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Expression of the *cryIB* crystal protein gene of *Bacillus thuringiensis*

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Summary. The *cryIB* gene of *Bacillus thuringiensis* subsp. *thuringiensis* HD-2 codes for a M_r 139492 protein that is lethal to certain lepidopteran larvae. We used primer extension to map transcriptional initiation sites and found that *cryIB* was transcribed from two sites that are activated at different times during sporulation. The presumed promoter regions for the two start sites are very similar to the two promoters preceding the *cryIA(a)* gene, and the in vivo transcriptional start sites were found to be identical. Variable amounts of the full-length *cryIB* protein were detected by immunoblotting of extracts of recombinant cells of *Escherichia coli;* larger amounts were found when the TTG translational start codon was changed to ATG and when an *htpR*$^-$ strain of *E. coli* was used as the recipient for transformation. When expressed in *E. coli*, the *cryIB* protein was found to be toxic to the larvae of *Artogeia rapae* (LC_{50} of 58 ng/cm^2) and exhibited little toxicity to the larvae of *Manduca sexta* ($LC_{50} > 5000$ ng/cm^2).

Key words: *Bacillus thuringiensis* – Lepidopteran larvae – Sporulation – Promoter – TTG translational start codon

Introduction

During sporulation, *Bacillus thuringiensis* synthesizes one or more proteinaceous crystalline inclusions that are toxic to certain insect larvae (reviewed by Höfte and Whiteley 1989). The many subspecies of *B. thuringiensis* that are toxic to lepidopteran larvae typically contain bipyramidal crystals composed of protoxins of 130–140 kDa encoded by one or more plasmid-borne genes. At the present time, six different genes coding for lepidopteran-specific protoxins can be distinguished on the basis of DNA sequence and host range (Höfte and Whiteley 1989). Three of these [*cryIA(a), cryIA(b)* and *cryIA(c)*] are closely related and are present in a number of subspecies; the remaining three genes (*cryIB, cryIC* and *cryID*) are not as widely distributed (Höfte et al. 1988). Kronstad and Whiteley (1986) reported that crystals from *B. thuringiensis* subsp. *thuringiensis* HD-2 contain two proteins: one with a deduced molecular mass of 130600 daltons encoded by the *cryIA(b)* gene and the other with a molecular mass originally estimated to be approximately 160 kDa. The gene coding for the latter peptide has been cloned from *B. thuringiensis* subsp. *thuringiensis* HD-2 and sequenced (Brizzard and Whiteley 1988). This gene, now designated *cryIB*, codes for a polypeptide with a deduced molecular mass of 139492 daltons.

In this paper, we report the location of the start sites for transcription of the *cryIB* gene in *B. thuringiensis*, the expression of the gene in *Escherihcia coli*, and present data concerning the toxicity of the gene product to lepidopteran insect larvae.

Materials and methods

Bacterial strains, plasmids and bacteriophage. B. thuringiensis subsp. *thuringiensis* strains HD-2 and HD-290 were obtained from B. Carlton (Ecogen, Langhorne, Pa.) and H.T. Dulmage (Cotton Insects Research, USDA, Brownsville, Tex.), respectively. The sources of various strains of *E. coli* were as follows: MC1000, from M.J. Casadaban (Univ. of Chicago, Chicago, Ill.); SG935 (*F*$^-$, *lac*^{am}, *trp*^{am}, *pho*^{am}, *mal*^{am}, *htpR*^{am}, *rpsL*, *supCts*, *tsx*::Tn*10 lon 100*), from S. Lory (Univ. of Washington, Seattle, Wash.); W3350 *htpR*^{ts}, from F.C. Neidhardt (Univ. of Michigan, Ann Arbor, Mich.); P2392 and LE-392 were obtained from commercial sources; JM103, JM83 and MV1193, from J. Vieira

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(Univ. of Minnesota, Minneapolis, Minn.); NS428 and MS433 have been described (Enquist and Sternberg 1979). Plasmid pBR322, phage lambda Embl-3, and plasmid pIC7 were obtained commerically; phage M13K07 and plasmids pUC118 and pUC119 were obtained from J. Vieira.

Purification and analysis of crystals. Crystals were purified from sporulated cultures of *B. thuringiensis* grown in G medium using two successive centrifugations in Renograffin gradients (Kronstad et al. 1983). The amino-terminal amino acid sequence of the crystal protein of strain HD-290-I was determined by Edman degradation in the laboratory of Dr. Kenneth A. Walsh (Dept. Biochemistry, University of Washington, Seattle, Wash.). Previously described methods were used to prepare antibodies to the 140 kDa polypeptide (Schnepf and Whiteley 1981). Crystal proteins were detected by immunoblotting using goat anti-rabbit alkaline phosphatase conjugate (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Cloning. High molecular weight plasmid DNA was purified from cultures of strain HD-2 grown in SPY medium as described (Kronstad et al. 1983). Plasmids in the 75 MDa range were isolated by sucrose density gradient centrifugation, digested with *Xba*I, ligated into pUC18 using T4 DNA ligase and transformed into *E. coli* JM83. Ampicillin-resistant transformants were screened by immunoblotting and a positive clone (p511) containing a 1.6 kilobase (kb) insert was obtained. Partially purified 75 Mda plasmid DNA was digested with *Bam*HI, ligated into *Bam*HI/*Eco*RI doubly digested phage EMBL-3 DNA. The recombinant phage was packaged, and used to infect *E. coli* P2392 (Sambrook et al. 1989). A 1 kb *Eco*RI-*Xba*I fragment from p511 was used as a hybridization probe to screen the library; a positive clone (lambda-211) was obtained containing a 16 kb *Bam*HI fragment. Phage lambda-211 was plaque-purified on *E. coli* LE-392, and phage lambda-211 DNA was extracted. The 16 kb *Bam*HI insert was subcloned into pBR322 which had been digested with *Bam*HI and treated with calf intestinal phosphatase; the resultant plasmid was designated pBLB1.

DNA sequencing. A restriction map of the region of pBLB1 used for DNA sequencing is shown in Fig. 1. The *Eco*RI-*Eco*RV fragment was subcloned into plasmid pIC7, producing p215. For sequencing, plasmid p215 was digested with *Eco*RI and *Hin*dIII, and subcloned into pUC118 to give p218. For sequencing the opposite strand, pUC119 was digested with *Sal*I, filled-in using the Klenow fragment of DNA polymerase I, and then digested with *Hin*dIII. Plasmid p215 was digested with *Eco*RI, filled-in, digested with *Hin*dIII, and cloned into the *Hin*dIII-digested, blunt-ended pUC119 to produce p219 (not shown in Fig. 1). Deletions for sequencing were generated by unidirectional digestion with exonuclease III as described by Henikoff (1987). Single-stranded template DNA was prepared by conventional methods (Sambrook et al. 1989). DNA sequencing was

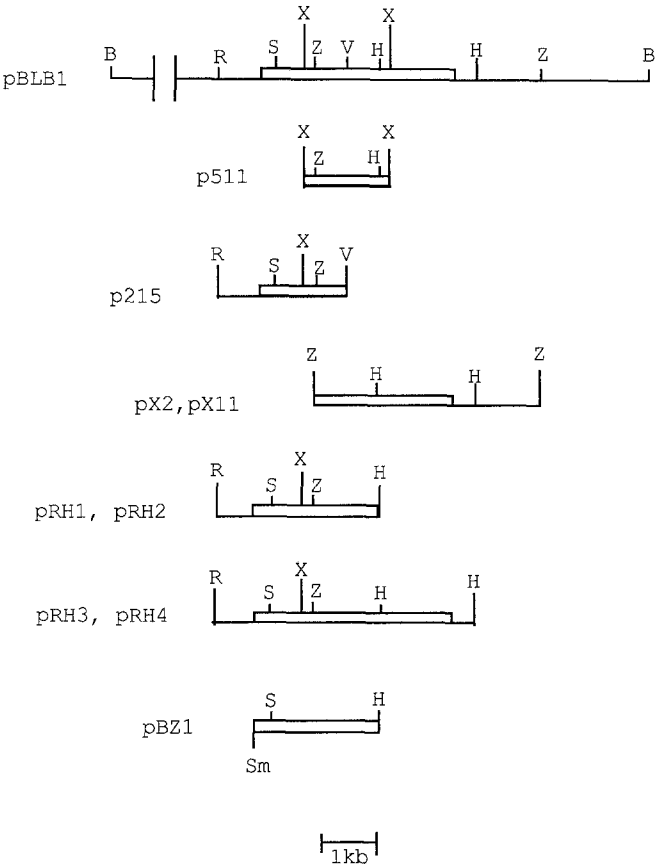


Fig. 1. Restriction maps of plasmids used in DNA sequencing, subcloning, and site-directed mutagenesis. Abbreviations used to indicate restriction sites: B, *Bam*HI; R, *Eco*RI; S, *Sac*II; X, *Xba*I; V, *Eco*RV; H, *Hin*dIII; Z, *Xho*I

performed using Sequenase (US Biochemicals) and [^{35}S]dATP (Williams et al. 1986) by the dideoxynucleotide method (Sanger et al. 1977); the reactions were analyzed by electrophoresis in 6% polyacrylamide/7 M urea gels buffered with TRIS-borate-EDTA.

Site-directed mutagenesis and reconstruction of cryIB. The plasmid pRH1 was constructed by inserting the *Xho*I-*Hin*dIII fragment of p511 into p215. The TTG translation start site was modified in two ways using oligonucleotide-directed mutagenesis (Kunkel et al. 1987): (1) the TTG start codon was modified to an ATG, to yield pRH2; and (2) a *Sma*I site was introduced immediately after the ATG codon to allow fusion with *lacZ* sequences containing the promoter and ribosome binding site. The resulting plasmid, pBZ1, was identical to pRH2 in the *cryIB* coding region except for the sequence of the first four codons: *lacZ*-ATG GCC CGG G-*cryIB* (from the second base of the 4th codon to the *Hin*dIII site shown in Fig. 1). As a result, the amino acid sequence at the start of the gene was changed from f-MTSNRK... to f-MARDRK.... The changes in both sequences were verified by sequencing from the *Sac*II site to 100 (pBZ1) or 450 (pRH2) bases upstream from the start codon. The *Sac*II-*Hin*dIII fragment from the gene was then replaced by DNA from unmutagenized pRH1. To reconstruct full-length *cryIB* genes, the

*Hin*dIII fragment of pX11, containing the 3′ end of the gene, was cloned into pRH1 and pRH2 to form pRH3 and pRH4, respectively.

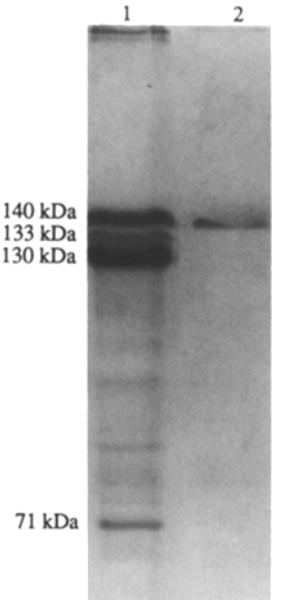
Transcription mapping. Strain HD-2 was grown in G-medium at 30° C and RNA samples were purified during sporulation as previously described (Wong et al. 1983). The oligonucleotide 5′-ACA-CAG-CAT-TAC-CTA-AG-3′ (complementary to nucleotides 97–113 of Fig. 4) was 5′ end-labeled with polynucleotide kinase and α-[^{32}P]ATP, annealed to the purified RNAs (Brown and Whiteley 1988), and used as a primer for reverse transcription by Moloney murine leukemia virus reverse transcriptase according to the conditions described by the manufacturer (Bethesda Research Laboratories). Base specific markers for determining the lengths of the extended primers were generated by sequencing (Sanger et al. 1977) the promoter regions using the same labeled primer described above. The reverse transcripts and control DNA sequencing reactions were analyzed as described in the section on DNA sequencing.

Preparation of inclusions from recombinant strains and insect bioassays. Inclusions of the truncated toxin synthesized by pBZ1 in *E. coli* MC1000 were purified from stationary phase cells by repeated sonication and washing in distilled water; protein was estimated by comparison with known amounts of purified *B. thuringiensis* HD-1-Dipel crystal proteins separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels and stained with Coomassie blue. Bioassays were performed with larvae (less than 1 day old) of *Manduca sexta* and *Artogeia rapae* (*Pieris rapae*) according to Schesser et al. (1977). The LC_{50} values and confidence limits were obtained by probit analysis (Finney 1971). *M. sexta* larvae were obtained from Drs. L. Riddiford and J. Truman (Dept. of Zoology, University of Washington, Seattle, Wash.) and *A. rapae* larvae were obtained from Dr. Duane Biever (United States Department of Agriculture, Agricultural Research Service, Yakima, Wash.).

Results and discussion

Composition of crystals

Earlier investigations (Kronstad and Whiteley 1986) demonstrated that crystal preparations from *B. thuringiensis* subsp. *thuringiensis* HD-2 contained a polypeptide with a greater apparent molecular mass than the peptides found in the crystals of many lepidopteran-specific strains. This larger peptide of ca. 140 kDa was also present in three other strains of the same subspecies, i.e. Berliner 1715, HD-290 and HD-120 (data not shown). Crystals from a spontaneous mutant strain of HD-290 (Fig. 2, lane 2) contained only the ca. 140 kDa polypeptide, whereas crystals from the parent strain (Fig. 2, lane 1) contained additional peptides of ca. 133 kDa, 130 kDa and 71 kDa encoded by *cryIA(a)*, *cryIA(b)* and *cryIIA*. Hybridization analyses using a probe specific for the *cryIA(a)* and *cryIA(b)* genes



(Kronstad and Whiteley 1986) revealed that the mutant strain HD-290-I lacked these genes (data not shown) suggesting that the latter strain had lost the large plasmids that bear the *cryIA(a)*, *cryIA(b)* and *cryIIA* genes. Presumably, the *cryIIA* gene was also lost, although this point was not tested directly. As demonstrated by Carlton and Gonzalez (1984), many strains of *B. thuringiensis* lose plasmids at a high rate.

The translational start site of the *cryIB* gene was identified using the apparently pure 140 kDa peptide from crystals of the mutant strain HD-290-I as substrate for amino acid sequence determination by Edman degradation. The 23 amino acid sequence determined for the HD-290-I crystal protein matched the deduced sequence of the HD-2 gene except that the initiating methionine (encoded by the rarely used TTG codon) was missing in the protein isolated from crystals. A potential ribosome binding site of 10 bases was found 5 bp upstream from the TTG codon; this 10 base sequence is exactly complementary to the sequence found at the 3′ end of *Bacillus* 16 S rRNA (McLaughlin et al. 1981).

Transcriptional start sites for cryIB *expressed in* B. thuringiensis

Crystal proteins are produced only during sporulation. To map the in vivo transcriptional start sites for the *cryIB* gene, RNAs isolated from strain HD-2 at the onset of the stationary phase and at different times during sporulation were hybridized to an end-labeled oligonucleotide primer and extended using reverse transcriptase (Materials and methods). No transcription of this gene could be detected in cells harvested at the end of the exponential phase of growth (Fig. 3, lane 1). Significant transcription was found from one of two adjacent sites (labeled P1 in Fig. 3). Two hours later (t_2), there was a slight increase in transcription from this site at $t_{3.5}$ and then a decreased transcription at t_5, $t_{6.5}$ and t_8

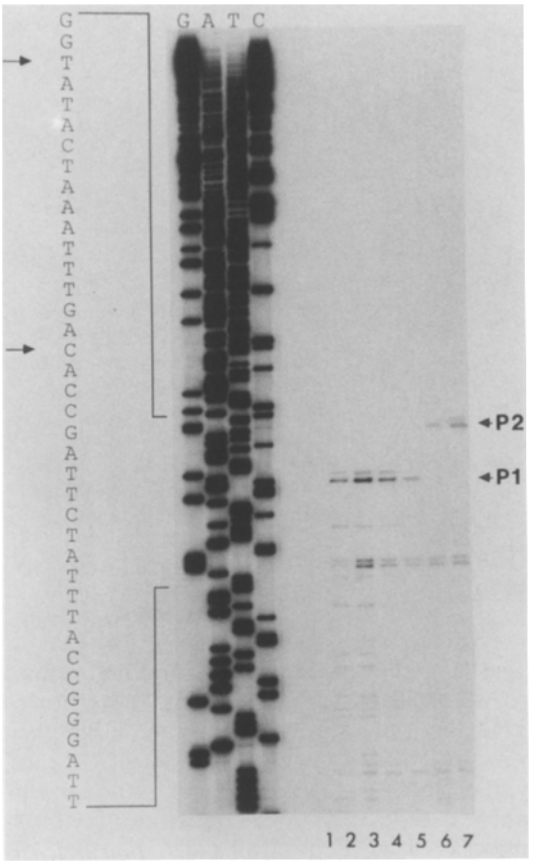


Fig. 3. Autoradiogram showing 5′ termini of *cryIB* mRNAs produced in *B. thuringiensis*. RNAs extracted from *B. thuringiensis* at: lane 1, the end of the exponential phase of growth; lane 2 at t_2; lane 3, at $t_{3.5}$; lane 4, at t_5; lane 5, at $t_{6.5}$; lane 6, at t_8; lane 7 at t_{20}. P1 and P2 indicate start sites of transcription. The first four lanes are sequencing tracks

(Fig. 3, lanes 3–6). Transcription from two adjacent sites upstream from the P1 start site (P2 in Fig. 3) was barely detected in the $t_{6.5}$ sample and was seen more clearly in the t_8 and t_{20} samples (Fig. 3, lanes 5–7). A pattern resembling that for the onset of transcription from P1 was observed for a pair of bands (seen below P1) except that these bands were also found at later time points. It is not known if these and other fainter bands represent inefficiently utilized start sites or are artifacts arising from the degradation of RNA or the use of reverse transcriptase. Overall, the pattern of transcription displaced in Fig. 3 is similar to that reported earlier for the transcription of the *cryIA(a)* gene of *B. thuringiensis* subsp. *kurstaki* HD-1-Dipel. The latter gene is transcribed from two overlapping promoters, Bt I and Bt II, that are activated at different times (Wong et al. 1983).

Figure 4 shows the DNA sequence upstream from the translational start codon (TTG) for *cryIB* and compares the promoter regions of the *cryIA(a)* (Wong et al. 1983) and *cryIB* genes. The P1 and P2 initiation sites mapped to the identical positions as the Bt I and Bt II start sites for *cryIA(a)*. This result is significant in that it facilitates the construction of consensus sequences for different forms of RNA polymerase recognizing crystal protein gene promoters (Brown and Whiteley 1990). The DNA sequences preceding the *cryIB* and *cryIA(a)* transcription initiation sites are very similar in the region −1 to −56 (45 of 56 bases are identical) and the presumed −10 and −35 regions of the P1 and P2 promoters of *cryIB* are almost identical with those of the Bt I and Bt II promoters of *cryIA(a)*. However, the *cryIB* promoter is 180 bases upstream of the translational start codon compared to 67 bases for the *cryIA(a)* promoter. The locations of the promoter regions for Bt I and Bt II have also been confirmed by deletion analysis (Schnepf et al. 1985; H.E. Schnepf et al., in preparation).

Although the transcriptional termination site of *cryIB* was not mapped, it seems likely that termination occurs following a thermodynamically stable potential stem

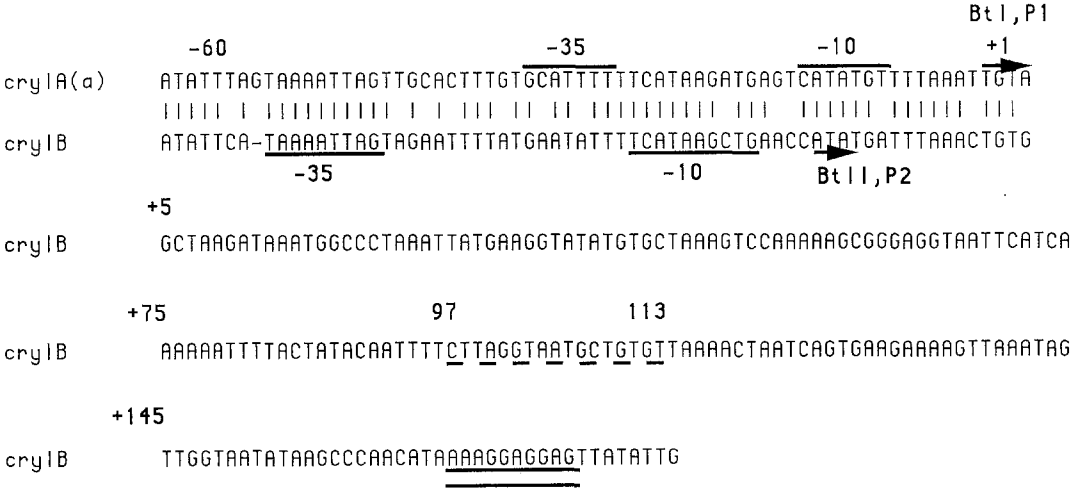


Fig. 4. The transcriptional start sites in *B. thuringiensis* for *cryIB* (taken from the data shown in Fig. 3 and *cryIA(a)*; Wong et al. 1983) are indicated with *horizontal half-arrows; single lines* above and below mark the −10 and −35 regions for the two promoters for each gene, the *double lines* below the sequence indicate the potential ribosome binding site that precedes the TTG translational start codon

(19 bp) and loop (4 bp) structure ($\Delta G = -22$ kCal) that is located 100 bp downstream from the translational stop codon; this inverted repeat is followed by 6 "T" residues (data not shown). Similar potential stem-loop structures resembling rho-independent terminators, have been found for the *cryIA* genes (Höfte and Whiteley 1989) including *cryIA(a)*. The transcriptional termination site for the latter gene was determined by S1 mapping (Schnepf et al. 1985).

Expression of cryIB *in* E. coli

Immunoblot analyses of extracts of *E. coli* strains JM83 or MC1000 containing the *cryIB* gene on plasmid pRH3 frequently showed only trace amounts of full-length *cryIB* and large amounts of two peptides of ca. 71 kDa and 65 kDa, whereas recombinant strains of *E. coli* JM83 bearing *cryIA* genes accumulate inclusions containing the full-length crystal proteins (Kronstad and Whiteley 1986; Schnepf et al. 1985). Additionally, as noted above, the translational start codon deduced from the DNA sequence of *cryIB* on pRH3 is TTG. These observations suggest that *cryIB* is more unstable in *E. coli* JM83 than the *cryIA* proteins and also suggest that either transcription or translation of *cryIB* may be less efficient in *E. coli* transformed with pRH3. To determine if the rarely used start codon contributed to the low level of expression in *E. coli*, it was changed to ATG (plasmid pRH4). Extracts of *E. coli* JM83 transformed with pRH4 contained larger amounts of full-length *cryIB* (data not shown).

We have encountered another instance of apparently poor expression of a crystal protein gene cloned in *E. coli*. We found that the product of the *cytA* gene, the 27 kDa cytolytic protein of *B. thuringiensis* subsp. *israelensis*, was detected in extracts of *E. coli* JM83 or MC1000 provided that the *E. coli* strain also expressed a 20 kDa protein encoded by a segment of DNA located ca. 4 kb upstream from *cytA* (McLean and Whiteley 1987). The effect of the 20 kDa peptide was post-transcriptional and required the intact 20 kDa peptide (Adams et al. 1989). However, if *cytA* was cloned into *E. coli* SG935 or W3350, each of which has a mutated *htpR* gene (the gene that regulates the heat shock response; Grossman et al. 1984), the 20 kDa peptide was no longer required (Visick and Whiteley 1991). One possible explanation for these observations is that the 20 kDa peptide protects *cytA* from proteolysis caused by *htpR*-regulated gene products (Straus et al. 1988).

To determine if proteins of the heat shock regulon influence the amounts of *cryIB* detected in *E. coli*, we cloned pRH4 (ATG start codon) and pRH3 (TTG start codon) into *E. coli* W3350 which has a temperature-sensitive *htpR* gene and examined extracts of the recombinant strains grown at 37° C (non-permissive temperature) and 30° C (permissive temperature). As shown in lane 5 of Fig. 5, neither the full-length 140 kDa CryIB protein nor its degradation products could be detected in the recombinant strain containing pRH3 grown at 30° C, however, full-length and some degraded forms

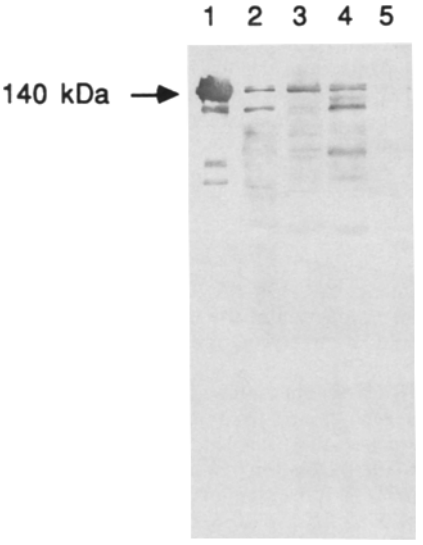


Fig. 5. Immunoblotting analysis for the presence of *cryIB* in extracts of recombinant *Escherichia coli* cells. Lane 1, crystals purified from *B. thuringiensis* subsp. *thuringiensis* HD-290-I (control); remaining lanes show proteins detected in 0.01 ml (lane 2) or 0.1 ml (lanes 3–5) of culture from the following *E. coli* strains: lanes 2 and 3, *htpR*^{ts} bearing pRH4 (ATG start codon) grown at 37° C (non-permissive temperature and 30° C (permissive temperature), respectively; lanes 4 and 5, *htpR*^{ts} bearing pRH3 (TTG start codon) grown at 37° C and 30° C, respectively

were found in extracts of the same strain grown at 37° C (lane 4). Extracts of the same *E. coli* strain transformed with pRH4 contained the full-length peptide when grown at either temperature (lanes 2 and 3), however, five- to ten-fold more *cryIB* antigen with a larger proportion of degradation products were found in extracts of the culture grown at 37° C (lane 2). These observations suggest that the apparently low expression of the *cryIB* gene on pRH3 in *E. coli* strain JM83 involves both inefficient translation initiation at the TTG start codon and protein degradation controlled by the heat shock regulon.

Toxicity of the cloned gene product

A *B. thuringiensis* strain thought to contain only *cryIB* was shown to be toxic to *Pieris brassicae* and to have little, if any, toxicity for several other *Lepidoptera*, including *Manduca sexta* (Höfte et al. 1988). However, as noted by Höfte and Whiteley (1989), such determinations may include additional comigrating proteins encoded by toxin genes too distantly related to be detected with existing DNA or antibody probes. The toxicity of the cloned *cryIB* gene was therefore estimated using inclusions produced by *E. coli* containing pBZ1, a construct in which the gene with the ATG start codon had been fused to the *lacZ* promoter to enhance expression (see Materials and methods). The inclusions had an LC_{50} of 58 ng/cm^2 on *Artogeia rapae* (*Pieris rapae*) with 95% confidence limits of 117 ng/cm^2 and 30 ng/cm^2. However, in agreement with prior results (Höfte et al. 1988), *M. sexta* was not killed by doses of 5000 ng/cm^2.

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Note added in proof

The nucleotide sequence data reported in this paper will appear in the EMBL, Gen Bank, and DDBJ Nucleotide Sequence Databases under the accession number X60950 *B. thuringiensis cryIB* gene.