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Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects

(lepidopteran pathogen/biological insect control)

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ABSTRACT **A novel vegetative insecticidal gene, *vip3A(a)*, whose gene product shows activity against lepidopteran insect larvae including black cutworm (*Agrotis ipsilon*), fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), tobacco budworm (*Heliothis virescens*), and corn earworm (*Helicoverpa zea*) has been isolated from *Bacillus thuringiensis* strain AB88. VIP3-insecticidal gene homologues have been detected in ≈15% of *Bacillus* strains analyzed. The sequence of the *vip3A(b)* gene, a homologue of *vip3A(a)* isolated from *B. thuringiensis* strain AB424 is also reported. Vip3A(a) and (b) proteins confer upon *Escherichia coli* insecticidal activity against the lepidopteran insect larvae mentioned above. The sequence of the gene predicts a 791-amino acid (88.5 kDa) protein that contains no homology with known proteins. Vip3A insecticidal proteins are secreted without N-terminal processing. Unlike the *B. thuringiensis* δ-endotoxins, whose expression is restricted to sporulation, Vip3A insecticidal proteins are expressed in the vegetative stage of growth starting at mid-log phase as well as during sporulation. Vip3A represents a novel class of proteins insecticidal to lepidopteran insect larvae.**

The isolation and characterization of new insecticidal activities is the basis of many pest control programs. *Bacillus thuringiensis*, a gram-positive soil bacterium, is well known for its ability to produce crystalline inclusions during sporulation which contain insecticidal proteins called δ-endotoxins. These inclusions are solubilized in insect midguts, releasing the δ-endotoxins that, upon proteolytic activation, exhibit a highly specific insecticidal activity (1). In the past decades, many *B. thuringiensis* strains with different insect host spectra have been identified and their δ-endotoxins used in formulations for biopesticides (2). Recently, the cloning of δ-endotoxin genes (3) and their expression in transgenic plants (4) has provided an alternative strategy for crop protection against insect damage.

Although *B. thuringiensis* δ-endotoxins are effective insecticidal proteins, there are several agronomically important insects that are less sensitive to their action (5). The lepidopteran black cutworm (BCW, *Agrotis ipsilon*) is an example. BCW is a worldwide pest that attacks more than 50 crops, including cereal grains (6). This pest is difficult to control because by the time the infestations are apparent, the susceptible stages (i.e., larvae) are past and damage may already be serious and irreversible.

Extensive screening programs are being carried out by various groups to search for *B. thuringiensis* strains with new insecticidal spectra. These evaluations have focused mainly on the identification of new δ-endotoxins that are expressed during sporulation (7). Our experimental approach focused on bacterial stages before sporulation, and has led to the identification of non-δ-endotoxins with insecticidal activities. We describe here the cloning and characterization of *vip3A(a)*, a vegetatively expressed gene from *B. thuringiensis* strain AB88 and its homologue, *vip3A(b)*, from *B. thuringiensis* strain AB424, whose encoded proteins exhibit potent insecticidal activity against BCW and other lepidopteran insects. The Vip3A-insecticidal proteins do not belong to the δ-endotoxin family and represent a novel insecticidal agent.

MATERIAL AND METHODS

Strains. *B. thuringiensis* strain AB88 was isolated from sour milk. *B. thuringiensis* strain 424 was isolated from mossy pine cones. Both AB88 and AB424 strains have been deposited in Agricultural Research Service Patent Culture Collection under the numbers B-21422 and B-21423, respectively. *Escherichia coli* strain DH5α was used as the host organism for recombinant DNA cloning, and plasmid pBluescript (Stratagene) was used as the cloning vector.

Protein Purification. Insecticidal protein was purified from supernatants of *B. thuringiensis* strain AB88. AB88 culture was grown for 12 h at 30°C in Terrific broth (12% tryptone/2.4% yeast extract/0.04% glycerol/0.17 m KH_2PO_4/0.72 M K_2HPO_4) and centrifuged at 5000 × *g* for 20 min. Proteins present in the supernatant were precipitated with ammonium sulfate (70% saturation) and collected by centrifugation at 5000 × *g* for 15 min. The pellet was then resuspended in the original volume of 20 mM Tris·HCl (pH 7.5) and dialyzed overnight at 4°C. The dialysate was titrated to pH 4.5 using 20 mM sodium citrate (pH 2.5). After 30 min incubation at room temperature, the sample was centrifuged at 3000 × *g* for 10 min. The resulting protein pellet was redissolved in 20 mM Bis·Tris·Propane (pH 9.0) and fractionated on a Poros HQ/N anion exchange column (PerSeptive Biosystems, Framingham, MA) using a linear gradient from 0 to 500 mM NaCl in 20 mM Bis·Tris·Propane (pH 9.0) at a flow rate of 4 ml/min. The insecticidal protein eluted at 250 mM NaCl.

Peptide Analysis and Oligonucleotide Synthesis. Fractions with insecticidal activity were fractionated in SDS/8–16% polyacrylamide gradient gels and transferred to poly(vinylidene difluoride) membranes (8). The most abundant protein band (molecular mass of ≈80 kDa) was subjected to N-terminal amino acid sequencing by repetitive Edman cycles on an Applied Biosystems model 476 pulsed-liquid sequencer. A 33-base oligonucleotide ACAATGATTAAAGATATTATGAAYATGATTTTT (Y being either C or T), corresponding

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Abbreviation: BCW, black cutworm.

Data depostion: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L48811 and L48812).

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to the sequence of N-terminal amino acids from position 28 to 38 (TGIKDIMNMIF), was synthesized. A preferred codon usage of *B. thuringiensis* was used in generating a consensus codon bias for the synthesis of the oligonucleotide.

Southern Blot Hybridization. Total DNA of strain AB88 was digested with different restriction enzymes, separated in a 0.7% agarose gel, and transferred to a nitrocellulose membrane (9). Filters were probed with the ^{32}P-labeled 33-base oligonucleotide described above. Hybridization was carried out at 42°C for 18 h using 10^6 cpm/ml. Filters were washed twice at 42°C in 2× standard saline citrate (SSC)/0.1% SDS (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.4) for 5 min, and twice at 50°C in 1× SSC/0.1% SDS for 10 min. The Southern blot analysis allowed us to do a size-selection library to clone the *vip3A* genes.

Molecular Cloning of the *vip3A(a)* and *vip3A(b)* Genes. Total DNA of *B. thuringiensis* strains AB88 and AB424 was isolated as described (9). For the *vip3A(a)* gene, a library of 4.0- to 5.0-kb size-fractionated *Xba*I fragments of total AB88 DNA was made in *E. coli* DH5α using pBluescript as cloning vector. For the *vip3A(b)* gene, a library of 4.5- to 6.0-kb size-fractionated *Eco*RI fragments of total AB424 DNA was transformed into *E. coli* DH5α. Recombinant clones were blotted to nitrocellulose filters and probed with the ^{32}P-labeled oligonucleotide according to Maniatis *et al.* (10). Hybridization and washing conditions were performed as described previously.

Bioassays. Cell-free supernatant of *B. thuringiensis* AB88 and AB424 cultures grown for 12 h at 30°C in TB medium were mixed with molten artificial diet (Bio-Serve, Frenchtown, NJ) and allowed to solidify. Neonate larvae of lepidopteran insects were placed on the solidified diet and kept at 30°C. Mortality was recorded after 6 days. Recombinant *E. coli* clones identified by hybridization were grown at 37°C overnight in Luria–Bertani medium supplemented with 100 μg of ampicillin per ml. Cultures (1 ml) were sonicated with three pulses of medium intensity for 20 s each. Sonicated cultures (500 μl) were added to molten diet and the combination was used to feed neonate larvae of lepidopteran insects. The amount of protein used in bioassays was determined according to Bradford (11).

Antiserum Production and Immunoblotting Methods. Antiserum against purified Vip3A(a) insecticidal protein was produced in rabbits. Nitrocellulose-bound protein (50 μg) was dissolved in dimethyl sulfoxide and emulsified with Freund's complete adjuvant (Difco). Two rabbits were given subcutaneous injections each month for 3 months. They were bled 10 days after the second and third injection, and the serum was recovered from the blood sample (12). Insecticidal crystal proteins (ICP) were isolated from *B. thuringiensis* strain GC91 according to Lecadet *et al.* (13). Antiserum against ICP was produced in rabbits and affinity purified according to Harlow and Lane (12). For Western blot analysis, proteins present in AB88 cultures were separated by SDS/PAGE and transferred to nitrocellulose (10). Nitrocellulose blots were treated with Vip3A(a) insecticidal protein antiserum (diluted 1/500) or ICP affinity-purified IgG (diluted 1/1000) and then with alkaline phosphatase-conjugated goat antirabbit IgG antiserum. Bromochloroindolyl-phosphate and nitroblue tetrazolium were used as substrate for the alkaline phosphatase reaction.

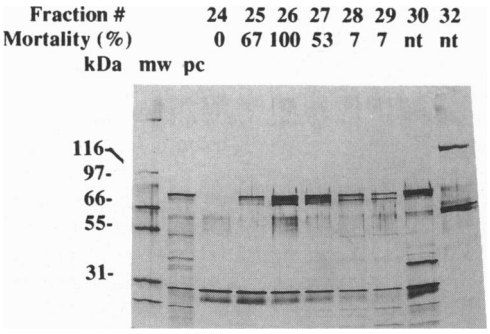

FIG. 1. SDS/PAGE of BCW active chromatographic fractions. Aliquots of the insecticidally active and adjoining chromatographic fractions were electrophoresed on an 8–16% polyacrylamide gel. The presence of an estimated 80-kDa protein in fractions 25–27 correlates well with the BCW mortality found in these fractions. mw, Molecular weight markers; pc, the sample after pH cut.

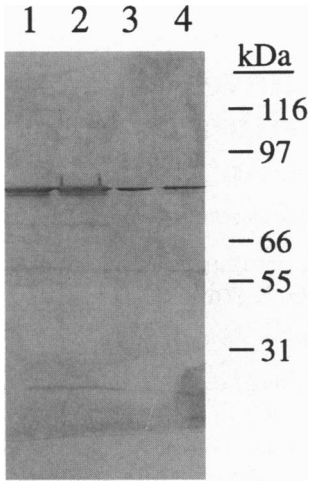

FIG. 2. Immunoblot analysis for the presence of the Vip3A(a) BCW insecticidal protein in supernatant (lanes 1 and 2) or cell pellet (lanes 3 and 4) of AB88 cultures. Cells were grown at 30°C in TB for 18 h (lanes 1 and 3) and 21 h (lanes 2 and 4). Fifty microliters of cell culture was centrifuged, and the resulting supernatant and cell pellet mixed with loading buffer and electrophoresed.

Cell Lysis Determination. Cell lysis occurring in the *Bacillus* cultures was estimated by assaying the release of the intracellular enzyme isocitrate dehydrogenase (14). Isocitrate dehydrogenase activity was determined as described (15). Thus, cell lysis was determined by measuring the percentage of the isocitrate dehydrogenase activity present in the supernatant in relation to the total isocitrate dehydrogenase activity of a culture (i.e., supernatant plus cell pellet).

RESULTS

BCW Insecticidal Activity. A number of *Bacillus thuringiensis* culture supernatants were assayed for insecticidal activity

Table 1. Effect of Vip3A insecticidal proteins on *Agrotis ipsilon* larvae

Treatment	Mortality, %
TB medium	5
AB88 supernatant	100
AB88 supernatant heated	5
AB424 supernatant	100
AB424 supernatant heated	4
AB424	100
LBAmp^{100}	7
E. coli pKS	10
E. coli AB88 (pCIB7104)	100
E. coli AB88 (pCIB7105)	100
E. coli AB424 (pCIB7106)	100
E. coli AB424 (pCIB7107)	100

Diet incorporation assays contained either supernatants of overnight cultures of AB88 or AB424 (1 mg of total protein per ml) or *E. coli* sonicated extracts (3 mg of total protein per ml). Heat inactivation involved treatment of supernatants at 95°C for 20 min. Mortality was scored 6 days after applying insect larvae. The results are the average of at least three trials with a minimum of 20 larvae per trial. LBAmp^{100}, Luria broth agar containing 100 μg of ampicillin per ml.

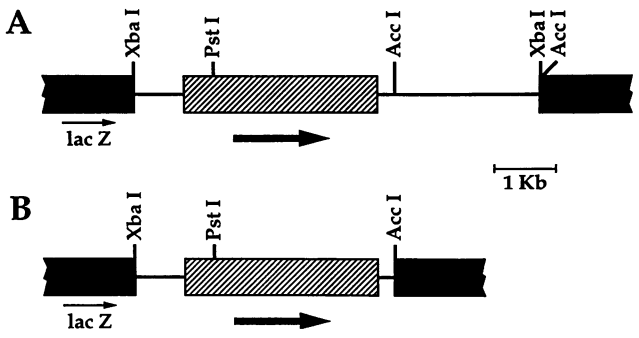

FIG. 3. Partial restriction maps of pCIB7104 (*A*) and pCIB7105 (*B*). Bold arrows show the direction of the Vip3A(a) insecticidal protein transcription. Hatched bars indicate insecticidal protein coding sequence. Solid bars indicate vector.

against BCW. Several supernatants from strains AB6, AB88, and AB424 were active against BCW, and the active component was characterized as a high molecular mass (>10 kDa) and heat-labile component, thus excluding the possibility of being a β-exotoxin. Subsequent purification from isolate AB88 identified a major protein of an estimated molecular mass of 80 kDa in chromatographic fractions, which correlated with BCW mortality (Fig. 1). The same protein was present in AB6 and AB424 supernatants as indicated by N-terminal amino acid analysis (data not shown).

The BCW insecticidal protein was detected mainly in the supernatant of an AB88 culture and was considerably less abundant in the cell pellet (Fig. 2). Based on isocitrate dehydrogenase activity, the extent of cell lysis in the sample was estimated to be 10–15%. Therefore the BCW insecticidal protein appears to be a secreted protein. Analysis of the N-terminal amino acid sequence of the cell pellet and supernatant forms of the BCW insecticidal protein indicated no sign of processing (data not shown). This is also evidenced by their identical sizes when run on a SDS/polyacrylamide gel (Fig. 2).

Cloning of the *vip3A* Insecticidal Protein Genes. Size-selected *Xba*I fragments of AB88 total DNA or *Eco*RI fragments of AB424 were ligated into pBluescript and transformed into *E. coli* DH5α. Four recombinants (two for each library) out of 400 colonies probed with a ^{32}P-labeled oligonucleotide specific to the BCW insecticidal gene of AB88 were positive. Insect bioassays on extracts of the positive recombinants exhibited toxicity to BCW larvae comparable to that of the AB88 and AB424 supernatants (Table 1). The plasmid pCIB7104 contained a 4.5-kb *Xba*I fragment of AB88 DNA (Fig. 3*A*). *E. coli* (pCIB7105), shown in Fig. 3*B*, was constructed by cloning the 3.5-kb *Xba*I–*Acc*I fragment of pCIB7104 into pBluescript. Plasmid pCIB7106 contained a 5.0-kb *Eco*RI fragment of AB424 DNA (data not shown). This

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673  AAGAATTAAG AATACAAGTT TACAAGAAAT AAGTGTTACA AAAAATAGCT GAAAAGGAAG
                                                          SD
733  ATGAACATGA ACAAGAATAA TACTAAATTA AGCACAAGAG CCTTACCAAG TTTTATTGAT
          M  N  K  N  N  T  K  L  S  T  R  A  L  P  S  F  I  D

793  TATTTTAATG GCATTTATGG ATTTGCCACT GGTATCAAAG ACATTATGAA CATGATTTTT
       Y  F  N  G  I  Y  G  F  A  T  G  I  K  D  I  M  N  M  I  F

853  AAAACGGATA CAGGTGGTGA TCTAACCCTA GACGAAATTT TAAAGAATCA GCAGTTACTA
       K  T  D  T  G  G  D  L  T  L  D  E  I  L  K  N  Q  Q  L  L

913  AATGATATTT CTGGTAAATT GGATGGGGTG AATGGAAGCT TAAATGATCT TATCGCACAG
       N  D  I  S  G  K  L  D  G  V  N  G  S  L  N  D  L  I  A  Q

973  GGAAACTTAA ATACAGAATT ATCTAAGGAA ATATTAAAAA TTGCAAATGA ACAAAATCAA
       G  N  L  N  T  E  L  S  K  E  I  L  K  I  A  N  E  Q  N  Q

1033 GTTTTAAATG ATGTTAATAA CAAACTCGAT GCGATAAATA CGATGCTTCG GGTATATCTA
       V  L  N  D  V  N  N  K  L  D  A  I  N  T  M  L  R  V  Y  L

1093 CCTAAAATTA CCTCTATGTT GAGTGATGTA ATGAAACAAA ATTATGCGCT AAGTCTGCAA
       P  K  I  T  S  M  L  S  D  V  M  K  Q  N  Y  A  L  S  L  Q

1153 ATAGAATACT TAAGTAAACA ATTGCAAGAG ATTTCTGATA AGTTGGATAT TATTAATGTA
       I  E  Y  L  S  K  Q  L  Q  E  I  S  D  K  L  D  I  I  N  V

1213 AATGTACTTA TTAACTCTAC ACTTACTGAA ATTACACCTG CGTATCAAAG GATTAAATAT
       N  V  L  I  N  S  T  L  T  E  I  T  P  A  Y  Q  R  I  K  Y

1273 GTGAACGAAA AATTTGAGGA ATTAACTTTT GCTACAGAAA CTAGTTCAAA AGTAAAAAAG
       V  N  E  K  F  E  E  L  T  F  A  T  E  T  S  S  K  V  K  K

1333 GATGGCTCTC CTGCAGATAT TCTTGATGAG TTAACTGAGT TAACTGAACT AGCGAAAAGT
       D  G  S  P  A  D  I  L  D  E  L  T  E  L  T  E  L  A  K  S

1393 GTAACAAAAA ATGATGTGGA TGGTTTTGAA TTTTACCTTA ATACATTCCA CGATGTAATG
       V  T  K  N  D  V  D  G  F  E  F  Y  L  N  T  F  H  D  V  M

1453 GTAGGAAATA ATTTATTCGG GCGTTCAGCT TTAAAAACTG CATCGGAATT AATTACTAAA
       V  G  N  N  L  F  G  R  S  A  L  K  T  A  S  E  L  I  T  K

1513 GAAAATGTGA AAACAAGTGG CAGTGAGGTC GGAAATGTTT ATAACTTCTT AATTGTATTA
       E  N  V  K  T  S  G  S  E  V  G  N  V  Y  N  F  L  I  V  L

1573 ACAGCTCTGC AAGCCCAAGC TTTTCTTACT TTAACAACAT GCCGAAAATT ATTAGGCTTA
       T  A  L  Q  A  Q  A  F  L  T  L  T  T  C  R  K  L  L  G  L
                   K                    P
1633 GCAGATATTG ATTATACTTC TATTATGAAT GAACATTTAA ATAAGGAAAA AGAGGAATTT
       A  D  I  D  Y  T  S  I  M  N  E  H  L  N  K  E  K  E  E  F

1693 AGAGTAAACA TCCTCCCTAC ACTTTCTAAT ACTTTTTCTA ATCCTAATTA TGCAAAAGTT
       R  V  N  I  L  P  T  L  S  N  T  F  S  N  P  N  Y  A  K  V

1753 AAAGGAAGTG ATGAAGATGC AAAGATGATT GTGGAAGCTA AACCAGGACA TGCATTGATT
       K  G  S  D  E  D  A  K  M  I  V  E  A  K  P  G  H  A  L  I

1813 GGGTTTGAAA TTAGTAATGA TTCAATTACA GTATTAAAAG TATATGAGGC TAAGCTAAAA
       G  F  E  I  S  N  D  S  I  T  V  L  K  V  Y  E  A  K  L  K

1873 CAAAATTATC AAGTCGATAA GGATTCCTTA TCGGAAGTTA TTTATGGTGA TATGGATAAA
       Q  N  Y  Q  V  D  K  D  S  L  S  E  V  I  Y  G  D  M  D  K

1933 TTATTGTGCC CAGATCAATC TGAACAAATC TATTATACAA ATAACATAGT ATTTCCAAAT
       L  L  C  P  D  Q  S  E  Q  I  Y  Y  T  N  N  I  V  F  P  N
                                     G
1993 GAATATGTAA TTACTAAAAT TGATTTCACT AAAAAAATGA AAACTTTAAG ATATGAGGTA
       E  Y  V  I  T  K  I  D  F  T  K  K  M  K  T  L  R  Y  E  V

2053 ACAGCGAATT TTTATGATTC TTCTACAGGA GAAATTGACT TAAATAAGAA AAAAGTAGAA
       T  A  N  F  Y  D  S  S  T  G  E  I  D  L  N  K  K  K  V  E

2113 TCAAGTGAAG CGGAGTATAG AACGTTAAGT GCTAATGATG ATGGGGTGTA TATGCCGTTA
       S  S  E  A  E  Y  R  T  L  S  A  N  D  D  G  V  Y  M  P  L

2173 GGTGTCATCA GTGAAACATT TTTGACTCCG ATTAATGGGT TTGGCCTCCA AGCTGATGAA
       G  V  I  S  E  T  F  L  T  P  I  N  G  F  G  L  Q  A  D  E

2233 AATTCAAGAT TAATTACTTT AACATGTAAA TCATATTTAA GAGAACTACT GCTAGCAACA
       N  S  R  L  I  T  L  T  C  K  S  Y  L  R  E  L  L  A  T

2293 GACTTAAGCA ATAAAGAAAC TAAATTGATC GTCCCGCCAA GTGGTTTTAT TAGCAATATT
       D  L  S  N  K  E  T  K  L  I  V  P  P  S  G  F  I  S  N  I

2353 GTAGAGAACG GGTCCATAGA AGAGGACAAT TTAGAGCCGT GGAAAGCAAA TAATAAGAAT
       V  E  N  G  S  I  E  E  D  N  L  E  P  W  K  A  N  N  K  N

2413 GCGTATGTAG ATCATACAGG CGGAGTGAAT GGAACTAAAG CTTTATATGT TCATAAGGAC
       A  Y  V  D  H  T  G  G  V  N  G  T  K  A  L  Y  V  H  K  D

2473 GGAGGAATTT CACAATTTAT TGGAGATAAG TTAAAACCGA AAACTGAGTA TGTAATCCAA
       G  G  I  S  Q  F  I  G  D  K  L  K  P  K  T  E  Y  V  I  Q

2533 TATACTGTTA AAGGAAAACC TTCTATTCAT TTAAAAGATG AAAATACTGG ATATATTCAT
       Y  T  V  K  G  K  P  S  I  H  L  K  D  E  N  T  G  Y  I  H

2593 TATGAAGATA CAAATAATAA TTTAGAAGAT TATCAAACTA TTAATAAACG TTTTACTACA
       Y  E  D  T  N  N  N  L  E  D  Y  Q  T  I  N  K  R  F  T  T

2653 GGAACTGATT TAAAGGGAGT GTATTTAATT TTAAAAAGTC AAAATGGAGA TGAAGCTTGG
       G  T  D  L  K  G  V  Y  L  I  L  K  S  Q  N  G  D  E  A  W

2713 GGAGATAACT TTATTATTTT GGAAATTAGT CCTTCTGAAA AGTTATTAAG TCCAGAATTA
       G  D  N  F  I  I  L  E  I  S  P  S  E  K  L  L  S  P  E  L

2773 ATTAATACAA ATAATTGGAC GAGTACGGGA TCAACTAATA TTAGCGGGAA TACACTCACT
       I  N  T  N  N  W  T  S  T  G  S  T  N  I  S  G  N  T  L  T

2833 CTTTATCAGG GAGGACGAGG GATTCTAAAA CAAAACCTTC AATTAGATAG TTTTTCAACT
       L  Y  Q  G  G  R  G  I  L  K  Q  N  L  Q  L  D  S  F  S  T

2893 TATAGAGTGT ATTTTTCTGT GTCCGGAGAT GCTAATGTAA GGATTAGAAA TTCTAGGGAA
       Y  R  V  Y  F  S  V  S  G  D  A  N  V  R  I  R  N  S  R  E

2953 GTGTTATTTG AAAAAAGATA TATGAGCGGT GCTAAAGATG TTTCTGAAAT GTTCACTACA
       V  L  F  K  K  R  Y  M  S  G  A  K  D  V  S  E  M  F  T  T
                E
3013 AAATTTGAGA AAGATAACTT CTATATAGAG CTTCCTCAAG GGAATAATTT ATATGGTGGT
       K  F  E  K  D  N  F  Y  I  E  L  P  Q  G  N  N  L  Y  G  G
                                     S
3073 CCTATTGTAC ATTTTTACGA TGTCTCTATT AAGTAAGATC GGGATCTATC
       P  I  V  H  F  Y  D  V  S  I  K  -
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FIG. 4. Nucleotide sequence of 2.4-kb *B. thuringiensis* DNA fragment encoding the BCW insecticidal protein. The deduced amino acid sequence for the open reading frame of the Vip3A(a) protein is presented underneath the nucleotide sequence. The Vip3A(b) protein is 98% identical to Vip3A(a), and the five different amino acids are indicated in italic type. The N-terminal and internal sequences of the protein identified by protein analysis are indicated in boldface type. The putative Shine–Dalgarno sequence is underlined.

fragment was further digested with *Hin*cII to produce a 2.8-kb *Eco*RI–*Hin*cII insert (pCIB 7107) which still encoded a functional insecticidal protein.

Analysis of the *vip3A* Insecticidal Genes. Sequences of the 4.5-kb *Xba*I fragment from AB88 [*vip3A(a)*] and of the 2.8-kb *Eco*RI–*Hin*cII fragment from AB424 [*vip3A(b)*] were determined and have been deposited in the GenBank data base (accession numbers L48811 and L48812, respectively). An open reading frame of the *vip3A(a)* gene extends from nucleotides 732 to 3105 (Fig. 4). This open reading frame encodes a peptide of 791 aa, corresponding to a molecular mass of 88,500 Da. A Shine–Dalgarno sequence is located 6 bases before the first methionine, and its sequence matches that of a strong Shine–Dalgarno sequence for *Bacillus* (16). The Vip3A(b) protein is 98% identical to Vip3A(a); the amino acid changes are indicated in Fig. 4. When blots of total DNA isolated from AB88 *B. thuringiensis* cells were probed with a 33-base fragment that spans the N-terminal region of the Vip3A insecticidal protein, single bands could be observed in different restriction digests (Fig. 5). This result was confirmed by using larger probes spanning the coding region of the gene (data not shown). A search of the GenBank data base revealed no homology with known proteins.

Expression of the Vip3A Insecticidal Proteins. The time course for expression of the Vip3A(a) insecticidal protein was analyzed by Western blot (Fig. 6). Samples from AB88 cultures were taken throughout its growth curve and sporulation (Fig. 6*A*). The Vip3A(a) insecticidal protein can be detected in nonconcentrated supernatants of AB88 cultures during logarithmic phase as early as 15 h after initiating the culture (Fig. 6*B*). It reached its maximum level during early stages of stationary phase and remained at high levels during and after sporulation (Fig. 6*B*). Similar results were obtained when supernatants of AB424 cultures were analyzed (data not shown). The levels of Vip3A(a) insecticidal protein reflected

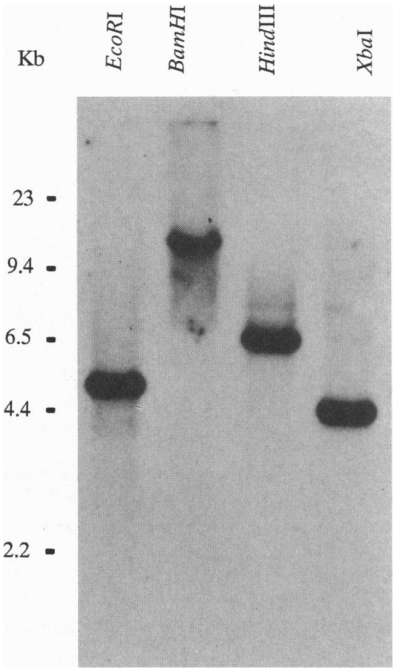

FIG. 5. Southern blot analysis. AB88 total DNA (1 μg) was digested with restriction enzymes, separated on a 0.7% agarose gel, and blotted onto a nitrocellulose filter. A filter containing the DNA samples was probed with a ^{32}P-labeled 33-base-long oligonucleotide spanning the N-terminal amino acids of the Vip3A(a) insecticidal protein. Hybridization and washes were performed as specified. Sizes of the λ DNA marker fragments are shown on the left.

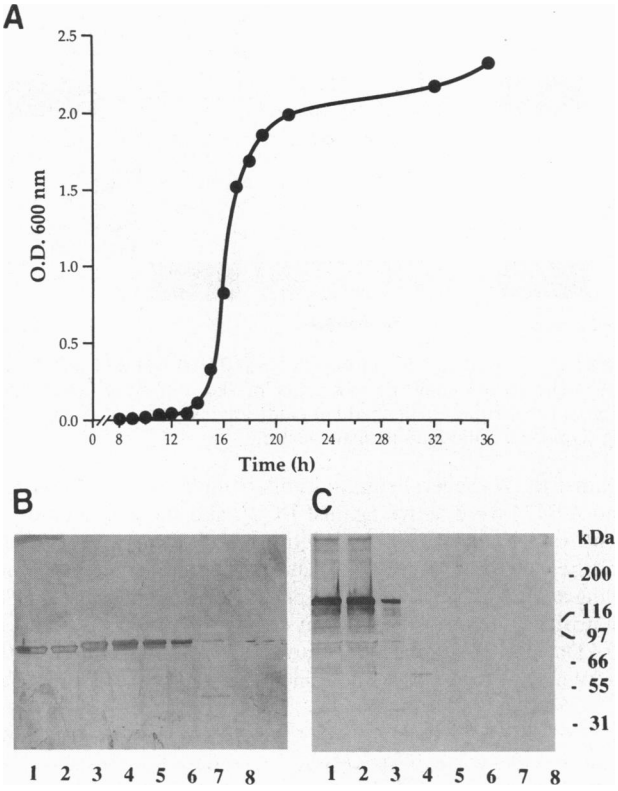

FIG. 6. Expression of the Vip3A(a) insecticidal protein in *B. thuringiensis* AB88. (*A*) Growth curve of shake flask culture. Time is indicated as hours after inoculation. (*B*) Immunoblot analysis for the presence of Vip3A(a) insecticidal protein in supernatant. (*C*) Immunoblot analysis for the presence of Cry I-type proteins in the cell pellet. These proteins are used as sporulation markers. For *B* and *C*, lanes 1–8 correspond to 72, 48, 21, 18, 17, 16, 15, and 13 h after inoculation, respectively. A total of 1.0 μg of protein was loaded, normalizing the protein content to the 13-h sample.

the expression of the *vip3A(a)* gene as determined by Northern blot analysis (data not shown). The initiation of sporulation was determined by direct microscopic observations and by analyzing cell pellets for the presence of δ-endotoxins. Cry-I-type proteins could be detected late in the stationary phase, during and after sporulation (Fig. 6*C*).

Identification of *vip3A*-Like Insecticidal Genes in Other *Bacilli*. To identify *Bacillus* containing genes related to the *vip3A(a)* gene of AB88, a collection of *Bacillus* isolates was screened by colony hybridization using a 1.2-kb internal *Hin*dIII fragment of the *vip3A(a)* gene as probe. Of the 463 *Bacillus* isolates screened, 60 contain *vip3A*-like genes. Further characterization of some of them (AB6 and AB426) showed that their supernatants contain an insecticidal protein similar to the Vip3A protein that are active against BCW.

Insecticidal Activity Spectrum of Vip3A Proteins. The activity spectrum of Vip3A insecticidal proteins was determined in insect bioassays in which recombinant *E. coli* carrying the *vip3A* genes were fed to larvae. In these assays, cells carrying the *vip3A(a)* or *vip3A(b)* gene showed different degrees of larvicidal activity against *A. ipsilon, Spodoptera frugiperda, Spodoptera exigua, Heliothis virescens*, and *Helicoverpa zea* (Table 2). Under the same experimental conditions, bacterial extracts containing Vip3A proteins did not show any activity against *Ostrinia nubilalis* larvae.

DISCUSSION

Novel BCW insecticidal activities in *Bacilli*. Novel insecticidal genes, *vip3A(a)* from *B. thuringiensis* strain AB88 and

Table 2. Effect of the Vip3A insecticidal protein on several lepidopteran insect larvae

Treatment	Dosage		Insect mortality, %					
	μg protein per cm²	ng Vip3A per cm²	BCW	FAW	BAW	TBW	CEW	ECB
LBAmp^{100} *E. coli* extracts	—	—	6	8	18	12	15	7
pSK	22.5	—	7	5	10	14	12	10
pCIB 7105	1.5	28	82	44	65	19	20	12
	3.8	70	96	78	95	25	22	10
	7.5	140	100	100	98	35	25	8
	15.0	280	100	100	100	45	35	10
	22.5	420	100	100	100	75	50	7

Diet incorporation assays contained *E. coli* extracts (3 mg of total protein per ml). The amount of Vip3A insecticidal protein was estimated by ELISA. Mortality was scored 6 days after applying insect larvae. The results are the average of at least three trials with a minimum of 20 larvae per trial. Some reported LC_{50} values of δ-endotoxins against these insects (5, 17) are the following: BCW, >6000 ng/cm² Cry-IA(c); beet armyworm (BAW, *S. exigua*), 50 ng/cm² Cry-IC; tobacco budworm (TBW, *H. virescens*), 4 ng/cm² Cry-IA(c); corn earworm (CEW, *H. zea*), 17 ng/cm² Cry-IA(b). FAW, fall armyworm (*S. frugiperda*); ECB, European corn borer (*O. nubilalis*), 27 ng/cm² CryIA(b).

vip3A(b) from *B. thuringiensis* strain AB424, have been cloned and characterized. They encode 791-aa polypeptides that exhibit insecticidal activity against BCW. The expression of the *vip3A(a)* and *vip3A(b)* genes in *E. coli* confirmed that they are sufficient to confer on *E. coli* the capacity to kill BCW. The spectrum of insecticidal activity of the Vip3A proteins includes four additional lepidopterans, fall armyworm, beet armyworm, tobacco budworm, and corn earworm. Thus, the Vip3A spectrum of insecticidal activity seems to be unique when compared to other reported insecticidal proteins (17).

BCW is an agronomically important insect quite resistant to δ-endotoxins. MacIntosh *et al.* (5) have reported that the δ-endotoxins CryIA(b) and CryIA(c) possess insecticidal properties against BCW with LC_{50}s of >80 μg and 18 μg of diet per ml, respectively. The Vip3A insecticidal proteins reported here provide 100% mortality when added at 62 ng of diet per ml. This amount of protein is at least 260-fold lower than the amounts of CryIA proteins needed to achieve just 50% mortality, and it is similar to the levels of δ-endotoxins used for insects susceptible to δ-endotoxins (17). δ-Endotoxins are believed to form lytic pores in the insect gut membranes (18). Based on the structure of the CryIIIA protein (19), they display long hydrophobic and amphipathic helices in the N-terminal region of the protein (domain I) which could penetrate the cell membrane. Vip3A insecticidal proteins have no structural homology with δ-endotoxins that would suggest a similar mechanism of action.

Vip3A Insecticidal Proteins Are Secreted Proteins. The Vip3A(a) insecticidal protein from AB88 is present mostly (at least 75% of the total) in supernatants of AB88 cultures. Protein secretion across the *Bacillus* cell wall usually involves an insertion of the precursor protein into the membrane followed by a translocation process (20). Secreted proteins typically contain a signal peptide that is cleaved before or after the translocation process has been completed. The N-terminal sequence of the Vip3A(a) insecticidal protein possesses a number of positively charged residues (from Asn-2 to Asn-7) followed by a hydrophobic core region (from Thr-8 to Ile-34), which is similar to other signal peptides described for *Bacillus* (21). However, unlike most of the known secreted proteins, the Vip3A(a) insecticidal protein is not N-terminally processed during export. Several reports (22, 23) have shown that some proteins are secreted or targeted to the periplasm of gram-negative bacteria with no processing. Taking together, these results suggest that some proteins might be secreted via a hitherto unknown mechanism.

Vip3A Insecticidal Proteins Are Expressed Vegetatively. The Vip3A insecticidal proteins can be detected in growth stages before sporulation, establishing a clear distinction from other described insecticidal proteins that belong to the δ-endotoxin family (3). Under our experimental conditions, the Vip3A(a) insecticidal protein was detectable in the supernatant of AB88 cultures as early as 15 h, whereas Cry-type proteins could not be detected until 36 h after culture initiation. When other strains (AB6 and AB424) were analyzed for the expression pattern of Vip3A insecticidal proteins, they were also present during vegetative stages. Although the expression of the *vip3A(a)* insecticidal gene starts during mid-logarithmic phase, the gene is actively expressed in sporulating cultures (data not shown). High level of expression in combination with high stability of the protein produces large amounts of the protein in supernatants of sporulating cultures.

The molecular and biological properties of the Vip3A insecticidal proteins have proven these proteins to be novel insecticidal agents that may complement and extend the use of known insecticidal proteins derived from *Bacillus*.

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