

## CLONING OF A *Bacillus thuringiensis* CRYSTAL PROTEIN GENE

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### ABSTRACT

The vector pUC18 and a shuttle vector containing the replication region of a resident plasmid of *B. thuringiensis* pHT3101, were used to clone a  $\delta$ -endotoxin gene of a newly isolated strain SPL407 (serotype H1). A 12 kb *Bam*HI/*Pst*I fragment was isolated from total DNA cloned in *E. coli* using pUC18 as vector. A 12 kb *Sma*I/*Pst*I fragment was isolated from this construct and cloned into vector pHT3101 digested with *Sma*I/*Pst*I. A 7 kb *Hpa*I/*Sma*I fragment from this second construct was cloned into pHT3101 digested with *Sma*I. The two latter plasmids were introduced into *E. coli* and *B. thuringiensis*. Analysis of plasmid DNA from the recombinant clones of *B. thuringiensis* indicated that it was not subject to molecular rearrangements and appeared structurally stable.

### INTRODUCTION

*Bacillus thuringiensis* has been extensively studied because of its entomopathogenic properties. Crystalline inclusions with insecticidal activity are found in *B. thuringiensis*. These inclusions contain one or more polypeptides called  $\delta$ -endotoxins.

Various  $\delta$ -endotoxins have been found in different *B. thuringiensis* strains and these are grouped into four major classes according to their amino acid sequences, and their specificities against Lepidoptera, Diptera or Coleoptera (Höfte and Whiteley, 1989; Lereclus *et al.*, 1989b).

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Delta endotoxins are encoded by genes located in large plasmids or in chromosomes and different genes may coexist in a single strains (Gonzalez *et al.*, 1982; Klier *et al.*, 1982; Lereclus *et al.*, 1982). Some of these genes have been cloned (for a review see Lereclus, 1988) and their regulatory systems have been analyzed in *Escherichia coli* (Schnepf *et al.*, 1987). A limiting factor in the study of the expression and regulation of these genes is the lack of an efficient technique for transformation in *B. thuringiensis*.

We report here the cloning of a  $\delta$ -endotoxin gene from a new strain of *B. thuringiensis*, SPL407, using plasmid pUC18 and a novel 6.6 kb shuttle vector containing the replication region of a resident plasmid of *B. thuringiensis* (Lereclus *et al.*, 1989a). The new recombinant plasmids thus constructed were later introduced into *B. thuringiensis* by electroporation.

## MATERIAL AND METHODS

**Strains:** The strain SPL407 (serotype H1) of *B. thuringiensis* was provided by Dr. Sergio B. Alves, Department of Entomology, ESALQ-USP, Brazil. The strain was serotyped by Dr. H. de Barjac, Laboratoire de Lutte Biologique II, Institut Pasteur, Paris. The acrySTALLIFEROUS mutant SPL407Cry<sup>-</sup> was obtained by curing at 42°C.

**Methods:** Total DNA was extracted from the wild-type and mutant strains as described by Msadek *et al.* (in press), subjected to single and double hydrolysis with the restriction endonucleases *Pvu* II, *Pst* I, *Bam*HI and then subjected to electrophoresis on 0.6% agarose gels. All enzymes were used as recommended by the manufacturers. The resulting DNA fragments were transferred onto cellulose nitrate sheets (Southern, 1975) and hybridized with an internal part of a  $\delta$ -endotoxin gene, previously cloned in the plasmid pHTA4, by Sanchis *et al.* (1988). This DNA fragment used as a probe was labelled with ( $\alpha^{32}$ P) - dATP, dCTP by nick-translation as described by Rigby *et al.* (1977).

The 12 kb *Bam*HI/*Pst* I DNA fragment containing the  $\delta$ -endotoxin gene was then cloned into two vectors, pUC18 and pHT3101, resulting in two recombinant plasmids. This fragment linked to pUC18 and digested with the restriction enzymes *Bam*HI and *Pst* I gave pHT407. Cloning of the 12 kb fragment in pHT3101 digested with the restriction enzymes *Sma*I and *Pst* I gave pHT408.

The 5 kbp DNA fragment upstream of the *Hpa*I site was deleted to determine whether this region could act as a negative transcriptional regulator in *B. thuringiensis*, as previously suggested by Schnepf *et al.* (1987). Thus, pHT407 was hydrolyzed with the enzymes *Sma*I/*Pst* I/*Hpa*I and the 7 kb DNA fragment containing the crystal gene was cloned in both orientations in pHT3101 digested with *Sma*I. Two derived plasmids, pHT409 and 410 were obtained. Single hydrolysis with the restriction en-

zymes *Bam*HI and *Sst*I was performed to determine the cloning orientation in these two recombinant plasmids.

All recombinant plasmids were introduced into *E. coli* JM83 as described by Lederberg and Cohen (1974). The colonies carrying the *cry* gene were selected by colony hybridization as described by Frunstein and Hogness (1975), using an internal part of a *cry* gene previously cloned in the plasmid pHTA2 by Sanchis *et al.* (1988) as a radioactive probe.

All hybridization experiments were performed at 42°C for 24 h in a solution containing 50% formamide: 5xSSC (saline sodium citrate): 1 x Denhardt (Denhardt, 1976). The filters were then washed at 42°C for 20 min using the following solutions: 50% formamide: 5xSSC, 5xSSC, 2xSSC and 0.5xSSC, before drying at room temperature.

The DNA recombinant *E. coli* JM83 clones were extracted according to the method described by Birnboim and Doly (1979) and introduced into *B. thuringiensis* car. *kurstaki* HDICry<sup>B</sup> by electroporation (Lereclus *et al.*, 1989a). The plasmid DNA of the *B. thuringiensis* recombinant clones was extracted (Birnboim and Doly, 1979, modified by Lereclus *et al.*, 1982) and used to transform *E. coli* JM83 again. The plasmids recovered after this transformation step were analyzed on 1% agarose gel to verify their entirety.

## RESULTS AND DISCUSSION

As described in Material and Methods, the DNA fragments hybridizing a crystal protein gene were detected by hybridization with the radioactively labelled plasmid pHTA4. Figure 1 shows that hybridization occurs with different DNA fragments resulting from several digestions with restriction enzymes (lanes 1, 2, 4, 5 and 6). This indicates the presence of more than one crystal protein gene sequences in the total DNA of the strain SPL407. A *Bam*HI/*Pst*I fragment of approximately 12 kbp, which was used for the subsequent cloning is shown in lane 4 B.

Figure 1 also shows that no hybridization occurred with strain SPL407Cry<sup>-</sup> (lane 3), demonstrating that the detected genes are not present in the acrySTALLIFEROUS strain. The genes are probably carried on plasmids that were eliminated by shifting the bacterial culture to 42°C. However, hybridization with the crystal gene was not obtained with plasmid DNA purified from wild type strain SPL407 (results not shown). Therefore these observations suggest that the  $\delta$ -endotoxin genes are not part of the chromosome but of large plasmids indistinguishable from the chromosomal DNA by the described experimental procedures.

A restriction map of the recombinant plasmid pHT407 obtained as described in Material and Methods, showed that the cloned 12 kbp DNA fragment corresponds

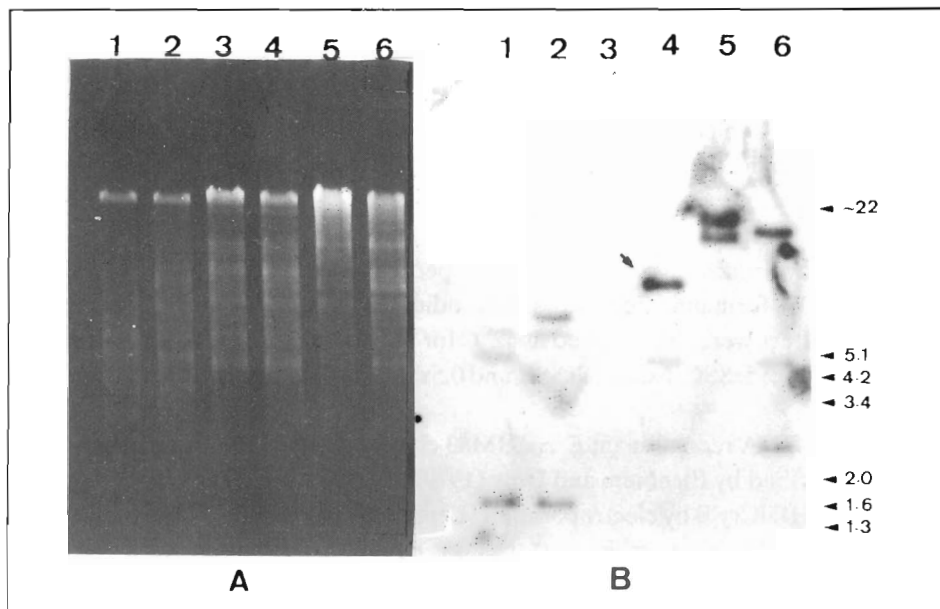


Figure 1 - Electrophoresis of total DNA from the strain SPL407 and the derivate SPL407Cry<sup>-</sup> after digestion with restriction enzymes (A) and autoradiogram (B). 1, SPL407/PvuII/PstI; 2, SPL407/PvuII; 3, SPL407Cry<sup>-</sup>/BamHI/PstI; 4, SPL407/BamI/PstI; 5, SPL407/BamHI; 6, SPL407/PstI.

to the *cryIAa* type of  $\delta$ -endotoxin genes (Höfte and Whiteley, 1989). The position and orientation of the *cry* gene on the recombinant plasmid pHT407 was deduced by analogy with the crystal gene sequenced by Schnepf *et al.* (1985).

Plasmid pHT3101 was constructed in order to obtain a vector that would express the crystal gene both in *E. coli* and in *B. thuringiensis*. Figure 2 shows the restriction map of the recombinant plasmids originating from cloning the *cry* gene in this vector and pUC18.

Restriction enzyme analysis showed that the *cryIAa* gene was cloned in an opposite orientation to the *lacZ* promoter of pUC18 in pHT409 and in the same orientation in pHT410. Both recombinant plasmids are 13.6 kbp in size.

Plasmids containing the crystal gene were expressed both in *E. coli* and *B. thuringiensis* as shown by Lereclus *et al.* (1989a). A 130 kDa protein was produced in both cases. The presence or absence of the 5 kb *PstI/HpaI* fragment upstream of the crystal gene had no effect on the level of expression of this gene in *B. thuringiensis* (Lereclus *et al.*, 1989a). Figure 3 shows that plasmids used in *B. thuringiensis* and then reintroduced into *E. coli*, did not undergo molecular rearrangements and appeared to be structurally stable.

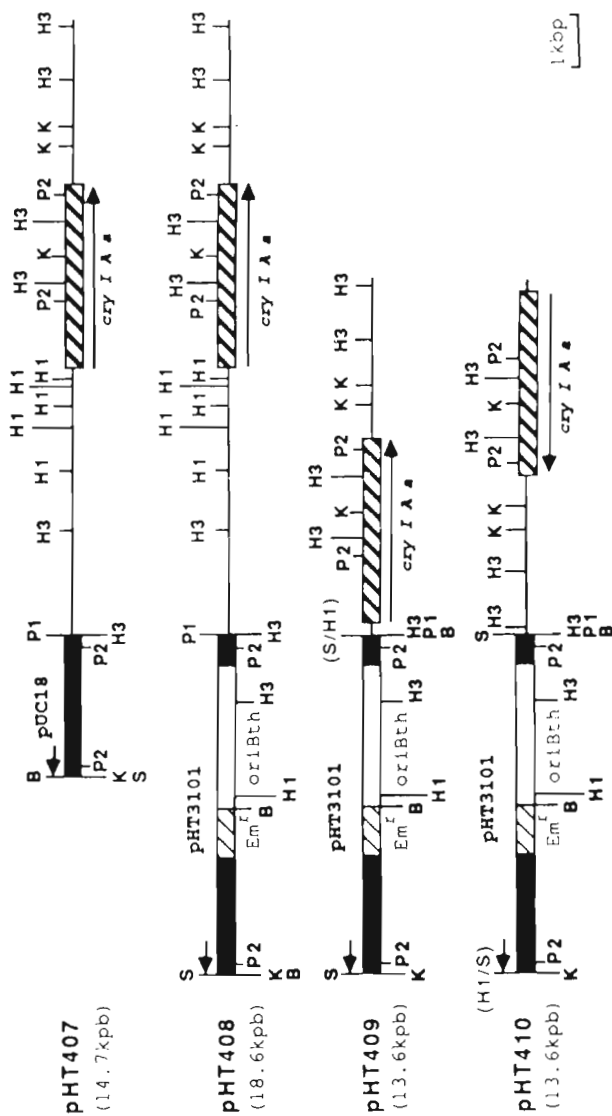


Figure 2 - Restriction map of the recombinant plasmids. The arrows below *cryIA* indicate the presumed direction of transcription. The arrows at the end of the *pUC18* and *pHT3101* plasmids represent the orientation of the *lacZ* promoter. Abbreviations used: B = *Bam*HI; H1 = *Hpa*I; H3 = *Hind*III; K = *Kpn*I; P1 = *Pst*I; P2 = *Pvu*III; S = *Sma*I.

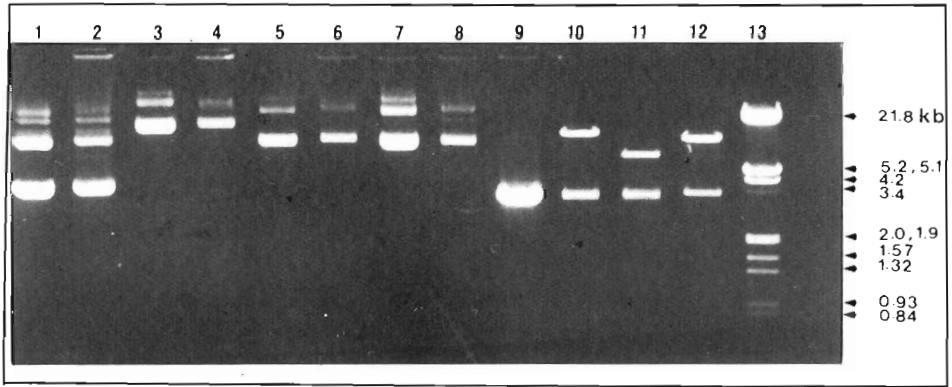


Figure 3 - Electrophoresis of the recombinant plasmids pHT408, 409 and 410 obtained after electroporation in *B. thuringiensis*. Columns 1, 3, 5 and 7: DNA isolated from *E. coli*. Columns 2, 4, 6 and 8 represent DNA after a growth step in *B. thuringiensis*. 1 and 2: plasmid pHT3101; 3, 4: pHT408; 5, 6: pHT409 and 7, 8: pHT410. Columns 9 to 12 represent the plasmids pHT3101, 408, 409 and 410, respectively, after digestion with *Bam*HI and *Pst*I. 13 -  $\lambda$  *Hind*III/*Eco*RI.

### ACKNOWLEDGMENTS

We thank Dr. R. Dedonder and Dr. M.-M. Lecadet, Laboratoire de Biochimie Microbienne, Institut Pasteur - Paris and Dr. R. Vencovsky, Institute of Genetics - ESALQ - Universidade de São Paulo, in whose laboratories this work was conducted.

We also wish to thank A. Edelman for revising the English manuscript.

Further financial support was granted by CAPES/PICD Program, Universidade Estadual de Londrina and CNPq.

Publication supported by FAPESP.

### RESUMO

Um fragmento de DNA contendo o gene da  $\delta$ -endotoxina de um novo isolado de *Bacillus thuringiensis*, o SPL407 (serotipo H1), foi clonado usando o vetor pUC18 e um novo vetor, o pHT3101, que contém a região de origem de replicação de um plasmídeo nativo de *B. thuringiensis*.

As clonagens foram feitas a partir do DNA total: na primeira um fragmento de DNA de 12 kb *Bam*HI/*Pst*I, foi clonado em *E. coli* usando o pUC18 como vetor; na segunda um fragmento de DNA de 12 kb *Sma*I/*Pst*I foi clonado no pHT3101 digerido por *Sma*I/*Pst*I; e na terceira um fragmento de DNA de 7 kb *Hpa*I/*Sma*I foi inserido no pHT3101 digerido por *Sma*I. Os dois plasmídios construídos usando o pHT3101 foram introduzidos em *E. coli* e em *B. thuringiensis*.

A análise dos plasmídios dos clones recombinantes de *B. thuringiensis* indicou que eles não sofreram rearranjos moleculares e parecem ser estruturalmente estáveis.

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(Received December 27, 1989)