Identification of an insecticidal crystal protein from *Bacillus thuringiensis* DSIR517 with significant sequence differences from previously described toxins

ANDREW P. GLEAVE,* REBECCA J. HEDGES and ANDREW H. BROADWELL

Molecular Biology Group, DSIR Plant Protection, Private Bag, Auckland, New Zealand

(Received 28 June 1991; revised 17 September 1991; accepted 11 October 1991)

The nucleotide (nt) sequence of a DNA segment containing the majority of a gene cloned from *Bacillus thuringiensis* DSIR517 encoding a 130 kDa insecticidal crystal protein has been determined. Sequence analysis reveals an open reading frame (ORF) of 3453 nt. The ATG initiation codon, which is preceded by a potential ribosome-binding site sequence, was confirmed by N-terminal amino acid sequencing. The ORF extends beyond the 3' terminus of the cloned fragment; however, the high degree of homology between the deduced amino acid sequence of this ORF and other Cry proteins suggests the clone lacks only five C-terminal amino acids. Making this assumption, the ORF of 3468 nt encodes a protein of 1156 amino acids with an estimated molecular mass of 129700 Da. Analysis of the deduced amino acid sequence reveals a number of features characteristic of Cry proteins. Alignment of the Cry 517 protein sequence with other Cry proteins suggests it is most closely related to the *cryIA-E* genes but sufficiently different to form a new *cryI* gene subclass.

Introduction

Bacillus thuringiensis is a Gram-positive soil bacterium characterized by the production of proteinaceous crystals during sporulation. The crystals are composed of proteins which exhibit highly specific insecticidal activity. *B. thuringiensis* strains active against larvae of certain members of Lepidoptera, Diptera or Coleoptera have been identified and over 40 *cry* genes encoding insecticidal crystal proteins (ICPs), active against these three insect orders, have been cloned and sequenced. These *cry* genes are classified according to their nucleotide sequence and the host specificity of their product (Hofte & Whiteley, 1989). Genes encoding lepidopteran-active proteins (130–140 kDa) are designated as *cryI*, as is a *cry* gene of *B. thuringiensis* subsp. *aizawai* IC-1 with lepidopteran and dipteran activity. Similarly, genes encoding lepidopteran-active and lepidopteran/dipteran-active proteins (65 kDa) are desig-

nated as *cryII*, coleopteran-active proteins (73 kDa) as *cryIII* and dipteran-active proteins (72–135 kDa) as *cryIV*.

There have been a number of reports of insect resistance to *B. thuringiensis* ICPs (McGaughey, 1985; McGaughey & Beeman, 1988; Tabashnik *et al.*, 1990). In one instance, such resistance was attributed to a reduction in binding affinity of the proteolytically activated toxin to the membrane of the susceptible insect epithelial midgut cells (Van Rie *et al.*, 1990). Binding of the toxin is necessary for subsequent cell lysis (Wolfersberger, 1990), which results in the death of the larva.

As part of a strategy to minimize the potential problems of resistance to *B. thuringiensis* ICPs we have initiated a programme to identify novel *B. thuringiensis* *cry* genes, with the aim of obtaining new proteins which bind to alternative membrane receptors on the insect midgut cells. Increasing the diversity of genes available, for either microbial insecticides or transgenic plants, would facilitate the simultaneous use of genes encoding insecticidal proteins which bind to different membrane receptors, and should both increase the efficiency of pest control and delay the emergence of resistance.

In this report we describe the determination of the nucleotide sequence of a novel *cry* gene cloned from *B. thuringiensis* DSIR517, isolated from an agricultural soil sample in New Zealand (Wigley & Chilcott, 1990). *B.*

* Author for correspondence. Tel. (09) 893 660; fax (09) 863 330.

Abbreviations: ICP, insecticidal crystal protein; nt, nucleotide(s); RBS, ribosome-binding site.

The nucleotide sequence data reported in this paper have been submitted to Genbank and have been assigned the accession number X58534.

thuringiensis DSIR517 produces crystals, composed of at least two 130–135 kDa proteins and a 60 kDa protein, which are toxic to the leafroller *Epiphyas postvittana* (Lepidoptera: Tortricidae), a major horticultural pest in New Zealand. The deduced amino acid sequence, encoded by the cloned gene, is compared with that of previously reported Cry proteins and discussed.

Methods

Bacterial strains and culture conditions. *Bacillus thuringiensis* DSIR517 was obtained from the DSIR *B. thuringiensis* culture collection. *E. coli* JM109 (Yanisch-Perron *et al.*, 1985) was used as the host for the Bluescript SK⁺ (Stratagene) and pGEM7Zf⁻ (Promega) derivatives. JM109 harbouring these derivatives were grown at 37 °C in Luria-Bertani medium (Maniatis *et al.*, 1982) containing ampicillin (50 µg ml⁻¹). Agar was added to 1.5% (w/v) to solidify the medium.

Plasmids and phage. Plasmids pSK517 and pGEM517, derivatives of Bluescript SK⁺ and pGEM7Zf⁻, respectively, containing a 5.2 kb *Eco*RI fragment encoding most of a *cry* gene from *B. thuringiensis* DSIR517 (A. H. Broadwell and coworkers, unpublished) were used as the source of DNA in nucleotide sequence determination. Plasmid DNA was isolated as described by Del Sal *et al.* (1988). Single-stranded template DNA for nucleotide sequencing was prepared essentially as described in the Stratagene sequencing manual, using R408 helper phage.

DNA manipulations. Transformation of *E. coli* was carried out using a modified procedure of Meyer *et al.* (1977). Restriction enzymes and T4 DNA ligase (Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs) were used according to the manufacturers recommendations. Exonuclease III/SI nuclease unidirectional deletions were generated as described for the Erase-a-Base System (Promega) with minor modifications. All other recombinant DNA techniques were as described in Maniatis *et al.* (1982).

Protein analysis and sequencing. Crystals of *B. thuringiensis* DSIR517 were purified by growing the cells to sporulation at 30 °C in CCY media (Stewart *et al.*, 1981). Spores and crystals were washed in 1 M-NaCl/10 mM-EDTA and separated by differential centrifugation through sucrose gradients. Polypeptides of purified crystals were separated by 5% (w/v) polyacrylamide gel electrophoresis using a Hoefer SE600 gel system. Polypeptides were electroblotted onto polyvinylidene difluoride membranes as described by Matsudaira (1987) and subjected to automated Edman degradation amino acid sequencing in an Applied Biosystems Gas Phase Sequencer (Model 470A). Recombinant protein preparations from λ gt11 lysogens, immunological screening of plaques and Western blotting were all carried out as described in the Promega Protocols and Applications Manual.

Nucleotide sequencing. Dideoxy chain termination sequencing (Sanger *et al.*, 1977) of single-stranded DNA templates was carried out using a Sequenase kit (United States Biochemical Corporation) incorporating [α -³⁵S]dATP (Amersham International). The universal 17-mer primer and custom-made 17-mer oligonucleotides synthesized on a Cyclone Plus DNA Synthesizer (Milligen) were used as primers. Both strands of the *cry-517* ORF were sequenced.

Nucleotide and amino acid sequence analysis. Sequence data analysis was carried out using the LINEUP, BESTFIT, GAP and GAPSHOW version 5 programs of the University of Wisconsin, Genetics Computer Group Sequence Analysis Package, on a MicroVAX 3400 computer.

Fig. 1. Restriction map of 5.2 kb *Eco*RI fragment cloned from *B. thuringiensis* DSIR 517. The position of the *cry-517* ORF and direction of transcription are indicated by the arrow. Restriction sites: B, *Bam*HI; C, *Cla*I; H, *Hind*III; Hc, *Hinc*II; K, *Kpn*I; RI, *Eco*RI; RV, *Eco*RV; S, *Sst*I.

Results and Discussion

Nucleotide sequence of the *cry-517* gene

Total genomic DNA, isolated from *B. thuringiensis* DSIR517, was digested with *Eco*RI and ligated to the *Eco*RI arms of λ gt11. Recombinant λ gt11 plaques were screened immunologically with antibody raised against purified crystals of *B. thuringiensis* DSIR517, to identify clones expressing a *cry* gene. Restriction mapping of positive clones indicated that they all contained a 5.2 kb *Eco*RI insert. To facilitate the determination of the nucleotide sequence of the *cry* gene cloned from *B. thuringiensis* DSIR517, the 5.2 kb *Eco*RI fragment (Fig. 1) was subcloned into the Bluescript vector SK⁺ (Stratagene) and pGEM7Zf⁻ (Promega), generating pSK517 and pGEM517, respectively. Exonuclease III/SI nuclease deletions were generated across the 5.2 kb insert in both directions and single-stranded templates of suitable clones were sequenced.

The nucleotide sequence of the cloned *cry-517* gene from *B. thuringiensis* DSIR517 consists of a protein coding region of 3453 nt, as shown in Fig. 2. The ATG initiation codon is located at nt 385, preceded by a potential RBS, 5'-GTAGGAGGAAAA-3'. Confirmation of the proposed initiation codon was established by Edman degradation amino acid sequencing, which indicated the five N-terminal amino acids of the 130 kDa protein of *B. thuringiensis* DSIR517 to be M-N-Q-N-K.

Sequence analysis reveals that the 3' terminus of the *cry-517* ORF lies beyond the end of the cloned fragment. The high degree of homology between the C-termini of the protein encoded by the cloned gene and those of other Cry I proteins implies that the clone lacks five C-terminal amino acids. Assuming that the clone lacks these 5 amino acids, the ORF (3468 nt) codes for a protein of approximately 129.7 kDa consisting of 1156 amino acid residues. This is consistent with the size of the protein as estimated by SDS-polyacrylamide gel electrophoresis (130 kDa).

CAGAAATCAACTTACAAGTATTAGGGATTTCCCGTTTCGACCCGTTTATACAAAAC TAG 60
 TATACTGCGATGGGTAGATATTCGTCTATTTCCCTTAAGTTTTTAAGATGCAGTATATGTA 120
 GATCATATTTTTAAATATAGCGTTTCATCAACTACTATATTATGTAATTGACGGTAATCAT 180
 ATTCAAGTGGTGATTACAAATCAAAATTAATGAAAGAACATGCTCAATCTGCTCAAATA 240
 TCTGTTTTTTATTTATGAGTAAAAACCGAAGTTTGTGGAACGTAAACTGTAATAAACGTA 300
 ATGGCGAAGATATATAGATGTCAATATAAAAAAGTTAACCCTAAATAATGTTTTAAAAATTT 360
 AAAAAATATGTAGGAGGAAAAATTATGAATCAAAATAAACACGGGAATTATTGGCGCTTCC 420
 M N Q N K H G I I G A S
 AATTGTGGTTGTGCATCTGATGATGTTGCGAAATATCCTTTAGCCAACAATCCATATTCA 480
 N C G C A S D D V A K Y P L A N N P Y S
 TCTGCTTTAAATTTAAATCTTGTCAAAATAGTAGTATTCTCAACTGGATTAACATAATA 540
 S A L N L N S C Q N S S I L N W I N I I
 GGCATGTCAGCAAAAGAAGCAGTATCTATTGGGACAACCATAGTCTCTCTTATCACAGCA 600
 G D A A K E A V S I G T T I V S L I T A
 CCTTCTCTTACTAGTTAAATTAATAGTATATGACCTTATAGGTAAGTACTAGGAGGT 660
 P S L T G L I S I V Y D L I G K V L G G
 AGTAGTGGACAATCCATATCAGATTTGTCTATATGTGACTTATTATCTATTATTGATTTA 720
 S S G Q S I S D L S I C D L L S I I D L
 CGGGTAAGTCAGAGTGTTTAAATGATGGGATTGAGATTTTAATGGTTCTGTACTCTTA 780
 R V S L T N G S V L N D G I A D F N G S V L L
 TACAGGAACATTTTAGAGGCTCTGGATAGCTGGAATAAGAATCCTAATTCTGCTTCTGCT 840
 Y R N Y L E A L D S W N K N P N S A S A
 GAAGAACTCCGTACTCGTTTTAGAATCGCCGACTCAGAATTTGATAGAATTTTAACCCG 900
 E E L R T R F R I A D S E F D R I L T R
 GGGTCTTTAACGAATGGTGGCTCGTTAGCTAGACAAAATGCCCAAATATTATTATTACCT 960
 G S L T N G G S L A R Q N A Q I L L L P
 TCTTTTGCAGAGCGCTGCATTTTCCATTTTATTACTACTAAGGATGCTACTAGATATGGC 1020
 S F A S A A F F H L L L L R D A T R Y G
 ACTAATTGGGGCTATACAATGCTACACCTTTTATAAATTATCAATCAAACTAGTAGAG 1080
 T N W G L Y N A T P F I N Y Q S K L V E
 CTTATTGAATATATACTGATTATTGCGTACATTGGTATAATCGAGGTTTCAACGAACTA 1140
 L I E L Y T D Y C V H W Y N R G F N E L
 AGACAACGAGGCACTAGTCTACGCTTGTTAGAAATTCATAGATATCGTAGAGAGATG 1200
 R Q R G T S A T A W L E F H R Y R R E M
 ACATTGATGGTATTAGATATAGTAGCATATTTTCAAGTCTTGATATTACTAATTACCC 1260
 T L M V L D I V A S F S S L D I T N Y P
 ATAGAAACAGATTTTCAGTTGAGTAGGGTCATTTATACAGATCCAATTGGTTTTGTACAT 1320
 I E T D F Q I E Y I Y T D P I G F V H
 CGTAGTAGTCTTAGGGGAGAAAGTTGGTTAGCTTTGTTAATAGAGCTAATTCTCAGAT 1380
 R S S L R G E S W F S F V N R A N F S D
 TTAGAAAATGCAATACCTAATCCTAGACCGTCTTGTTTAAATAATATGATTATATCT 1440
 L E N A I P N P R P S W F L N N M I I S
 ACTGGTTCACTTACATTGCGGTTAGCCCAAGTACTGATAGAGCGAGGGTATGGTATGGA 1500
 T G S L T P V S P S T D R A R V W Y G
 AGTCGAGATCGAATTTCCCTGCTAATTCACAATTTATTACTGAACTAATCTCTGGACAA 1560
 S R D R I S P A N S Q F I T E L I S G Q
 CATACGACTGCTACACAACTATTTTAGGGCGAAATATATTAGAGTAGATTCTCAAGCT 1620
 H T T A T Q T I L G R N I F R V D S Q A
 TGTAATTTAAATGATACCACATATGGAGTGAATAGGGCGGTATTTTATCATGATGCGAGT 1680
 G N L N D T T Y G V N R A V F Y H D A S
 GAAGGTTCTCAAAGATCCGTGTACGAGGGGTATATTGGAACAACTGGGATAGATAACCCCT 1740
 E G S Q R S V Y E G Y I R T T G I D N P
 AGAGTTCAAATATTAACACTTATTTACCTGGAGAAAATTCAGATATCCCAACTCCAGAA 1800
 R V Q N I N T Y L P G E N S D I P T P E
 GACTATCTCATATTAAGCACAACAATAAATTTAACAGGAGGACTTAGACAAGTAGCA 1860
 D Y T H I L S T T I N L T G G L R Q V A
 TCTAATCGCCGTTTCATCTTTAGTAATGTATGGTTGGACACATAAAAGTCTGGCTCGTAAC 1920
 S N R R S S L V M Y G W T H K S L A R N
 AATACCATTAAATCCAGATAGAATTACACAGATACCATTGACGAAGGTTGATACCCGAGGC 1980
 N T I N P D R I T Q I P L T K V D T R G
 ACAGGTGTTTCTTATGTGAATGATCCAGGATTTATAGGAGGAGCTCTACTTCAAAGGACT 2040
 T G V S Y V N D P G F I G G A L L Q R T

```

GACCATGGTTCGCTTGGAGTATTGAGGGTCCAATTTCCACTTCACTTAAGACAACAATAT 2100
D H G S L G V L R V Q F P L H L R Q Q Y
CGTATTAGAGTCCGTTATGCTTCTACAAACAAATATTTCGATTGAGTGTGAATGGCAGTTTC 2160
R I R V R Y A S T T N I R L S V N G S F
GGTACTATTTCTCAAAATCTCCCTAGTACAATGAGATTAGGAGAGGATTTAAGATACGGA 2220
G T I S Q N L P S T M R L G E D L R Y G
TCTTTTGCTATAAGAGAGTTTAATACTTCTATTAGACCCACTGCAAGTCCCGACCAAATT 2280
S F A I R E F N T S I R P T A S P D Q I
CGATTGACAAATAGAACCATCTTTTATTAGACAAGAGGCTCTATGTAGATAGAATTGAGTTTC 2340
R L T I E P S F I R Q E V Y V D R I E F
ATTCCAGTTAATCCGACGCGAGAGGCGAAAGAGGATCTAGAAGCAGCAAAAAAAGCGGTG 2400
I P V N P T R E A K E D L E A A K K A V
GCGAGCTTGTTCACGCGACAAGGGACGGATTACAAGTAAATGTGAAAGATTATCAAGTC 2460
A S L F T R T R D G L Q V N V K D Y Q V
GATCAAGCGGCAAAATTTAGTGTCTGCTTATCAGATGAACAAATATGGGTATGACAAAAAG 2520
D Q A A N L V S C L S D E Q Y G Y D K K
ATGTTATTGGAAGCGGTACGTGCGGCAAAACGACTTAGCCGAGAACGCAACTTACTTCAG 2580
M L L E A V R A A K R L S R E R N L L Q
GATCCAGATTTTAATACAATCAATAGTACAGAAGAAATGGATGGAAGCAAGTAACGGC 2640
D P D F N T I N S T E E N G W K A S N G
GTTACTATTAGTGAGGGCGGGCCATTCTATAAAGGCCGTGCAATTCAGCTAGCAAGTGCA 2700
V T I S E G G P F Y K G R A I Q L A S A
CGAGAAAATTACCCAACATACATCTATCAAAAAGTAGATGCATCGGAGTTAAAGCCGTAT 2760
R E N Y P T Y I Y Q K V D A S E L K P Y
ACACGTTATAGACTGGATGGGTTCGTGAAGAGTAGTCAAGATTAGAAATTGATCTCATT 2820
T R Y R L D G F V K S S Q D L E I D L I
CACCATAAAGTCCATCTTGTGAAAAATGTACCAGATAATTTAGTATCTGATACCTTAC 2880
H H H K V H L V K N V P D N L V S D T Y
CCAGATGATTCTGTAGTGAATCAATCGATGTCAGGAACAACAGATGGTAAATGCGCAA 2940
P D D S C S G I N R C Q E Q Q M V N A Q
CTGGAACAGAGCATCATCATCCGATGGATTGCTGTGAAGCAGCTCAAACACATGAGTTT 3000
L E T E H H H P M D C C E A A Q T H E F
TCTTCTATATTGATACAGGGGATTAAATTCGAGTGTAGACCAGGGAATCTGGGCGATC 3060
S S Y I D T G D L N S S V D Q G I W A I
TTTAAAGTTCGAACAACCGATGGTTATGCGACGTTAGGAAATCTTGAATTGGTAGAGGTC 3120
F K V R T T D G Y A T L G N L E L V E V
GGACCGTTATCGGGTGAATCTTTAGAACGTGAACAAAGGGATAATACAAATGGAGTGCA 3180
G P L S G E S L E R E Q R D N T K W S A
GAGCTAGGAAGAAAGCGTCAGAAACAGATCGCGTGTATCAAGATGCCAAACATCCATC 3240
E L G R K R A E T D R V Y Q D A K Q S I
AATCATTTATTTGTGGATTATCAAGATCAACAATTAATCCAGAAATAGGGATGGCAGAT 3300
N H L F V D Y Q D Q Q L N P E I G M A D
ATTATGGACGCTCAAAATCTTGTGCGATCAATTTAGATGTATATAGCGATGCCGTACTG 3360
I M D A Q N L V A S I S D V Y S D A V L
CAAATCCCTGGAATTAATATGAGATTACACAGAGCTGTCCAATCGCTTACAACAGCA 3420
Q I P G I N Y E I Y T E L S N R L Q Q A
TCGTATCTGTATACGTCTCGAAATGCGGTGCAAAATGGGGACTTTAACAACGGGCTAGAT 3480
S Y L Y T S R N A V Q N G D F N N G L D
AGCTGGAATGCAACAGCGGGTGCATCGGTACAACAGGATGGCAATACGCATTCTTAGTT 3540
S W N A T A G A S V Q Q D G N T H F L V
CTTTCTCATTGGGATGCACAAGTTTCTCAACAATTTAGAGTGCAGCCGAATTGTAAATAT 3600
L S H W D A Q V S Q Q F R V Q P N C K Y
GTATTACGTGTAACAGCAGAGAAAGTAGGCGGCGGAGACGGATACGTGACTATCCGGGAT 3660
V L R V T A E K V G G G D G Y V T I R D
GATGCTCATCATACAGAAACGCTTACATTTAATGCATGTGATTATGATATAAATGGCAG 3720
D A H H T E T L T F N A C D Y D I N G T
TACGTGACTGATAATACGTATCTAACAAAAGAGTGGTATTCCATCCGGAGACACAACAC 3780
Y V T D N T Y L T K E V V F H P E T Q H
ATGTGGGTAGAGGTAAATGAAACAGAAGGTGCATTTTCATATAGATAGTATTGAATTC 3837
M W V E V N E T E G A F H I D S I E F

```

Fig. 2. Nucleotide sequence of crystal protein gene *cry-5/7* and the deduced amino acid sequence. The one-letter amino acid code is shown below the nucleotide sequence. A potential RBS, upstream of the ATG initiation codon (nt 385) is indicated by the underlined letters.

cry517	AQILLPLSPASAAFFHLLLRDSTRYGTNW	YTDYCVHYNRGFNEL	...RQRGTSATAMLEPHRYRREMTLVLDIVASFSSLDITNYP	...IETDFQLSRVIYT
cryIA (a)	YQVPLLSVYQAANLHLSVLRDVSFVGQRN	YTDYAVRWYNTGLERV	...WGPDSRDWVRYNQFRRELTITVLDIVALFSDYDRTYP	...IRTVSGLTREIYT
cryIA (b)	YQVPLLSVYQAANLHLSVLRDVSFVGQRN	YTDHAWRWYNTGLERV	...WGPDSRDWVRYNQFRRELTITVLDIVALFSDYDRTYP	...IRTVSGLTREIYT
cryIA (c)	YQVPLLSVYQAANLHLSVLRDVSFVGQRN	YTDYAVRWYNTGLERV	...WGPDSRDWVRYNQFRRELTITVLDIVALFSDYDRTYP	...IRTVSGLTREIYT
cryIB	QEVPLLMVYAQAANLHLSVLRDVSFVGQRN	YSDYCVHYNRGFNEL	...RGTNAASWVRYNQFRRELTITVLDIVALFSDYDRTYP	...INTSAGLTREIYT
cryIC	FEVPLLSVYAQAANLHLSVLRDVSFVGQRN	YADHCANTYNRGLNML	...PKSTYQWITYNRLRLDITVLDIAAFPPNYDNRYP	...IQPVGQLTREIYT
cryID	YEVALLSVYQAANLHLSVLRDVSFVGQRN	YTNHCVDYTNQGLRRL	...EGRFLSDWVRYNQFRRELTITVLDIVAFPPNYDRTYP	...IQPVGQLTREIYT
cryIE	YQVPLLSVYQAANLHLSVLRDVSFVGQRN	YTDYAVRWYNTGLERV	...WGPDSRDWVRYNQFRRELTITVLDIVALFSDYDRTYP	...IRTVSGLTREIYT
cryIIIA	YEVPLLSVYQAANLHLSVLRDVSFVGQRN	YTDHAWRWYNTGLERV	...WGPDSRDWVRYNQFRRELTITVLDIVALFSDYDRTYP	...IRTVSGLTREIYT
cryIIIB	FEVPLLSVYAQAANLHLSVLRDVSFVGQRN	YTDHAWRWYNTGLERV	...WGPDSRDWVRYNQFRRELTITVLDIVALFSDYDRTYP	...IRTVSGLTREIYT
cryIVA	YEVPLLSVYQAANLHLSVLRDVSFVGQRN	YTDHAWRWYNTGLERV	...WGPDSRDWVRYNQFRRELTITVLDIVALFSDYDRTYP	...IRTVSGLTREIYT
cryIVB	YEVPLLSVYQAANLHLSVLRDVSFVGQRN	YTDHAWRWYNTGLERV	...WGPDSRDWVRYNQFRRELTITVLDIVALFSDYDRTYP	...IRTVSGLTREIYT
cryIVC	YEVPLLSVYQAANLHLSVLRDVSFVGQRN	YTDHAWRWYNTGLERV	...WGPDSRDWVRYNQFRRELTITVLDIVALFSDYDRTYP	...IRTVSGLTREIYT
cryIIA	YQVPLLSVYQAANLHLSVLRDVSFVGQRN	YTDHAWRWYNTGLERV	...WGPDSRDWVRYNQFRRELTITVLDIVALFSDYDRTYP	...IRTVSGLTREIYT

cry517	YGWTHKSLARNNTINPDRIQIPLTKVDTRGTGVSYNDPGFIGGALL	QYRIRVRYAS	VYVDRIEFIPVN
cryIA (a)	FSWQERSAEFNNIPSSQITQIPLTKSTNLGSGTSVVKGPFGTGGDIL	RYRVIRIYAS	VYIDRIEFVPAE
cryIA (b)	FSWIERSAEFNNIPSSQITQIPLTKSTNLGSGTSVVKGPFGTGGDIL	RYRVIRIYAS	VYIDRIEFVPAE
cryIA (c)	FSWIERSAEFNNIPSSQITQIPLTKSTNLGSGTSVVKGPFGTGGDIL	RYRVIRIYAS	VYIDRIEFVPAE
cryIB	YSWTHRSADRTWTIGPNRITQIPVKASELPGQTTVVRGPGFTGGDIL	RYRVIRIYAS	VYIDRIEFVPAE
cryIC	FSWTHRSADRTWTIGPNRITQIPVKASELPGQTTVVRGPGFTGGDIL	RYRVIRIYAS	VYIDRIEFVPAE
cryID	FSWTHRSADRTWTIGPNRITQIPVKASELPGQTTVVRGPGFTGGDIL	RYRVIRIYAS	VYIDRIEFVPAE
cryIE	FSWTHRSADRTWTIGPNRITQIPVKASELPGQTTVVRGPGFTGGDIL	RYRVIRIYAS	VYIDRIEFVPAE
cryIIIA	FSWTHRSADRTWTIGPNRITQIPVKASELPGQTTVVRGPGFTGGDIL	RYRVIRIYAS	VYIDRIEFVPAE
cryIIIB	FSWTHRSADRTWTIGPNRITQIPVKASELPGQTTVVRGPGFTGGDIL	RYRVIRIYAS	VYIDRIEFVPAE
cryIVA	FSWTHRSADRTWTIGPNRITQIPVKASELPGQTTVVRGPGFTGGDIL	RYRVIRIYAS	VYIDRIEFVPAE
cryIVB	FSWTHRSADRTWTIGPNRITQIPVKASELPGQTTVVRGPGFTGGDIL	RYRVIRIYAS	VYIDRIEFVPAE
cryIVC	FSWTHRSADRTWTIGPNRITQIPVKASELPGQTTVVRGPGFTGGDIL	RYRVIRIYAS	VYIDRIEFVPAE

Fig. 3. Comparison of the conserved amino acid sequence blocks encoded by the *cry-517* gene with those encoded by other *cry* genes. Amino acids marked by the shading are identical or conservatively changed for at least 10 of the 13 sequences. References for sequences can be found in Hofte & Whiteley (1989), except for *cryIE* (Visser *et al.*, 1990) and *cryIIIB* (Sick *et al.*, 1990).

No significant homology was found between sequences upstream of the *cry-517* translational start and the proposed promoter sequences for previously reported *B. thuringiensis* *cry* genes (Wong *et al.*, 1983; Brown & Whiteley, 1988, 1990). The absence of such sequences suggests that expression of the *cry-517* gene requires alternative RNA polymerase-associated σ factors to those used by RNA polymerase when transcribing the *cry* genes published to date. Alternatively, the *cry-517* gene may be expressed as a downstream region of an operon. Although the possibility of the *cry-517* gene being expressed as an operon cannot be discounted, the absence of any significant ORF in the 385 bp 5' to the ATG start of the *cry-517* gene suggests this is unlikely.

Conserved amino acid sequences of Cry 517

The deduced amino acid sequence of the *cry-517* gene shows the presence of the five highly conserved blocks (C_1 – C_5), which are present in the toxic portion of all published *B. thuringiensis* Cry proteins, with the exception of Cry II and Cry IV D (Hofte & Whiteley, 1989). The conserved blocks of the Cry 517 protein are located at amino acid residues 185–215 (C_1), 237–306 (C_2), 503–550 (C_3), 571–580 (C_4) and 645–656 (C_5). Fig. 3 shows an alignment of the conserved blocks of representatives of each of the classes of *B. thuringiensis* Cry proteins with those of Cry 517. Comparison of the amino acid sequence of these aligned blocks indicates that C_1 , C_2 and C_3 of Cry 517 show 54–70% similarity (identical

and conservative amino acid changes) to Cry I and Cry III proteins and only 46–57% similarity to Cry IVA–C proteins. The relatively shorter C_4 and C_5 blocks show a high degree of similarity (>70%) to all published Cry I, III and IVA–C protein sequences.

The overall pattern of amino acid similarity between Cry proteins is maintained by Cry 517. There are however, a number of amino acids in the conserved blocks which are conserved among the published Cry proteins but not conserved in Cry 517 nor are they altered to similar amino acids. One such difference occurs in block C_1 , four in C_2 and four in C_3 . Blocks C_1 and C_2 are thought to be involved directly in toxicity, whereas C_3 – C_5 are indirectly involved in host specificity (Wu & Aronson, 1990). Because of these differences in the conserved blocks, it is conceivable that the Cry 517 protein may have considerably different toxicity levels and host spectrum from other Cry proteins. Detailed analysis of the host spectrum and levels of toxicity of the *cry-517* gene product is required to establish the significance of these variations.

The hydrophobicity profile of the Cry 517 amino acid sequence indicates a hydrophobic region upstream of the C_1 block between amino acid residues 57–94. This hydrophobic region is characteristic of Cry proteins, and probably spans the membrane of insect midgut epithelial cells, as has been proposed for the corresponding hydrophobic region of the Cry IA(a) protein (Schnepf *et al.*, 1985).

Fig. 4. Amino acid sequence comparisons of the *cry-517* gene product with those of other *cryI* genes. The comparisons extend from the N-terminus of the protein through to the end of the conserved block C_5 . The top line represents the Cry 517 amino acid sequence, the bottom line the Cry amino acid sequence with which it is being compared, as listed to the left of the diagram. Vertical lines represent perfectly conserved amino acids between the two sequences. Gaps introduced to obtain maximum alignment are represented by blocks above and below the vertical lines. The locations of conserved amino acid blocks (1–5) from Fig. 3 are indicated.

In addition, five potential trypsin cleavage sites, which could act as the proteolytic cleavage sites necessary for activation of the protoxin in the insect midgut, are located in and around block C_5 , between amino acid residues 649–670.

Alignment of Cry 517 protein sequence with other Cry I proteins

Alignment of the Cry 517 amino acid sequence with other Cry proteins indicates that Cry 517 is more closely related to Cry IA–E, showing 37–39% homology throughout the entire sequence, than to Cry II (23%), Cry III (30%) or Cry IVA–C (33–34%). Fig. 4 shows a diagrammatic representation of the homology between Cry 517 and Cry IA–E proteins up to the end of the block C_5 (i.e. the minimal toxic portion of the protein). The level of homology throughout this segment is 29–31%, whereas the homology beyond this region to the C-terminus is 43–45%. Optimal alignment of the C-terminal portion of the Cry 517 protein (amino acid residues 657–1156) with Cry IA–E proteins requires the

introduction of two significant gaps into the Cry 517 sequence suggesting a deletion of some 68 amino acids. This is similar to the deletion of 26 amino acids observed when aligning the C-terminal portion of Cry IA(b) with other Cry I proteins (Geiser *et al.* 1986).

Amino acid conservation between Cry 517 and other Cry I proteins extends throughout the molecule, but as previously discussed, is significantly higher in the regions of the five conserved blocks and the C-terminal region of the protein, which is cleaved off during protoxin activation. A high degree of heterogeneity occurs in the regions between the conserved blocks, most significantly between C_2 and C_3 which shows less than 22% identity to other Cry I proteins. This region has been implicated in the determination of host specificity of several Cry proteins (Ge *et al.*, 1989; Schnepf *et al.* 1990).

The N-terminal region of the Cry 517 protein differs from other Cry I proteins in containing a considerably greater number of amino acids in the region between the initial methionine and the C_1 block. However this may be of little significance as it has been demonstrated that

part of the N-terminus of Cry I proteins is not required for insecticidal activity (Hofte *et al.*, 1986). The important feature of the N-terminal region of the protein is probably the spatial relationship between the hydrophobic region and C₁ block. The number of amino acids between these two regions of the Cry 517 protein approximates to that of other Cry I proteins suggesting that the spatial relationship is maintained.

Although the *cry-517* gene appears to have at least a distant ancestral relationship to other *cryI* genes there are no significantly long stretches of homology to suggest that part of the gene has a closer evolutionary relationship to one *cryI* gene rather than another or, as is suggested for *cryIE* (Visser *et al.*, 1990), that *cry-517* is a result of a recombination event between two known *cry* genes.

Insecticidal activity of the cry-517 gene product

Western blot analysis of the Cry 517 protein from recombinant λ gt11 lysates indicated a low level of expression of the *cry-517* gene in *E. coli*. This may have been due to the lack of an *E. coli* promoter sequence. Alternatively, the low level expression may have been due to the absence of a transcriptional terminator, 3' to the *cry-517* ORF, resulting in unstable *cry-517* mRNA transcripts. The latter explanation has been observed with other genes lacking transcriptional terminators (Wong & Chang, 1985). Due to this low level of expression of the Cry 517 protein in *E. coli*, some difficulty was encountered in obtaining adequate protein for extensive toxicity studies. However, preliminary bioassays showed that 100 μ l of recombinant λ gt11 lysate incorporated into 1 ml of artificial diet (Malone & Wigley, 1990) caused 70% mortality of first instar larvae of *Epiphyas postvittana* (Lepidoptera: Tortricidae) in 6 d, whereas controls showed less than 10% mortality, demonstrating that the recombinant Cry 517 protein is biologically active.

The estimated molecular mass of 129 700 Da, and the fact that the amino acid sequence appears more closely related to Cry I proteins than to Cry II, III or IV proteins, suggests that according to the Hofte & Whiteley (1989) classification system, the *cry-517* gene is a *cryI*-type but is sufficiently different to form a new subclass. Although further characterization of the gene product, particularly toxicity data, is required before this gene can be assigned a *cry* number, we tentatively refer to the *cry-517* gene as *cryIG*.

We are grateful to D. Christie (University of Auckland) for the N-terminal amino acid determination and to R. L. S. Forster for his critical reading of this manuscript.

References

- BROWN, K. L. & WHITELEY, H. R. (1988). Isolation of a *Bacillus thuringiensis* RNA polymerase capable of transcribing crystal protein genes. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 4166–4170.
- BROWN, K. L. & WHITELEY, H. R. (1990). Isolation of a second *Bacillus thuringiensis* RNA polymerase that transcribes from a crystal protein gene promoter. *Journal of Bacteriology* **172**, 6682–6688.
- DEL SAL, G., MANFIOLETTA, G. & SCHNEIDER, C. (1988). A one tube plasmid DNA miniprep preparation suitable for sequencing. *Nucleic Acids Research* **16**, 9878.
- GE, A. Z., SHIVAROVA, V. I. & DEAN, D. H. (1989). Location of the *Bombyx mori* specificity domain on a *Bacillus thuringiensis* δ endotoxin protein. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 4037–4041.
- GEISER, M., SCHWEITZER, S. & GRIMM, C. (1986). The hypervariable region in the genes coding for entomopathogenic crystal proteins of *Bacillus thuringiensis*: Nucleotide sequence of the *kurhd1* gene of subsp. *kurstaki* HD1. *Gene* **48**, 109–118.
- HOFTE, H. & WHITELEY, H. R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Reviews* **53**, 242–255.
- HOFTE, H., DE GREVE, H., SEURINCK, J., JANSSENS, S., MAHILLON, J., AMP, C., VANDERKERCKHOVE, J., VANDERBRUGGEN, H., VAN MONTAGU, M. & VAECK, M. (1986). Structural and functional analysis of a cloned delta endotoxin of *Bacillus thuringiensis* Berliner 1715. *European Journal of Biochemistry* **161**, 273–280.
- MCGAUGHEY, W. H. (1985). Insect resistance to biological insecticide *Bacillus thuringiensis*. *Science* **229**, 193–195.
- MCGAUGHEY, W. H. & BEEMAN, R. W. (1988). Resistance to *Bacillus thuringiensis* in colonies of Indian meal moth and Almond moth (Lepidoptera: Pyralidae). *Journal of Economic Entomology* **81**, 28–33.
- MALONE, L. A. & WIGLEY, P. J. (1990). A practical method for rearing Argentine Stem Weevil, *Listronotus bonariensis*, (Coleoptera: Curculionidae) in the laboratory. *New Zealand Entomologist* **13**, 87–88.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MATSUDAIRA, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *Journal of Biological Chemistry* **262**, 10035–10038.
- MEYER, R., FIGURSKI, D. & HELINSKI, D. R. (1977). Physical and genetic studies with restriction endonucleases on the broad-host range plasmid RK2. *Molecular and General Genetics* **152**, 129–135.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SCHNEFF, H. E., WONG, H. C. & WHITELEY, H. R. (1985). The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. *Journal of Biological Chemistry* **260**, 6264–6272.
- SCHNEFF, H. E., TOMCZAK, K., ORTEGA, J. P. & WHITELEY, H. R. (1990). Specificity-determining regions of a lepidopteran-specific insecticidal protein produced by *Bacillus thuringiensis*. *Journal of Biological Chemistry* **265**, 20923–20930.
- SICK, A., GAERTNER, F. & WONG, A. (1990). Nucleotide sequence of a coleopteran-active gene from a new isolate of *Bacillus thuringiensis* subsp. *tolworthi*. *Nucleic Acids Research* **18**, 1305.
- STEWART, G. S. A. B., JOHNSTON, K., HAGELBERG, E. & ELLAR, D. J. (1981). Commitment of bacterial spores to germinate. A measure of the trigger reaction. *Biochemical Journal* **196**, 101–106.
- TABASHNIK, B. E., CUSHING, N. L., FINSON, N. & JOHNSON, M. W. (1990). Field development of resistance to *Bacillus thuringiensis* in Diamondback moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology* **83**, 1672–1676.
- VAN RIE, J., MCGAUGHEY, W. H., JOHNSON, D. E., BARNETT, B. D. & VAN MELLAERT, H. (1990). Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. *Science* **247**, 72–74.

- VISSER, B., MUNSTERMAN, E., STOKER, A. & DIRKSE, W. G. (1990). A novel *Bacillus thuringiensis* gene encoding a *Spodoptera exigua*-specific crystal protein. *Journal of Bacteriology* **172**, 6783–6788.
- WIGLEY, P. J. & CHILCOTT, C. N. (1990). *Bacillus thuringiensis* isolates active against the New Zealand pasture pest, *Costelytra zealandica* (Coleoptera: Scarabaeidae). In *Proceedings of the Vth International Colloquium on Invertebrate Pathology and Microbial Control*, p. 344. Edited by D. E. Pinnock. Adelaide.
- WOLFERSBERGER, M. (1990). Specificity and mode of action of a *Bacillus thuringiensis* insecticidal crystal protein to lepidopteran larvae: Recent insights from studies using midgut brush border membrane vesicles. In *Proceedings of the Vth International Colloquium on Invertebrate Pathology and Microbial Control*, pp. 278–282. Edited by D. E. Pinnock. Adelaide.
- WONG, H. C. & CHANG, S. (1985). Identification of a positive retroregulator that functions in *Escherichia coli* and *Bacillus subtilis*. In *Molecular Biology of Microbial Differentiation*, pp. 104–109. Edited by J. A. Hoch & P. Setlow. Washington, DC: ASM.
- WONG, H. C., SCHNEPF, H. E. & WHITELEY, H. R. (1983). Transcriptional and translational start sites for the *Bacillus thuringiensis* crystal protein. *Journal of Biological Chemistry* **172**, 6682–6688.
- WU, D. & ARONSON, A. I. (1990). Use of mutagenic oligonucleotides for defining regions of *Bacillus thuringiensis* δ endotoxin involved in toxicity. In *Proceedings of the Vth International Colloquium on Invertebrate Pathology and Microbial Control*, pp. 273–277. Edited by D. E. Pinnock. Adelaide.
- YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.