraction was performed as described by Cabanes et al. (2000). After extraction, RNA samples were quickly dried and resuspended in DEPC-treated water (0.01%) and stored at –80ºC after quantification. RNA samples were analyzed by spectrophotometry (BECKMAN, model DU-640B) (Sambrook & Russel, 2001), and RNA integrity was analyzed by electrophoresis in a 1.2% agarose gel containing 6.7% formaldehyde, according to Soares & Bonaldo (1997).

Production of cDNA probes for macroarray hybridization - Complementary DNA was synthesized by reverse transcription of the RNA extracted from *B. thuringiensis* var. *kurstaki* HD-1 samples from different developmental phases.

Thirty micrograms of total RNA (in 6 μL) was added to 156 pmols of Random Primer $Pd(N)_6$ (Amersham Pharmacia Biotec Inc.). Samples were incubated at 75ºC for 10 min to allow primers to anneal, and then transferred to ice bath. Five microliters of First Strand Buffer (5X, BRL) (Invitrogen), 2.5 μL of DTT (100 mM), 2.0 μL of RNAguard (Invitrogen), 2.5 μL of ATG (10 mM each) and 5 μL of ^{33}P αdCTP (50 μCi) were then added. The mix was then heated to 42ºC and 1.25 μL of Superscript II (Invitrogen, 200 U μL^{-1}) was added. Samples were gently stirred and incubated at 37ºC for 20 min. Then, 1.25 μL of dCTP (10 mM) was added to the mix and the mix was incubated at 37ºC for approximately 2 h. Samples were then denatured at 94ºC for 5 min and incubated at 37ºC for 15 min after adding 1.4 μL NaOH (5M). After incubation, 1.8 μL HCl (3.94M) and 7.0 μL of Tris-HCl (1M) pH 7.5 were added. The total volume of the probe was measured in a scintillator and purified using a G-50 column (Amersham Pharmacia Biotec Inc.). After purification, the probe volume was measured again. The incorporation efficiency of purified probe should range from 30 to 50%, compared to the unpurified version.

Macroarray hybridization

Pre-hybridization - Membranes were pre-hybridized according to a Southern protocol (Sambrook & Russel, 2001) for 4 h at 42ºC. Hybridization cylinders containing 10 ml of solution were used, and each cylinder contained only one membrane.

Hybridization - The hybridization solution was prepared as recommended for a Southern protocol (Sambrook & Russel, 2001) and kept at 42ºC until use. After purification and denaturing, the probe was added to a 50 mL tube containing the hybridization solution for 42 h at 42ºC.

Membrane washing - After hybridization, probes were discarded and membranes were washed under high stringency conditions (Desprez et al., 1998). Membranes were then sealed and exposed to a phosphoimaging screen for approximately 72 h.

Detection and measurement of hybridization signals - Hybridization signals were obtained from 30 genes from the three development phases of this bacterium (treatments). Each gene was spotted three times onto each membrane, and two different membranes were used, with a completely randomized design, and therefore there were six repetitions of each treatment.

After the membranes were exposed, the phosphoimaging screens were analyzed with a Cyclone Phosphor-Imager (Packard Instruments), and hybridization signals were measured using the Optiquant software, and expressed in DLU (Digital Light Units).

The probe hybridization data were normalized by subtracting background-related values from regions of the image that did not correspond to DNA spots. The genes expression levels were subject to analysis of variance (ANOVA), being their means compared by a Tukey test ($p \leq 0.05$). These analyses were performed with the SAS statistical program (SAS Institute, 1995).

RESULTS AND DISCUSSION

Gene expression analysis - PCR products were analyzed by electrophoresis in an agarose gel in order to determine their size and purity. This procedure is considered one of the most important steps in large-scale gene expression studies (Deyholos & Galbraith, 2001). It confirms that a unique amplicon is present and provides an estimate of the concentration of the amplified product.

Three developmental phases of *B. thuringiensis kurstaki* HD-1 were analyzed in order to investigate the expression of 30 genes. The sequences of these genes can be found in GenBank, which also includes recent citations of other sequences obtained by well-known researchers, allowing specific primers to be designed for the detection of these genes. The genes that were chosen for the present study play important biological roles in this bacterium and encode the Cry proteins. These proteins mediate highly specific toxic activities, allowing larvae from insect pest species to be controlled by this bacterium. The other genes evaluated in this study are involved in basic metabolic processes, and act to initiate the process that ultimately leads to *cry1* gene expression. Our results indicate that two important genes related to physiological processes that take place before sporulation are differentially expressed at phases in bacterial development.

Two *B. thuringiensis* genes exhibited significant variations in their levels of expression (Figure 1); one gene was related to sigma factors (sigma^{35}), and the other was related to the *cry* gene (*cry2Ab*). The sigma factor is expressed during all three phases examined, laying an important role in transcription. RNA polymerase begins transcription by recognizing a specific region of DNA, the promoter. Promoter recognition by RNA polymerase is mediated by a sigma factor, which attaches to the enzyme, and identifies promoter sites. The action of several types of promoters and sigma factors regulate cell functions, allowing a balance of physiological activities to be maintained. Therefore, the observation that sigma^{35} factor is expressed at all the development stages in this study confirms its direct involvement in the *cry1Aa* promoter site recognition of *B. thuringiensis*, as reported by Adams et al. (1991).

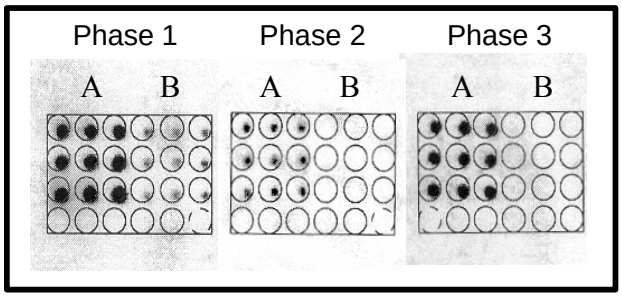

Figure 1 - Hybridization of *B. thuringiensis* genes with *B. thuringienis kurstaki* HD-1 cDNAs at three developmental phases. The image was produced by the Cyclone Phosphor-Imager (Packard Instruments). **A**: sigma^{35}. **B**: *cry2Ab*

The sigma^{35} holoenzyme is also related to other *cry* genes, as observed by Tounsi & Jaoua (2002), who suggested that the transcription of a *cry1Ia* promoter in *B. thuringiensis* var. *kurstaki* is mediated by this holoenzyme. They described this enzyme as an RNA polymerase specific to the sporulation phase associated with the BtI promoter. However, the sigma^{38}–dependent BtII is apparently not involved in *cry1Ia* transcription. Despite the fact that both promoters are active during the medium and late sporulation stages of *B. thuringiensis* var. *kurstaki* HD-1-Dipel, sigma^{35} was expressed in the sporulation, vegetative and stationary phases in this study, but was expressed at lower levels in the stationary phases (Figure 1).

The high expression of the sigma^{35} factor from the log phase onwards suggests that the activation of *cry* genes begins in this phase. This is because these genes are related to sporulation, which depends on the activation of several genes in cascade, including the sigma^{35} factor. This hypothesis is also supported by the fact that transcription from the *amy*E promoter increased substantially as cell growth reached the log phase. The gene that encodes sigma^{35} is a significant promoter, when one observes cry genes expression, indicating that the presence of other promoters might change the expression in each phase in which the gene is expressed.

Expression of *cry1Ac* begins in the *B. thuringiensis* log phase (Chak et al., 1994). *cry1Ac* expression in *B. thuringiensis kurstaki* Tt14 is regulated by the *amy*E promoter, which is dependent on a specific sigma factor (sigma^{43}) (Yang et al., 2003). Other studies have shown that *cry* gene expression is dependent on sigma^{28} or sigma^{35} factors (Nicholson et al., 1987).

The c*ry2Ab* gene was also expressed in the log phase in *B. thuringiensis kurstaki* HD-1, although at much lower levels than the gene encoding sigma^{35}. Despite the fact that c*ry2Ab* has been described as Diptera-specific, it has also been reported to have effects upon Lepidoptera (Schnepf et al., 1998). Its expression in the log phase, together with the gene encoding sigma^{35} factor, suggests that this holoenzyme is not responsible for recognition of the *cry2Ab* promoter site. This explains why it was not possible to detect the expression of the *cry1* genes at this stage of development. The sigma^{35} factor is responsible for promoter site recognition; there would be no expression of these genes before expression of the gene encoding the sigma factor. In the log phase, the *cry2Ab* gene is transcribed using an alternative promoter unrelated to sigma^{35}. This unknown alternative promoter may also activate *cry2Ab* expression at earlier developmental phases, before the *cry1A* genes are expressed.

There was a difference in the levels of expression of the sigma factor and *cry2Ab* (Tukey test, $p < 0.01$) (Tables 2 and 3). The highest mean of expression was observed in the log phase for the gene encoding sigma^{35}, and the second highest expression was during the sporulation phase (Table 3). *Cry2Ab* was only expressed at significant levels in the log phase, but the expression of this gene was still low when compared with the sigma-encoding gene during all developmental phases examined (Tables 2 and 3).

The expression of *cry2Ab* is highly relevant even at low levels, since this gene has previously been studied using transgenic varieties of cotton, maize and tomato, which simultaneously express *cry1Ac* and *cry2Ab,* the so-called 'second generation' of commercial transgenic genotypes. A combination of *cry* genes is being used to minimize selection pressures and to avoid Bt-resistant insect pests. The use of *cry2Ab* allows monitoring programs and preventive resistance control measures to be implemented, because cross-resistance with the *cry2Ab* and *cry1Ac* combination is much lower than when using the combination of *cry1Ac* and *cry1Aa*, as observed for the pink bollworm (Tabashnik et al., 2002) and *cry1Ac* and *cry1Aa* reported in *Heliotis virescens* with transgenic Bt maize (Fuentes et al., 2003).

In the log phase, only the gene encoding sigma^{35} was expressed at high levels. This result contrasts to the results from a previous study that reported that both *cry1Aa* and sigma^{35} are expressed in the log phase (Schnepf et al., 1998). However, this study reporting simultaneous expression of *cry1Aa* and sigma^{35} used an *in vivo* fusion of the *cry1Aa'-'lacZ* genes, whereas gene expression was analyzed in the natural state without gene fusion in our study, which may explain the differences in the results between these two studies.

In addition to sigma^{35}, we also attempted to analyze the expression of the gene that encodes sigma^{28} using a specific set of primers. However, no sigma^{28} expression was observed in any of the developmental phases, thus suggesting that sigma^{28} is not involved in the recognition of the *cry* promoters studied here.

Based on the finding that no other genes were significantly expressed during the developmental stages studied in these experiments, we suggest that new studies should consider the development phases defined in this work and even divide them into shorter time periods in order to attempt to explain the lack of expression of genes that are supposedly related to sporulation and virulence factors in *B. thuringiensis* var. *kurstaki* HD1-Dipel. It is also important to mention that the absence of expression of other genes using macroarray techniques may be simply due to the lack of information about the time intervals between the activations of the *cry* genes. For example, in *B.*

Table 2 - Variance analysis (ANOVA) for expression genes (DLU) relative to exponential, logarithmic and stationary developmental phases.

Sources of Variation	Degrees of Freedom	Mean Squares (DLU)	F value	P value
Experiment (EX)	1	5142374052.00	368.58	< 0.0001
Gene (GN)	1	78220040180.00	5606.42	< 0.0001
Phase (PS)	2	17437460829.00	1249.83	< 0.0001
EX*GN	1	2562660381.00	183.68	< 0.0001
EX*PS	2	19135176304.00	1371.51	< 0.0001
GN*PS	2	13192121728.00	945.54	< 0.0001
EX*GN*PS	2	16906193773.00	1211.75	< 0.0001

Table 3 - Comparison between sigma^{35} and *cry2AB* gene expression means at exponential, logarithmic and stationary developmental phases.

Genes	Phases	Means
sigma^{35}	Phase 1 (log)	95443.33 a
sigma^{35}	Phase 2 (stationary)	13608.47 c
sigma^{35}	Phase 3 (sporulation)	58211.04 b
cry2Ab	Phase 1 (log)	7119.35 d
cry2Ab	Phase 2 (stationary)	974.01 e
cry2Ab	Phase 3 (sporulation)	-2302.95 e

Means followed by different letters in the column are different (Tukey test, $p < 0.01$).

subtilis, seven stages have been defined during sporulation, and sporulation is mediated by activation of genes through a signaling cascade. About 800 genes are involved in sporulation in *B. subtilis* and other species from this genus (Mandelstam, 1969).

CONCLUSION

During the log developmental phase of the *B. thuringiensis kurstaki*-HD-1 bacterium, the genes encoding sigma^{35} and *cry2Ab* were expressed at the highest levels.

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