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(21) International Application Number: PCT/US98/00357 (22) International Filing Date: 9 January 1998 (09.01.98) (30) Priority Data: 60/035,361 10 January 1997 (10.01.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/035,361 (CON) Filed on 10 January 1997 (10.01.97) (71)(72) Applicants and Inventors: OSMAN, Yehia, A. [EG/EG]; Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center, 9 Gamaa Street, Giza-12619 (EG). MADKOUR, Magdy, A. [EG/EG]; Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center, 9 Gamaa Street, Giza-12619 (EG). BULLA, Lee, A., Jr. [US/US]; University of Wyoming, Laramie, WY 82071-3944 (US). (74) Agents: SPRUILL, W., Murray et al.; Bell Seltzer Intellectual Property Law Group Alston & Bird LLP, P.O. Drawer 34009, Charlotte, NC 28234 (US).		(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, GH, GM, GW, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: BACILLUS THURINGIENSIS ISOLATES WITH BROAD SPECTRUM ACTIVITY (57) Abstract A broad spectrum <i>Bacillus</i> strain is provided which displays activity against at least two orders of insects. Novel proteins active against corn rootworm and nematodes and methods of isolation are additionally provided. Also provided is a purified crystal protein complex isolated from a <i>Bacillus</i> strain that demonstrates insecticidal properties. In addition, the nucleotide and amino acid sequence of the CryII gene isolated from this strain is provided.		

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BACILLUS THURINGIENSIS ISOLATES
WITH BROAD SPECTRUM ACTIVITYBACKGROUND OF THE INVENTION

Bacillus thuringiensis is a gram-positive soil bacterium characterized by its ability to produce crystalline inclusions during sporulation. The crystalline inclusions can, in some subspecies, account
5 for 20 to 30 percent of the dry weight of the sporulated cell and may be composed of more than one protein. Crystals are composed primarily of a single polypeptide, a protoxin, which may also be a component of the spore coat. The protoxin genes are located
10 mainly on large plasmids, although chromosomally encoded endotoxins have been reported.

The crystal proteins exhibit a highly specific insecticidal activity. Many *B. thuringiensis* strains with different insect host spectra have been
15 identified. They are classified into different serotypes or subspecies based on their flagellar antigens.

The protoxin does not exhibit its insecticidal activity until after oral intake of the crystalline body. The crystal is dissolved in the
20 intestinal juice of the target insects. In most cases, the actual target component (toxin) is released from the protoxin as a result of proteolytic cleavage caused by the action of proteases from the digestive tract of
25 the insects. The activated toxin interacts with the midgut epithelium cells of susceptible insects.

Electrophysiological and biochemical evidence suggests that the toxins generate pores in the cell membrane, thus disrupting the osmotic balance.
30 Consequently, the cells swell and lyse. For several *B. thuringiensis* toxins, specific high-affinity binding sites have been demonstrated to exist on the midgut epithelium of susceptible insects. Nucleotide

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sequences have been recorded for a large number of *B. thuringiensis* (*Bt*) crystal protein genes. Several sequences are nearly identical, and have been designated as variations of the same gene. The crystal
5 protein (Cry) genes specify a family of related insecticidal proteins. The genes are divided into major classes and subclasses characterized by both the structural similarities and the insecticidal spectra of the encoded proteins. The classification, explained by
10 Höfte and Whiteley (1989) Microbiol. Rev., 53:242-255, placed the known insecticidal crystal proteins into four major classes. The four major classes were Lepidoptera-specific (I), Lepidoptera- and Diptera-specific (II), Coleoptera-specific (III), and Diptera-
15 specific (IV) genes. Additional classes have since been added.

The Cry1 genes are undoubtedly the best-studied crystal proteins. The Cry1 proteins are typically produced as 130 to 140 kDa protoxin proteins
20 which are proteolitically cleaved to produce active toxin proteins about 60 to 70 Kda. The active portion or toxic domain is localized in the N-terminal half of the protoxin. Six groups of Cry1 proteins were known in 1989 when the Höfte and Whiteley article was
25 published. These groups were designated IA(a), IA(b), IA(c), IB, IC, and ID. Since 1989, additional proteins have been discovered and classified as Cry1E, Cry1F, Cry1G, Cry1H, and Cry1X.

The spectrum of insecticidal activity of an
30 individual protoxin from *Bt* tends to be quite narrow. That is, a given crystal protein is active against only a few insects. None of the crystal proteins active against Coleopteran larvae such as colorado potato beetle (*Leptinotarsa decemlineata*) or yellow mealworm
35 (*Tenebrio molitor*) have demonstrated significant effects on members of the genus *Diabrotica* particularly *D. virgifera virgifera*, the western corn

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rootworm (WCRW) or *D. longicornis barberi*, the northern corn rootworm.

Insect pests are a major factor in the loss of the world's commercially important agricultural crops. Broad spectrum chemical pesticides have been used extensively to control or eradicate pests of agricultural importance. However, there is substantial interest in developing effective alternative pesticides.

Microbial pesticides have played an important role as alternatives to chemical pest control. The most extensively used microbial product is based on the bacterium *Bacillus thuringiensis*. However, as noted above, the majority of Bt strains have a narrow range of activity. There is therefore needed microbial strains with a broad range of insecticidal activity for use as broad spectrum insecticides and as a source for additional toxin genes and proteins.

SUMMARY OF THE INVENTION

A broad spectrum *Bacillus* strain is provided. The strain is active against insects from at least the orders Lepidoptera, Diptera, and Coleoptera. Additionally, the strain is active against nematodes, and rootworms. Disclosed in this invention is the isolation and partial purification of the crystal protein complex from this strain. The crystal protein complex is demonstrated to be active against rootworms and other pests. Genes, proteins, and their use, as well as the use of the strain are provided. The complete nucleotide sequence of a Cry5-like gene herein designated as Cry1I which was isolated from this strain is also provided.

The methods and compositions of the invention may be used in a variety of systems for controlling pests, particularly plant pests.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Fig. 1A shows an electron micrograph with a light micrograph insert; Fig. 1B shows an electron micrograph with the different kinds of crystal shapes as indicated by the arrows or arrow heads. The three types of crystal shapes may correlate with the three kinds of insecticidal activity toward at least three insect orders, namely: Lepidoptera, Diptera and Coleoptera.

Figure 2: Plasmid profile of BtC-18. Lane a: DNA molecular weight standards; Lane b: *B. thuringiensis* subsp. *kurstaki*; Lane c: *Bacillus thuringiensis* subsp. *aizawia*; and, Lane d: strain C-18. BtC-18 also has different plasmid DNA profiles from the other known *B. thuringiensis* subspecies, such as subspecies *israelensis* and *tenebrionis*, (data not shown).

Figure 3: Protective effect of BtC-18 against the root nematode *Meloidogyne incognata*. In panel A: the roots of infected tomato plant with the nematode (positive control). panel B: the roots of tomato plants infected with nematode eggs before BtC-18 was applied, and panel C: roots of plant treated first with BtC-18 and then infected with nematode eggs.

Figure 4: PCR product profile of BtC-18. In Fig 4A: Lane a contains DNA molecular weight standards; Lane b, *B. thuringiensis* subsp. *kurstaki*; Lane c, BtC-18; Lane d: *B. thuringiensis* subsp. *israelensis*; Lane e, BtC-18. In Fig 4B: Lane a contains DNA standards; Lane f, *Bacillus thuringiensis* subsp. *tenebrionis*; Lane g, BtC-18. The results show that BtC-18 produced the same PCR product profile as *B. thuringiensis* subsp. *kurstaki*, but different PCR products with Dipteran and Coleopteran DNA primers.

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Figure 5. Nucleotide and amino acid sequence of the CryII gene isolated from BtC-18.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Compositions and methods for controlling
5 plant pests are provided. In particular, novel broad
spectrum *Bacillus* strains having a wide range of
insecticidal activity are provided. The strains are
useful as insecticidal agents. In addition, the
crystal protein complex from one of these strains has
10 been purified and is shown to have insecticidal
properties. Methods of its purification are discussed
and evidence of its activity against rootworm is
disclosed. Also disclosed is the entire nucleotide
sequence of a Cry5-like gene herein designated as CryII
15 which was isolated from this strain.

The *Bacillus* strain of the invention has a
broad spectrum of activity. By broad spectrum it is
intended that the strains are active against insects
from more than one order, preferably against insects
20 from several orders, more preferably active against
insects from at least three orders. Additionally, the
strains are active against noninsect pests. For
purposes of the present invention, pests include but
are not limited to insects, fungi, bacteria, nematodes,
25 mites, ticks, and the like. Insect pests include
insects selected from the orders Coleoptera, Diptera,
Hymenoptera, Lepidoptera, Mallophaga, Homoptera,
Hemiptera, Orthoptera, Thysanoptera, Dermaptera,
Isoptera, Anoplura, Siphonaptera, Trichoptera, etc.,
30 particularly Coleoptera, Lepidoptera, and Diptera.

In one embodiment, the invention encompasses
the Bt isolate known as BtC-18, deposited December 31,
1996 as ATCC Accession No. 55922. Protein toxins, and
DNA which encodes the protein toxins are additionally
35 encompassed. The subject invention also includes
variants of the Bt isolate which have substantially the

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same pesticidal properties as the exemplified isolate. These variants include mutants and recombinant isolates. Procedures for making mutants are well known in the art and include ultraviolet light and

5 nitrosoguanidine.

The *Bacillus thuringiensis* isolate C-18 produces more than one kind of crystal protein during sporulation. Microscopic examination of the sporulated cells revealed at least three different shapes of
10 crystals: bipyramidal similar to the lepidopteran-specific *B. thuringiensis* subsp. *kurstaki*; circular or irregular as that produced by dipteran-specific *B. thuringiensis* subsp. *israelensis*; and rhomboid-shaped similar to the coleopteran-specific *B. thuringiensis*
15 subsp. *tenebrionis*.

The presence of the different types of crystals indicated that the bacterium can kill insects belonging to more than one order of insects. Bioassays confirmed that conclusion. The spore-crystal complex
20 of C-18 killed insects from at least three orders, Lepidoptera, Diptera, and Coleoptera. Generally, Bt's produce only a single crystal and therefore have limited insect activity. Of the Bt's which have been reported to kill insects from two orders, the activity
25 is not stable. In contrast, the present Bt has been shown to be highly stable.

Of particular interest is the corn rootworm activity exhibited by the *Bacillus* strains of the invention. Generally, Bt's have little or no rootworm
30 activity. In contrast, the present strain exhibits substantial rootworm activity. By substantial activity is intended that the protein is capable of killing the target insect when present in at least microgram (μg) quantities.

35 Methods are available in the art for the identification and isolation of the protein or proteins associated with insecticidal activity, i.e., rootworm

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activity. Generally, proteins can be purified by conventional chromatography, including gel-filtration, ion-exchange, and immunoaffinity chromatography, by high-performance liquid chromatography, such as
5 reversed-phase high-performance liquid chromatography, ion-exchange high-performance liquid chromatography, size-exclusion high-performance liquid chromatography, high-performance chromatofocusing and hydrophobic interaction chromatography, etc., by electrophoretic
10 separation, such as one-dimensional gel electrophoresis, two-dimensional gel electrophoresis, etc. Such methods are known in the art. See for example, Ausubel et al. (1988) *Current Protocols in Molecular Biology*, Vols. 1 & 2, (eds.) John Wiley &
15 Sons, NY.

Additionally, antibodies can be prepared against substantially pure preparations of the protein. See, for example, Radka et al. (1983) *J. Immunol.*, 128:2804; and Radka et al. (1984) *Immunogenetics*,
20 19:63. Any combination of methods may be utilized to purify protein having pesticidal properties, particularly rootworm activity. As the protocol is being formulated, insecticidal activity is determined after each purification step to assure the presence of
25 the toxin of interest.

Methods are available in the art to assay for insect activity. Generally, the protein is mixed and used in feeding assays. See, for example Marrone et al. (1985) *J. of Economic Entomology*, 78:290-293.

30 Such purification steps will result in a substantially purified protein fraction. By "substantially purified" or "substantially pure" is intended protein which is substantially free of any compound normally associated with the protein in its
35 natural state. "Substantially pure" preparations of protein can be assessed by the absence of other detectable protein bands following SDS-PAGE as

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determined visually or by densitometry scanning. Alternatively, the absence of other amino-terminal sequences or N-terminal residues in a purified preparation can indicate the level of purity. Purity
5 can be verified by rechromatography of "pure" preparations showing the absence of other peaks by ion exchange, reverse phase or capillary electrophoresis. The terms "substantially pure" or "substantially
10 purified" are not meant to exclude artificial or synthetic mixtures of the proteins with other compounds. The terms are also not meant to exclude the presence of minor impurities which do not interfere with the biological activity of the protein, and which may be present, for example, due to incomplete
15 purification.

Once purified protein is isolated, the protein, or the polypeptides of which it is comprised, can be characterized and sequenced by standard methods known in the art. For example, the purified protein,
20 or the polypeptides of which it is comprised, may be fragmented as with cyanogen bromide, or with proteases such as papain, chymotrypsin, trypsin, lysyl-C endopeptidase, etc. (Oike et al. (1982) *J. Biol. Chem.*, 257:9751-9758; Liu et al. (1983) *Int. J. Pept. Protein
25 Res.*, 21:209-215). The resulting peptides are separated, preferably by HPLC, or by resolution of gels and electroblotting onto PVDF membranes, and subjected to amino acid sequencing. To accomplish this task, the peptides are preferably analyzed by automated
30 sequencers. It is recognized that N-terminal, C-terminal, or internal amino acid sequences can be determined. From the amino acid sequence of the purified protein, a nucleotide sequence can be synthesized which can be used as a probe to aid in the
35 isolation of the gene encoding the pesticidal protein.

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Similar protocols can be utilized to isolate nematode active proteins, rootworm active proteins, or proteins having activity to other pests.

It is recognized that the pesticidal proteins
5 may be oligomeric and will vary in molecular weight,
number of protomers, component peptides, activity
against particular pests, and in other characteristics.
However, by the methods set forth herein, proteins
active against a variety of insects or pests may be
10 isolated and characterized.

Once the purified protein has been isolated
and characterized it is recognized that it may be
altered in various ways including amino acid
substitutions, deletions, truncations, and insertions.
15 Methods for such manipulations are generally known in
the art. For example, amino acid sequence variants of
the pesticidal proteins can be prepared by mutations in
the DNA. Such variants will possess the desired
pesticidal activity. Obviously, the mutations that
20 will be made in the DNA encoding the variant must not
place the sequence out of reading frame and preferably
will not create complementary regions that could
produce secondary mRNA structure. See, EP Patent
Application Publication No. 75,444.

25 In this manner, the present invention
encompasses the active rootworm proteins, and other
insecticidal and pesticidal proteins, as well as
components and fragments thereof. That is, it is
recognized that component protomers, polypeptides or
30 fragments of the proteins may be produced which retain
pesticidal activity. These fragments include truncated
sequences, as well as N-terminal, C-terminal, internal
and internally deleted amino acid sequences of the
proteins.

35 Most deletions, insertions, and substitutions
of the protein sequence are not expected to produce
radical changes in the characteristics of the

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pesticidal protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays.

The proteins or other component polypeptides described herein may be used alone or in combination. That is, several proteins may be used to control different insect pests.

The pesticidal proteins of the invention can be used in combination with Bt toxins or other insecticidal proteins to increase insect target range. Other insecticidal principles include protease inhibitors (both serine and cysteine types), lectins, α -amylase and peroxidase. This co-expression of more than one insecticidal principle in the same transgenic plant can be achieved by genetically engineering a plant to contain and express all the genes necessary. Alternatively, separate plants can be transformed with different components. By crossing the plants, progeny are obtained which express all of the genes of interest.

It is recognized that there are alternative methods available to obtain the nucleotide and amino acid sequences of the present proteins. For example, to obtain the nucleotide sequence encoding the pesticidal protein, cosmid clones, which express the pesticidal protein, can be isolated from a genomic library. From larger active cosmid clones, smaller subclones can be made and tested for activity. In this manner, clones which express an active pesticidal protein can be sequenced to determine the nucleotide sequence of the gene. Then, an amino acid sequence can be deduced for the protein. For general molecular methods, see, for example, Molecular Cloning, A Laboratory Manual, Second Edition, Vols. 1-3, Sambrook et al. (eds.) Cold Spring Harbor Laboratory Press, Cold

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Spring Harbor, NY (1989), and the references cited therein.

The present invention also encompasses nucleotide sequences from other *Bacillus* strains and organisms other than *Bacillus*, where the nucleotide sequences are isolatable by hybridization with the *Bacillus* nucleotide sequences of the invention. Proteins encoded by such nucleotide sequences can be tested for pesticidal activity. The invention also encompasses the proteins encoded by the nucleotide sequences. Furthermore, the invention encompasses proteins obtained from organisms other than *Bacillus* wherein the protein cross-reacts with antibodies raised against the proteins of the invention. Again the isolated proteins can be assayed for pesticidal activity by the methods disclosed herein or others well-known in the art.

In this manner, the insecticidal genes of the present invention include those coding for proteins homologous to, and having essentially the same biological properties as, the insecticidal genes disclosed herein, and particularly the rootworm active gene. This definition is intended to encompass natural allelic variations in the genes. Cloned genes of the present invention can be of other species of origin. Thus, DNAs which hybridize to the present insecticidal genes are also an aspect of this invention. Conditions which will permit other DNAs to hybridize to the DNA disclosed herein can be determined in accordance with known techniques. For example, hybridization of such sequences may be carried out under conditions of reduced stringency, medium stringency or even stringent conditions (e.g., conditions represented by a wash stringency of 35-40% Formamide with 5x Denhardt's solution, 0.5% SDS and 1x SSPE at 37°C.; conditions represented by a wash stringency of 40-45% Formamide with 5x Denhardt's solution, 0.5% SDS, and 1x SSPE at

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42°C.; and conditions represented by a wash stringency of 50% Formamide with 5x Denhardt's solution, 0.5% SDS and 1x SSPE at 42°C., respectively, to DNA encoding the insecticidal genes disclosed herein in a standard hybridization assay. See J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989) (Cold Spring Harbor Laboratory)). In general, sequences which code for insecticidal protein and hybridize to the insecticidal gene disclosed herein will be at least 75% homologous, 85% homologous, and even 95% homologous or more with the sequences. Further, DNAs which code for insecticidal proteins, or sequences which code for an insecticidal protein coded for by a sequence which hybridizes to the DNAs which code for insecticidal genes disclosed herein, but which differ in codon sequence from these due to the degeneracy of the genetic code, are also an aspect of this invention. The degeneracy of the genetic code, which allows different nucleic acid sequences to code for the same protein or peptide, is well known in the literature. See, e.g., U.S. Pat. No. 4,757,006 to Toole et al. at Col. 2, Table 1.

The hybridization probes may be cDNA fragments or oligonucleotides, and may be labeled with a detectable group as discussed hereinbelow. Pairs of probes which will serve as PCR primers for the insecticidal gene or a protein thereof may be used in accordance with the process described in U.S. Pat. Nos. 4,683,202 and 4,683,195.

Once the nucleotide sequences encoding the pesticidal proteins of the invention have been isolated, they can be manipulated and used to express the protein in a variety of hosts including other organisms, including microorganisms and plants.

The pesticidal genes of the invention can be optimized for enhanced expression in plants. See, for example, EPA 0359472; EPA 0385962; WO 91/16432; Perlak

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et al. (1991) *Proc. Natl. Acad. Sci. USA*, 88:3324-3328; and Murray *et al.* (1989) *Nucleic Acids Research* 17:477-498. In this manner, the genes can be synthesized utilizing plant preferred codons. That is the preferred codon for a particular host is the single codon which most frequently encodes that amino acid in that host. The maize preferred codon, for example, for a particular amino acid may be derived from known gene sequences from maize. Maize codon usage for 28 genes from maize plants is found in Murray *et al.* (1989) *Nucleic Acids Research*, 17:477-498, the disclosure of which is incorporated herein by reference. Synthetic genes can also be made based on the distribution of codons a particular host uses for a particular amino acid.

In this manner, the nucleotide sequences can be optimized for expression in any plant. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used.

In like manner, the nucleotide sequences can be optimized for expression in any microorganism. For *Bacillus* preferred codon usage, see, for example US Patent No. 5,024,837 and Johansen *et al.* (1988) *Gene*, 65:293-304.

Methodologies for the construction of plant expression cassettes as well as the introduction of foreign DNA into plants are described in the art. Such expression cassettes may include promoters, terminators, enhancers, leader sequences, introns and other regulatory sequences operably linked to the pesticidal protein coding sequence.

Methodologies for the construction of plant expression cassettes are described in the art. The construct may include any necessary regulators such as terminators, (Guerineau *et al.* (1991) *Mol. Gen. Genet.*, 226:141-144; Proudfoot (1991) *Cell*, 64:671-674;

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Sanfacon et al. (1991) *Genes Dev.*, 5:141-149; Mogen et al. (1990) *Plant Cell*, 2:1261-1272; Munroe et al. (1990) *Gene*, 91:151-158; Ballas et al. (1989) *Nucleic Acids Res.*, 17:7891-7903; Joshi et al. (1987) *Nucleic Acid Res.*, 15:9627-9639); plant translational consensus sequences (Joshi, C.P. (1987) *Nucleic Acids Research*, 15:6643-6653), enhancers, introns (Luehrsen and Walbot (1991) *Mol. Gen. Genet.*, 225:81-93) and the like, operably linked to the nucleotide sequence. It may be beneficial to include 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. See, for example, (Elroy-Stein et al. (1989) *PNAS USA*, 86:6126-6130), Allison et al. (1986), Macejak and Sarnow (1991) *Nature*, 353:90-94, Jobling and Gehrke (1987) *Nature*, 325:622-625, Gallie et al. (1989) *Molecular Biology of RNA*, pp. 237-256, Lommel et al. (1991) *Virology*, 81:382-385, and Della-Cioppa et al. (1987) *Plant Physiology*, 84:965-968.

It is further recognized that the components of the expression cassette may be modified to increase expression. For example, truncated sequences, nucleotide substitutions or other modifications may be employed. See, for example Perlak et al. (1991) *Proc. Natl. Acad. Sci. USA*, 88:3324-3328; Murray et al. (1989) *Nucleic Acids Research*, 17:477-498; and WO 91/16432.

For tissue specific expression, the nucleotide sequences of the invention can be operably linked to tissue specific promoters.

Methods are available in the art for the introduction and stable incorporation of the expression cassettes into plants. Suitable methods of transforming plant cells include microinjection (Crossway et al. (1986) *Biotechniques*, 4:320-334), electroporation (Riggs et al. (1986) *Proc. Natl. Acad.*

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- Sci. USA*, 83:5602-5606, *Agrobacterium* mediated transformation (Hinchee et al. (1988) *Biotechnology*, 6:915-921), direct gene transfer (Paszkowski et al. (1984) *EMBO J.*, 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford et al., U.S. Patent 4,945,050; WO91/10725 and McCabe et al. (1988) *Biotechnology*, 6:923-926). Also see, Weissinger et al. (1988) *Annual Rev. Genet.*, 22:421-477; Sanford et al. (1987) *Particulate Science and Technology*, 5:27-37
- 10 (onion); Christou et al. (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe et al. (1988) *Bio/Technology*, 6:923-926 (soybean); Datta et al. (1990) *Biotechnology*, 8:736-740 (rice); Klein et al. (1988) *Proc. Natl. Acad. Sci. USA*, 85:4305-4309
- 15 (maize); Klein et al. (1988) *Biotechnology*, 6:559-563 (maize); WO91/10725 (maize); Klein et al. (1988) *Plant Physiol.*, 91:440-444 (maize); Fromm et al. (1990) *Biotechnology*, 8:833-839; and Gordon-Kamm et al. (1990) *Plant Cell*, 2:603-618 (maize); Hooydaas-Van Slogteren &
- 20 Hooykaas (1984) *Nature (London)*, 311:763-764; Bytebier et al. (1987) *Proc. Natl. Acad. Sci. USA*, 84:5345-5349 (Liliaceae); De Wet et al. (1985) In *The Experimental Manipulation of Ovule Tissues*, ed. G.P. Chapman et al. pp. 197-209. Longman, NY (pollen); Kaeppler et al.
- 25 (1990) *Plant Cell Reports*, 9:415-418; and Kaeppler et al. (1992) *Theor. Appl. Genet.*, 84:560-566 (whisker-mediated transformation); D'Halluin et al. (1992) *Plant Cell*, 4:1495-1505 (electroporation); Li et al. (1993) *Plant Cell Reports*, 12:250-255 and Christou and Ford
- 30 (1995) *Annals of Botany*, 75:407-413 (rice); Osjoda et al. (1996) *Nature Biotechnology*, 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

Alternatively, the plant plastid can be

35 transformed directly. Stable transformation of plastids have been reported in higher plants, see, for example, Svab et al. (1990) *Proc. Nat'l. Acad. Sci.*

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USA, 87:8526-8530; Svab & Maliga (1993) *Proc. Nat'l Acad. Sci. USA*, 90:913-917; Staub & Maliga (1993) *EMBO J.*, 12:601-606. The method relies on particular gun delivery of DNA containing a selectable marker and
5 targeting of the DNA to the plastid genome through homologous recombination. Additionally, plastid transformation can be accomplished by trans-activation of a silent plastid-borne transgene by tissue-specific expression of a nuclear-encoded and plastid-directed
10 RNA polymerase. Such a system has been reported in McBride et al. (1994) *Proc. Natl. Acad. Sci., USA*, 91:7301-7305.

The cells which have been transformed may be grown into plants in accordance with conventional ways.
15 See, for example, McCormick et al. (1986) *Plant Cell Reports*, 5:81-84 (1986). These plants may then be grown, and either pollinated with the same transformed strainer or different strains, and the resulting hybrid having the desired phenotypic characteristic
20 identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

25 The *Bacillus* strains of the invention may be used for protecting agricultural crops and products from pests. Alternatively, a gene encoding the pesticide may be introduced via a suitable vector into a microbial host, and said host applied to the
30 environment or plants or animals. Microorganism hosts may be selected which are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest. These microorganisms are selected so as to be capable
35 of successfully competing in the particular environment with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the

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polypeptide pesticide, and, desirably, provide for improved protection of the pesticide from environmental degradation and inactivation.

A number of methods are available for
5 introducing a gene expressing the pesticidal protein into the microorganism host under conditions which allow for stable maintenance and expression of the gene. For example, expression cassettes can be constructed which include the DNA constructs of
10 interest operably linked with the transcriptional and translational regulatory signals for expression of the DNA constructs, and a DNA sequence homologous with a sequence in the host organism, whereby integration will occur, and/or a replication system which is functional
15 in the host, whereby integration or stable maintenance will occur.

Transcriptional and translational regulatory signals include but are not limited to promoter, transcriptional initiation start site, operators,
20 activators, enhancers, other regulatory elements, ribosomal binding sites, an initiation codon, termination signals, and the like. See, for example, US Patent 5,039,523; US Patent No. 4,853,331; EPO 0480762A2; Sambrook et al. supra; Molecular Cloning, a
25 Laboratory Manual, Maniatis et al. (eds) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982); Advanced Bacterial Genetics, Davis et al. (eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1980); and the references cited therein.

30 General methods for employing the strains of the invention in pesticide control or in engineering other organisms as pesticidal agents are known in the art. See, for example US Patent No. 5,039,523 and EP 0480762A2.

35 The *Bacillus* strains of the invention or the microorganisms which have been genetically altered to contain the pesticidal gene and protein may be used for

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protecting agricultural crops and products from pests. In one aspect of the invention, whole, i.e., unlysed, cells of a toxin (pesticide)-producing organism are treated with reagents that prolong the activity of the toxin produced in the cell when the cell is applied to the environment of target pest(s).

Alternatively, the pesticides are produced by introducing a heterologous gene into a cellular host. Expression of the heterologous gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. These cells are then treated under conditions that prolong the activity of the toxin produced in the cell when the cell is applied to the environment of target pest(s). The resulting product retains the toxicity of the toxin. These naturally encapsulated pesticides may then be formulated in accordance with conventional techniques for application to the environment hosting a target pest, e.g., soil, water, and foliage of plants. See, for example EPA 0192319, and the references cited therein.

The active ingredients of the present invention are normally applied in the form of compositions and can be applied to the crop area or plant to be treated, simultaneously or in succession, with other compounds. These compounds can be both fertilizers or micronutrient donors or other preparations that influence plant growth. They can also be selective herbicides, insecticides, fungicides, bacteriocides, nematocides, molluscocides or mixtures of several of these preparations, if desired, together with further agriculturally acceptable carriers, surfactants or application-promoting adjuvants customarily employed in the art of formulation. Suitable carriers and adjuvants can be solid or liquid and correspond to the substances ordinarily employed in formulation technology, e.g. natural or regenerated

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mineral substances, solvents, dispersants, wetting agents, tackifiers, binders or fertilizers.

Preferred methods of applying an active ingredient of the present invention or an agrochemical composition of the present invention which contains at least one of the pesticidal proteins produced by the bacterial strains of the present invention are leaf application, seed coating and soil application. The number of applications and the rate of application depend on the intensity of infestation by the corresponding pest.

The following experiments are offered by way of illustration and not by way of limitation.

Experimental

15

Introduction

Bacillus thuringiensis (Bt) strain C-18 is a gram-positive sporeforming bacterium that produces parasporal crystals which have multiple toxicity against three orders of insects: Lepidoptera, Coleoptera and Diptera as well as to nematodes. C-18 is unique because of its capacity to kill such a wide range of agriculturally and biomedically important pests. No other Bt strain or isolate has been reported to have such a wide host range. The vast majority of Bt's can kill insects belonging to only one order of insects.

Isolation

C-18 was isolated in Egypt from dead pink bollworm larvae harvested from cotton bolls grown in cotton fields in Egypt. Standard bacteriological procedures were used to isolate gram-positive, spore forming bacteria. The larvae were washed twice with sterile deionized water, transferred to fresh sterile water (1ml), macerated with a sterile glass-rod before being subjected to a heat treatment (5 min. at 100°C).

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The heat treatment killed all the vegetative and non-sporulating microbes. 100ul samples were then streaked onto LB-agar plates and incubated at 30°C overnight. Individual colonies were then picked, streaked onto
5 fresh sporulating medium and incubated at 30°C for 48 hours.

The resulting colonies were stained with endospore stain and examined microscopically. All standard bacteriological techniques including
10 physiological and biochemical reactions were employed to determine the identity of the isolates and only those which matched *Bacillus thuringiensis* (Bt) were subjected to detailed analysis and evaluation. Among the isolates selected for determining insecticidal and
15 nematocidal activities was C-18.

A unique aspect of the isolate C-18 was its ability to produce more than one kind of protein crystal during sporulation. Microscopic examination of the stained sporulated cells revealed at least three
20 different shapes of crystals: bipyrimidial similar to the lepidopteran-specific *B. thuringiensis* subsp. *kurstaki*; circular or irregular as that produced by *B. thuringiensis* subsp. *israelensis*; and, rhomboid-shaped similar to the *B. thuringiensis* subsp. *tenebrionis*
25 (Fig. 1 A&B).

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Insect & Nematode Bioassays

The insecticidal activity of C-18 was measured using bioassays involving a variety of insects that represent the Lepidoptera, Coleoptera and Diptera orders of insects. Initial insect bioassays were performed with whole bacterial cells and included the raspberry silkworm *Philosamia ricini* (Lepidopteran), the mosquito *Culex pipiens* (Dipteran), and the flour beetle *Tribolium* spp. (Coleopteran). Nematode bioassays were done with the tomato nematode *Melodogyne incognata*. Positive results from these bioassays Table 1 prompted a more comprehensive analysis of the insecticidal and nematocidal activities of C-18 parasporal crystals. As can be seen in Table 1, C-18 is effective against a broad spectrum of insects. Also parasporal crystals purified from C-18 exhibited the same nematocidal activity as the whole organism (Fig. 1).

A more comprehensive bioassay system using larger numbers of insect representing the three orders mentioned above was then performed.

In Table 1, bioassays were divided into three groups. Group I contains the results of the lepidopteran insects (cotton leafworm *Spodoptera littoralis*, cotton bollworm *Pectinophora gossypiella*, tobacco hornworm *Manduca sexta*, raspberry silk worm *Philosamia ricini*, and a corn borer *Sesamia cratica*). Group II includes dipteran insects (mosquitoes *Culex pipiens*, *Aedes aegypti*, *Aedes albopictus*, *Aedes triseriatus*, and a blue tongue virus vector: *Culicoides variipennis*). Group III represents the coleopteran insects (flour beetle *Tribolium* spp., Colorado potato beetle *Leptinotarsa decemlineata*, western corn rootworm *Diabrotica virgifera* and southern corn rootworm *D. undecimpunctata howardi*).

The bacterium was also tested against the nematode *Melodogyne incognata* (Figure 3).

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Table 1.
Pesticidal Activities of an Egyptian Isolate
Bacillus thuringiensis (BtC-18)

5	INSECT	BtC-18	BTK	BTI	BTT1
<u>Group I: Lepidopteran-insects</u>					
	<i>Spodoptera littoralis</i> ¹	10.00	40.00	0.0	0.0
	<i>Pectinophera gossypiella</i> ¹	0.45	40.00	0.0	0.0
10	<i>Manduca sexta</i> ¹	0.45	7.00	0.0	0.0
	<i>Philosamia ricini</i> ¹	0.43	3.00	0.0	0.0
	<i>Sessemia cratia</i> ¹	20.00	30.00	0.0	0.0
<u>Group II: Dipteran-insects</u>					
15	<i>Culex pipiens</i> ²	7.00	0.0	5.50	0.0
	<i>Aedes aegypti</i> ²	200.00	0.0	40.00	0.0
	<i>Aedes albopictus</i> ²	36.00	0.0	nd	0.0
	<i>Aedes triseriatus</i> ²	360.00	0.0	nd	0.0
	<i>Culicoides variipennis</i> ²	18.00	0.0	NO	0.0
20	<u>Group III: Coleopteran-insects</u>				
	<i>Tribolium sp.</i> ³	35.00	0.0	0.0	30.00
	Colorado Potato Beetle ¹	50.00	0.0	0.0	25.00
	Western Corn Rootworm ¹	350.00	0.0	0.0	8,000.00
25	Southern Corn Rootworm ³	350.00	0.0	0.0	10,000.00
<u>Footnotes:</u>					
	¹ The LC50 is expressed in ng/cm. ²				
	² The LC50 is expressed in ng/ml				
	³ The LC50 is expressed in ng/g				
30	* BtC-18 also exhibited toxic activity against Nematodes				

Microscopic Examination of C-18

C-18 produces at least three morphological types of parasporal crystals (Fig. 1). The three types or shapes are: (i) bipyrimidal - characteristic of

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those crystals that are toxic to lepidopterans, (ii) rounded, amorphous clusters - characteristic of dipteran-specific crystals, and (iii) rhomboid - characteristic of coleopteran-specific crystals.

- 5 Presence of these three morphological crystal types correlate with the three kinds of insecticidal activities normally associated singularly with all other Bt's described in the scientific literature.

Profile of Proteins Extracted from Vegetative &
10 Sporulating Cells of C-18

The molecular characterization of BtC-18 was determined at the protein and the gene levels.

- Bacillus thuringiensis* has two phases of growth, the vegetative phase and the sporulation phase. The
15 bacterium produces a specific protein pool during each phase of growth. The proteins are the expression products of active genes at the specific stage of development. These proteins can be separated and visualized on a sodium dodecylsulphate-polyacrylamide
20 gel by electrophoresis (SDS-PAGE).

- Proteins of vegetative and sporulating cells of the various strains of Bt described in the literature have common and characteristic banding patterns when analyzed by SDS-polyacrylamide gel
25 electrophoresis. To determine whether C-18 has a distinctive protein profile of its own, proteins extracted from both vegetative and sporulating cells were examined by this technique and compared with Bt subspecies kurstaki (lepidopteran-specific),
30 israeliensis (dipteran-specific), and tenebrionis (coleopteran-specific). C-18 does, indeed, have distinctive protein profiles when compared with several other commonly known subspecies of Bt.

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Immunochemical Staining of Crystal Proteins

Western analysis of purified crystal proteins from C-18 was performed and proteins identified by this technique were compared with the same three
5 subspecies of Bt mentioned above. Although there are some similarities among the four organisms, C-18 does exhibit a distinctive and characteristic crystal protein profile.

Plasmid DNA Profiles

10 Genes responsible for encoding insecticidal proteins that constitute parasporal crystals of Bt usually are associated with plasmid DNA although there is evidence that such genes are chromosomally linked as well. The plasmids purified from C-18 are displayed in
15 Fig. 2. The plasmid profile of C-18 is distinctive when compared to the profiles of the same three subspecies of Bt indicated above.

This bacterium contains large number of plasmid DNA molecules. The majority of the toxin genes
20 have been reported to be carried on one of the large plasmids, however, few have been reported on the chromosome of some Bts. The plasmid profile of C-18 has been used as a tool to differentiate between bacterial species. The plasmid profile of the BtC-18
25 was found to be different from the other subspecies of *B. thuringiensis* tested as indicated in Fig. 2.

Polymerase Chain Reaction (PCR)

To determine the existence of genes (Cry genes) in C-18 that may encode different insecticidal
30 proteins (Cry proteins), specific DNA primers were constructed and used in PCR analysis of C-18 genomic DNA. The primers were custom designed against conserved regions in the genomes of the same subspecies used above. Amplification of various DNA fragments
35 revealed that C-18 contains similar Cry1 (Lepidopteran-specific) genes as Bt subsp. *kurstaki* but different Cry

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genes that those of Bt subspecies *israelensis* and *tenebrionis* (Fig. 4).

The proof of the existence or absence of the three genes encoding the three crystals produced by BtC-18 was proven by the use of specific DNA primers (three sets, each consisting of two pairs specific for one gene) designed against conserved regions in the genomes of *B. thuringiensis* subsp. *kurstaki* (Lep1A, Lep1B, Lep2A, and Lep2B), *B. thuringiensis* subsp. *israelensis* (Dip1A, Dip1B, Dip2A, and Dip2B) and *B. thuringiensis* subsp. *tenebrionis* (Col1A, Col1B, Col2A, and Col2B).

The primers were used in the polymerase chain reaction (PCR) to give specific product profiles. The two pairs of Lep primers amplify a 0.49kb and a 0.908kb DNA fragment. The Dip primers amplify a 0.797kb and 1.290kb DNA fragment. The Col primers amplify a 0.649kb and 1.060kb DNA fragments (Fig. 4).

Western blot analysis of the crystal proteins produced by BtC-18 confirm that the crystal proteins of BtC-18 are different from the subspecies *israelensis* and *tenebrionis*.

Purification of the C18 Crystal Protein Complex and Insecticidal Properties

The BtC18 crystal complex was purified from the sporulated culture broth by Renographin gradient from the methods discussed in Lee et al. (1995) *Biochem. Biophys. Res. Comm.* 126: 953-960. The purified crystals were washed several times with de-ionized water to get rid of any contaminants. The crystal complex was subjected to differential solubilization at various pH's according to the methods of Dai and Gill (1993) *Insect Biochem. Molec. Biol.* 23:273-283 and Hofte et al. (1986) *Eur. J. Biochem.* 161:273-280 and fractions (F1, F2, F3, F4, F5, and F6) obtained from this procedure were bioassayed against

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corn rootworms (southern and western) as representative of coleopteran insects and tobacco hornworm as lepidopteran insects. All fractions killed both representative insects, but with varying degrees.

- 5 Fractions F2, F4, and F6 killed rootworms very efficiently. $5\mu\text{g}/\text{cm}^2$ of the fractions killed 80-90% of the tested insects. 100% kill was recorded at $10\mu\text{g}/\text{cm}^2$. All fractions killed the tobacco hornworm albeit with variable efficiencies.

10 Binding of BtC18 toxins to specific brush border membrane proteins from the midgut of different insects

- Brush border membrane vesicles (BBMV) were prepared from: corn rootworms (WCRW and SCRW), tobacco hornworm (MS), and European corn borer (ECB) according to methods used by Wolfersberger et al. (1987) Comp. Biochem. Physiol. 86A:301-308. Specific amounts (20-MS, 50-ECB, and 120-W/SCRW $\mu\text{g}/\text{lane}$) of BBMV's were loaded and separated by SDS-PAGE. The separated proteins were electro-blotted onto PVDF nylon membranes and were reacted with ^{125}I labeled fractions (F2, F4, F5, and F6) from BtC18, which bind to specific receptors from the BBMV. The radioactive membranes are then exposed on film. Where there's a band there is an interaction with the specific receptor. Evident in this analysis is the broad based interaction of F6 radiolabelled proteins with the various brush border membrane proteins extracted from these pests (Table 2). Based on this observation, F6 appears to have the largest range of activity.

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Table 2.

BtC18 Protein fraction	BBMV Binding Protein (kDa)			
	MS	ECB	SCRW	WCRW
F2	210	nd	nd	nd
F4	210	nd	nd	nd
	84	nd	nd	nd
	45	nd	nd	nd
F5	210	nd	nd	nd
	120	nd	nd	nd
	60	nd	nd	nd
	55	nd	nd	nd
F6	210	210	210	nd
	120	84	nd	180
	60	nd	150	150
	45	nd	50	50
	40	nd	42	42

Identification of Cry genes

10 DNA primers available in the scientific literature enabled the identification of the types of genes present in this BtC-18 isolate. Some of the primers were designed to differentiate between the members of the same family of genes such as Cry1 family, Cry2
 15 genes, Cry3 genes, Cry4 genes and Cry5 gene. These primers were used in PCR analysis to determine the number and kinds of the different genes present in the isolate BtC-18. Several genes were determined to be present in BtC-18. The identified genes were Cry1Aa,

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Cry1Ab, Cry1Ac, Cry1B, Cry1C, Cry1D, Cry2A, Cry2B, and a Cry5-like gene designated as Cry1I.

These genes can be grouped into three major families: i) Cry1 family genes encode for proteins toxic to lepidopteran insects only, Cry1Aa, Cry1Ab, Cry1Ac, Cry1C, Cry1D, ii) Cry2 genes encode proteins toxic to lepidopteran and dipteran insects, Cry2A and Cry2B, and iii) Cry1B and Cry5 genes encodes proteins toxic against lepidopterous and coleopterans insects. A pool of at least nine genes were present in this single bacterium, which covers the widest range of insecticidal activity recorded in the scientific literature.

Identification of Additional Genes with Potential Insecticidal Activity

In the same manner as the above example, additional genes were discovered to exist in BtC18 which are potentially responsible for giving this strain its broad spectrum of insecticidal activity. Cry1F, Cry1G, Cry1K, and Cry1M were found as well as nematode specific genes, designated nem1, nem3, nem5 and nem7. In addition, a vegetative toxin was discovered in BtC18 which gives a PCR generated band of the size expected for strain BtHD1. This vegetative toxin is produced during vegetative growth and kills black cut worm of corn (BCW).

Cloning of Genes

All the genes detected by PCR in BtC-18, mentioned above, were cloned into BlueScript sk+ II plasmid vector. Custom made DNA primers against the toxin domains of the respective genes were used in PCR to amplify and target the N-terminus part of the genes. The amplification products were eluted from the gel, biocleaned and ligated to blunt ended pBlueScript. E.

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coli was subsequently transformed with this vector, and, recombinants containing the target genes were identified. The positive recombinants were determined using the specific DNA primers and PCR to identify the expected gene products. Recombinants clones were expressed in *E. coli* and the total cellular proteins of the recombinants were analyzed by SDS-PAGE. For better expression, the genes were cloned into an expression plasmid pTrc99.

10 CryII Gene

Analysis of the CryII expression products by SDS-PAGE was performed. Two proteins were produced, a 70 kDa protein and a smaller 58 to 60 kDa protein which are not produced by *E. coli* transformed with pTrc99 alone. The CryII gene was subcloned for the purpose of restriction mapping and DNA sequencing. Three nucleotide sequences of three different segments of the CryII gene from BtC-18 were obtained using the dideoxy chain termination method (sequences not shown). The three segments were the 5' and 3' ends of the gene as well as a middle segment. The nucleotide sequence of these three segments of the CryII gene from BtC-18 were compared using Blast server to nucleotide sequences of the published sequences of several different Cry5 genes. By this method identities and homologies of the nucleotide and amino acid sequences were determined. The closest relative to the C18 CryII gene is that of entomocidus. C18 CryII shares 97% nucleotide identity and 92% amino acid identity with the entomodidus Cry5. The complete nucleotide sequence of the C18 CryII has been obtained and is represented in Figure 5.

CryII activity against rootworm

The C18 CryII gene was inserted into the expression vector pTrc99 with IPTG induction. *E. coli* strain BL21 was transformed with vector plus CryII insert and empty vector as a negative control. Once

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the bacteria had grown to the appropriate cell density, they were pelleted and resuspended in 11 ml of buffer (10mM MgSO₄, 0.3% Tween-20, 2mM PMSF). The cellular suspension was sonicated and protein extracted. The concentration of the protein extract was ~45mg/ml. WCRW (Western Corn Rootworm) larvae were challenged with various concentrations of the protein extract and mortality scored after three days. Results of this assay demonstrates a dramatic increase in WCRW mortality compared to the negative control at doses as low as 2.3 $\mu\text{g}/\text{cm}^2$. At these levels mortality of the larvae exposed to the extract containing Cry1I_p is up 55% compared to the mortality of the larvae unexposed. At 11.8 $\mu\text{g}/\text{cm}^2$ the mortality is up 80%. See Table 3. This is clear evidence that Cry1I is one of the constituents in the BtC18 insecticidal arsenal that demonstrates anti-rootworm activity.

Table 3.

Extract vol.	Dead (WCRW)	Live (WCRW)	% Mortality	Est. Cry1I dose ($\mu\text{g}/\text{cm}^2$)
0.1 ml	6	2	75	2.3
0.5 ml	8	0	100	11.8
1.0 ml	8	0	100	22.3
2.0 ml	8	0	100	44.7
Control	3	12	20	0

Purification of Insecticidal Proteins from BtC-18

FPLC Separation of BtC-18 crystal proteins

Protein fractions from BtC-18 were dialyzed, solubilized and then purified on a Mono Q column following the FPLC procedure recommended by the manufacturer:

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1. Portions of the fractions were loaded onto the column and eluted using NaCl gradients.

2. Each individual fraction was bioassayed against different insects, analyzed by SDS-PAGE, and
5 ligand binding studies were performed using the midguts of different insects to identify specific receptor proteins.

Isolation of Crystal Proteins from BtC-18.

The bacterium BtC-18 was cultured in liquid
10 sporulation medium (T3-medium) at 30°-32°C and the crystal proteins were purified according to Lee et al. (1985) *Biochem. Biophys. Res. Commun.*, 126:953-960 using the following procedure:

1. The cells were grown to the spore stage. The
15 crystal proteins were released into the medium following autolysis of the cells.

2. The spore-crystal complexes were separated by centrifugation (10,000 rpm, for 10 min at 4 C). The complex was washed twice each with 1M potassium
20 chloride in deionized water.

3. The pellet was resuspended in distilled water and homogenized with a Dounce homogenizer followed by centrifugation to separate the protein complexes. This step was repeated two times.

25 4. The pellet (2-4g) was suspended in a 68% Renografin gradient (9.6 ml of 2% Triton X-100 and 20.4 ml of Renografin). The mixture was homogenized and subjected to sonication 10 times (20 pulses each time).

5. The mixture was centrifugated at 27,000 rpm
30 for 15 hours. The viscous top band on the gradient contained the crystal proteins. The purity of the crystal proteins was checked by microscopic examination.

6. The crystal pellet was resuspended in 30 ml of
35 55% Renografin, 1% Triton X-100, homogenized with a

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glass Dounce homogenizer and sonicated 10 times as above.

7. The mixture was centrifugated again at 27,000 rpm for 15h and the crystal proteins were precipitated as a white pellet.

8. The supernatant was carefully removed and the pellet was washed three times with distilled water before lyophilization.

Solubilization of the Crystal Proteins from BtC-18

10 Solubilization of the crystal proteins was accomplished according to the methods of Dai et al. (1993) *Insect Biochem. Mol. Biol.*, 23:273-283 and Höfte et al. (1986) *Eur. J. Biochem.*, 161:273-280.

15 1. 50 µg of the purified crystal proteins were dissolved in 5 ml of the following solution:

Sodium carbonate (pH 9.5)	50 mM
Dithiothreitol (DTT)	10 mM
Phenylmethylsulfonyl Fluoride (PMSF)	5 mM
Ethylenediaminetetraacetate, disodium	10 mM

20 The mixture was incubated at 37°C for 80 min. with continuous shaking.

2. The mixture was spun at 14K rpm for 15 min. and the supernatant was analyzed by SDS-PAGE.

25 3. The pellet was dissolved in the following solution:

Sodium hydroxide (pH12)	50mM
Dithiothreitol (DTT)	10mM
Phenylmethylsulfonyl fluoride (PMS)	5mM

and incubated at 37 C/1 hour.

30 4. The supernatant was collected again by centrifugation at 14k for 15 min.

5. The fractions were dialyzed against Tris-HCl (50 mM, pH 9) containing 50 mM NaCl.

35 After purification, the proteins are assayed for activity against insects of interest.

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All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent
5 applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been
10 described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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IN THE CLAIMS

What is claimed is:

1. A stable Bacillus strain which is capable of killing insects from at least two orders.

5 2. The strain of claim 1, wherein said strain kills insects from at least three orders.

3. The strain of claim 2, wherein said orders are selected from the group consisting of Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga,
10 Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anophora, Siphonaptera, and Trichoptera.

4. The strain of claim 3, wherein said orders are Coleoptera, Lepidoptera and Diptera.

15 5. The strain of claim 4, wherein said strain is active against nematodes.

6. The strain of claim 4, wherein said strain is active against rootworm.

7. The strain of claim 4, wherein said strain is
20 BtC-18.

8. A toxin protein having rootworm activity wherein said protein is isolated by
isolation of proteins from strain BtC-18,
separation of proteins on a column,
25 collection of fractions from said column, and assaying said fractions for rootworm activity.

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9. A DNA sequence encoding the protein of claim 8.
10. A DNA sequence which hybridizes to the DNA sequence of claim 9 under stringent hybridization conditions.
11. A plant transformed with the DNA sequence of claim 9.
12. Seed of the plant of claim 11.
13. A substantially purified protein having the amino acid sequence set forth in Figure 5.
14. An isolated nucleotide sequence encoding the protein of claim 13.
15. The nucleotide sequence of claim 14, said sequence having the sequence set forth in Figure 5.
16. A nucleotide sequence that hybridizes to the sequence of claim 15 under stringent hybridization conditions.
17. A chimeric gene comprising a promoter operable in a plant cell operably linked to a nucleotide sequence encoding the protein of Cry1I, said protein having the amino acid sequence set forth in Figure 5.
18. The chimeric gene of claim 17 wherein said sequence encoding the Cry1I gene is the sequence set forth in Figure 5.
19. A plant stably transformed with the gene of claim 17.

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20. A plant stably transformed with the gene of claim 18.

21. Seed of the plant of claim 19.

22. Seed of the plant of claim 20.

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FIGURE 1A

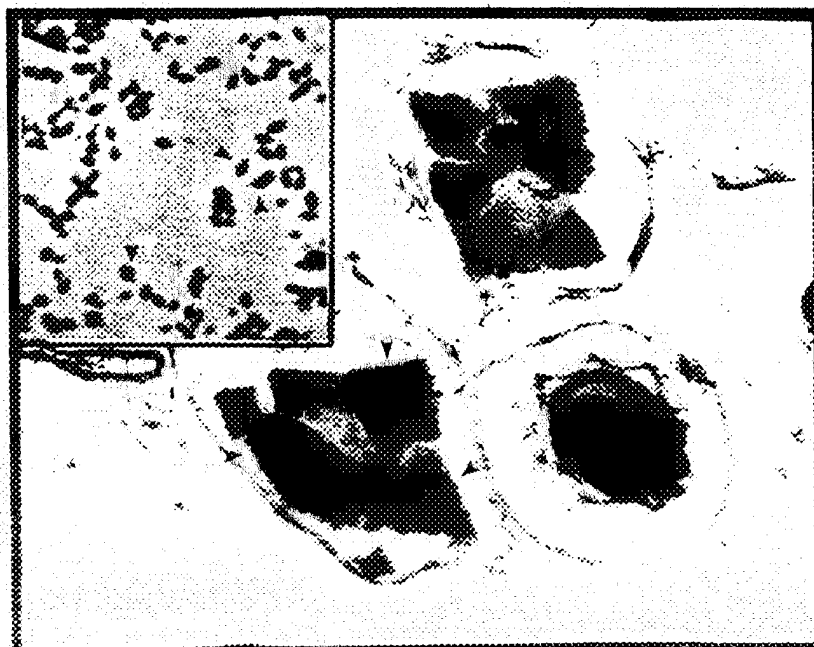


FIGURE 1B



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FIGURE 2

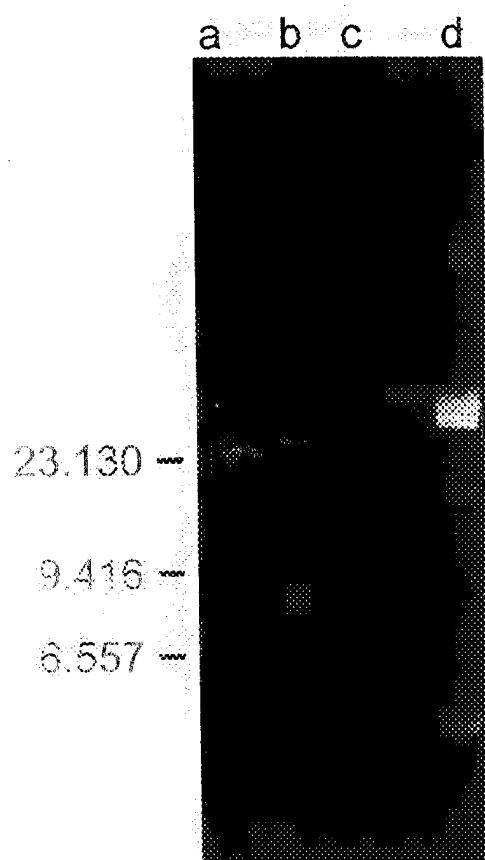


FIGURE 3A



FIGURE 3B



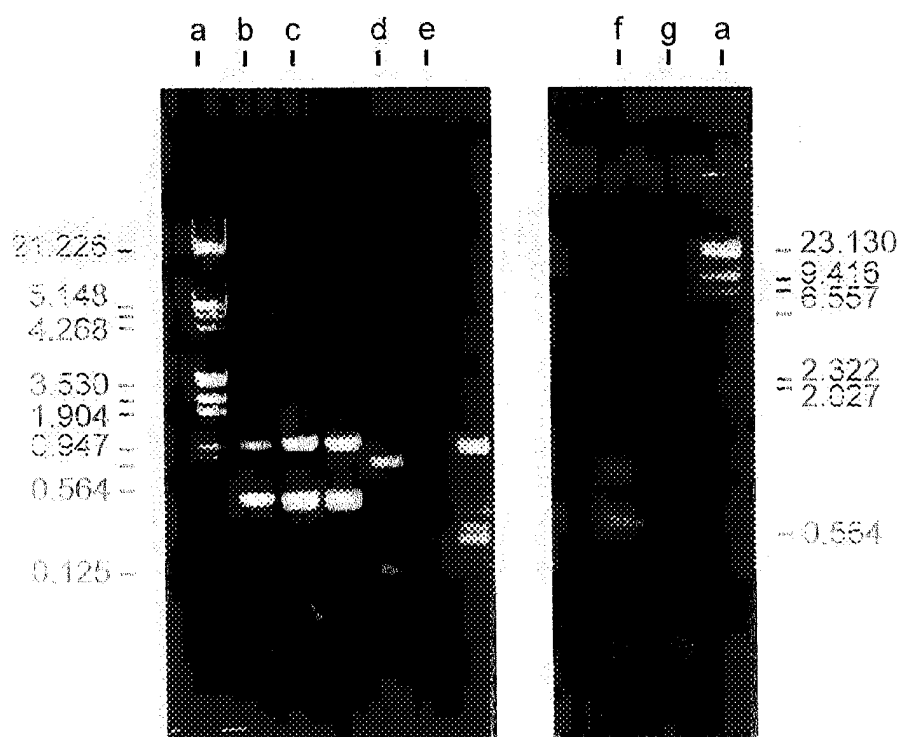
FIGURE 3C



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FIGURE 4A

FIGURE 4B



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FIGURE 5

			10			20			30			40					
	*		*	*		*	*	*	*	*	*	*	*	*	*	*	*
ATG	AAA	CTA	AAG	AAT	CCA	GAT	AAG	CAT	CAA	ACG	TTG	TCT	AGC	AAT	GCG		
Met	Lys	Leu	Lys	Asn	Pro	Asp	Lys	His	Gln	Thr	Leu	Ser	Ser	Asn	Ala>		
50			60			70			80			90					
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAG	GTA	GAT	AAA	ATC	GCG	ACG	GAT	TCA	CTA	AAA	AAT	GAA	ACA	GAT	ATA		
Lys	Val	Asp	Lys	Ile	Ala	Thr	Asp	Ser	Leu	Lys	Asn	Glu	Thr	Asp	Ile>		
100			110			120			130			140					
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GAA	TTG	AAA	AAT	ATG	AAT	AAT	GAA	GAT	TAT	TTG	AGA	ATG	TCT	GAG	CAT		
Glu	Leu	Lys	Asn	Met	Asn	Asn	Glu	Asp	Tyr	Leu	Arg	Met	Ser	Glu	His>		
150			160			170			180			190					
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GAG	AGT	ATT	GAT	CCG	TTT	GTT	AGT	GCA	TCA	ACA	ATT	CAA	ACG	GGT	ATT		
Glu	Ser	Ile	Asp	Pro	Phe	Val	Ser	Ala	Ser	Thr	Ile	Gln	Thr	Gly	Ile>		
200			210			220			230			240					
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GGA	ATT	GCT	GGT	AAG	ATT	CTT	GGT	ACT	CTA	GGC	GTT	CCT	TTT	CCT	GGA		
Gly	Ile	Ala	Gly	Lys	Ile	Leu	Gly	Thr	Leu	Gly	Val	Pro	Phe	Pro	Gly>		
250			260			270			280								
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CAA	ATA	GCT	AGC	CTC	TAT	AGT	TTT	ATC	TTA	GGC	GAG	CTT	TGG	CCT	AAG		
Gln	Ile	Ala	Ser	Leu	Tyr	Ser	Phe	Ile	Leu	Gly	Glu	Leu	Trp	Pro	Lys>		
290			300			310			320			330					
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GGG	AAA	AGT	CAA	TGG	GAA	ATC	TTT	ATG	GAA	CAT	GTA	GAA	GCG	ATT	ATT		
Gly	Lys	Ser	Gln	Trp	Glu	Ile	Phe	Met	Glu	His	Val	Glu	Ala	Ile	Ile>		
340			350			360			370			380					
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAT	CGA	AAA	ATA	TCA	ACT	TAT	GCA	AGA	AAT	AAA	GCA	CTT	ACG	GAC	TTG		
Asn	Arg	Lys	Ile	Ser	Thr	Tyr	Ala	Arg	Asn	Lys	Ala	Leu	Thr	Asp	Leu>		
390			400			410			420			430					
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAA	GGA	TTA	GGA	GAT	GCC	TTA	GCT	GTC	TAC	CAT	GAA	TCG	CTT	GAA	AGT		
Lys	Gly	Leu	Gly	Asp	Ala	Leu	Ala	Val	Tyr	His	Glu	Ser	Leu	Glu	Ser>		
440			450			460			470			480					
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
TGG	GTT	GGA	AAT	CGT	AAT	AAC	ACT	CGA	GCG	AGG	AGT	GTA	GTC	AAG	AAC		
Trp	Val	Gly	Asn	Arg	Asn	Asn	Thr	Arg	Ala	Arg	Ser	Val	Val	Lys	Asn>		

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FIGURE 5 (cont.)

			490			500			510			520			
	*		*		*	*		*	*		*	*		*	
CAA	TAT	ATC	GCA	TTA	GAA	CTG	ATG	TTT	GTT	CAA	AAA	CTA	CCT	TCT	TTT
Gln	Tyr	Ile	Ala	Leu	Glu	Leu	Met	Phe	Val	Gln	Lys	Leu	Pro	Ser	Phe>
530			540			550			560			570			
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GCA	GTA	TCT	GGT	GAG	GAA	GTA	CCA	TTA	TTA	CCG	ATA	TAT	GCC	CAA	GCT
Ala	Val	Ser	Gly	Glu	Glu	Val	Pro	Leu	Leu	Pro	Ile	Tyr	Ala	Gln	Ala>
580			590			600			610			620			
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GCC	AAT	TTA	CAT	TTG	TTG	TTA	TTA	AGA	GAT	GCA	TCT	ATT	TTT	GAA	AAG
Ala	Asn	Leu	His	Leu	Leu	Leu	Leu	Arg	Asp	Ala	Ser	Ile	Phe	Glu	Lys>
630			640			650			660			670			
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAT	GGG	GGA	TTA	TCA	GCT	TCA	GAA	ATT	TCA	ACA	TTT	TAT	AAC	CGT	CAA
Asn	Gly	Gly	Leu	Ser	Ala	Ser	Glu	Ile	Ser	Thr	Phe	Tyr	Asn	Arg	Gln>
680			690			700			710			720			
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GTC	GAA	CGA	ACA	AGA	GAT	TAT	TCC	TAC	CAT	TGT	GTG	AAA	TGG	AAT	AAT
Val	Glu	Arg	Thr	Arg	Asp	Tyr	Ser	Tyr	His	Cys	Val	Lys	Trp	Asn	Asn>
730			740			750			760						
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ACA	GGC	CTA	AAT	AAC	TTG	AGG	GCT	ACA	AAT	GGC	CAA	AGT	TGG	GTT	CGT
Thr	Gly	Leu	Asn	Asn	Leu	Arg	Ala	Thr	Asn	Gly	Gln	Ser	Trp	Val	Arg>
770			780			790			800			810			
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
TAT	AAT	CAA	TTT	CGT	AAA	GAT	ATC	GAG	TTA	ATG	GTA	TTA	GAT	TTA	GTT
Tyr	Asn	Gln	Phe	Arg	Lys	Asp	Ile	Glu	Leu	Met	Val	Leu	Asp	Leu	Val>
820			830			840			850			860			
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CGC	GTA	TTC	CCA	AGC	TAT	GAT	ACA	CTT	GTA	TAT	CCT	ATT	AAA	ACC	ACT
Arg	Val	Phe	Pro	Ser	Tyr	Asp	Thr	Leu	Val	Tyr	Pro	Ile	Lys	Thr	Thr>
870			880			890			900			910			
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
TCA	CAA	CTT	ACA	AGA	GAA	GTA	TAT	ACA	GAC	GCA	ATT	GGG	ACC	GTC	GAT
Ser	Gln	Leu	Thr	Arg	Glu	Val	Tyr	Thr	Asp	Ala	Ile	Gly	Thr	Val	Asp>
920			930			940			950			960			
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CCG	AAT	CAA	GCT	CTT	CGA	AGT	ACG	ACT	TGG	TAT	AAT	AAT	AAT	GCA	CCT
Pro	Asn	Gln	Ala	Leu	Arg	Ser	Thr	Thr	Trp	Tyr	Asn	Asn	Asn	Ala	Pro>
970			980			990			1000						
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

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FIGURE 5 (cont.)

TCG	TTC	TCT	GCC	ATA	GAG	GCT	GCT	GTT	ATC	CGA	AGT	CCA	CAC	CTA	CTT
Ser	Phe	Ser	Ala	Ile	Glu	Ala	Ala	Val	Ile	Arg	Ser	Pro	His	Leu	Leu>
1010			1020				1030			1040			1050		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GAT	TTT	CTA	GAA	AAA	GTT	ACA	ATT	TAC	AGC	TTA	TTA	AGT	CGG	TGG	AGT
Asp	Phe	Leu	Glu	Lys	Val	Thr	Ile	Tyr	Ser	Leu	Leu	Ser	Arg	Trp	Ser>
1060			1070				1080			1090			1100		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAT	ACT	CAG	TAT	ATG	AAT	ATG	TGG	GGA	GGA	CAT	AGA	CTT	GAA	TCC	CGC
Asn	Thr	Gln	Tyr	Met	Asn	Met	Trp	Gly	Gly	His	Arg	Leu	Glu	Ser	Arg>
1110			1120				1130			1140			1150		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CCA	ATA	GGA	GGG	GCA	TTA	AAT	ACC	TCA	ACA	CAA	GGA	TCT	ACC	AAT	ACT
Pro	Ile	Gly	Gly	Ala	Leu	Asn	Thr	Ser	Thr	Gln	Gly	Ser	Thr	Asn	Thr>
1160			1170				1180			1190			1200		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
TCG	ATT	AAT	CCA	GTA	ACA	TTA	CAG	TTC	ACG	TCT	CGA	GAC	TTC	TAT	AGG
Ser	Ile	Asn	Pro	Val	Thr	Leu	Gln	Phe	Thr	Ser	Arg	Asp	Phe	Tyr	Arg>
1210			1220				1230			1240					
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ACT	GAA	TCA	TGG	GCA	GGG	CTG	AAT	TTA	TTT	TTA	ACT	CAA	CCT	GTT	ATT
Thr	Glu	Ser	Trp	Ala	Gly	Leu	Asn	Leu	Phe	Leu	Thr	Gln	Pro	Val	Ile>
1250			1260				1270			1280			1290		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GGA	GTG	CCT	AGA	GTT	GAT	TTC	CAT	TGG	AAA	TTT	CCC	ACG	CTA	CCA	ATA
Gly	Val	Pro	Arg	Val	Asp	Phe	His	Trp	Lys	Phe	Pro	Thr	Leu	Pro	Ile>
1300			1310				1320			1330			1340		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GCA	TCT	GAT	AAT	TTT	TAT	TAT	CTA	GGG	TAT	GCT	GGA	GTT	GGT	ACG	CAA
Ala	Ser	Asp	Asn	Phe	Tyr	Tyr	Leu	Gly	Tyr	Ala	Gly	Val	Gly	Thr	Gln>
1350			1360				1370			1380			1390		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
TTA	CAA	GAT	TCA	GAA	AAT	GAA	TTA	CCA	CCT	GAA	ACA	ACA	GGA	CAG	CCA
Leu	Gln	Asp	Ser	Glu	Asn	Glu	Leu	Pro	Pro	Glu	Thr	Thr	Gly	Gln	Pro>
1400			1410				1420			1430			1440		
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AAT	TAT	GAA	TCA	TAT	AGT	CAT	AGA	TTA	TCC	CAT	ATA	GGA	CTC	ATT	TCA
Asn	Tyr	Glu	Ser	Tyr	Ser	His	Arg	Leu	Ser	His	Ile	Gly	Leu	Ile	Ser>
1450			1460				1470			1480					
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GGA	TCC	CAC	GTG	AAA	GCA	TTG	GTA	TAT	TCT	TGG	ACA	CAT	CGT	AGT	GCA
Ala	Ser	His	Val	Lys	Ala	Leu	Val	Tyr	Ser	Trp	Thr	His	Arg	Ser	Ala>

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FIGURE 5 (cont.)

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1490      1500      1510      1520      1530
*      *      *      *      *
GAT CGT ACA AAT ACA ATT GAG CCA AAT AGC ATT ACA CAA ATA CCA TTA
Asp Arg Thr Asn Thr Ile Glu Pro Asn Ser Ile Thr Gln Ile Pro Leu>

1540      1550      1560      1570      1580
*      *      *      *      *
GTA AAA GCG TTC AAT CTG TCT TCA GGT GCC GCT GTA GTG AGA GGA CCA
Val Lys Ala Phe Asn Leu Ser Ser Gly Ala Ala Val Val Arg Gly Pro>

1590      1600      1610      1620      1630
*      *      *      *      *
GGA TTT ACA GGT GGG CAT ATC CTT CGA AGA ACG AAA TCT GGT ACA TTT
Gly Phe Thr Gly Gly His Ile Leu Arg Arg Thr Lys Ser Gly Thr Phe>

1640      1650      1660      1670      1680
*      *      *      *      *
GGG CAT ATA CGA GTA AAT ATT AAT CCA CCA TTT GCT CAA AGA TAT CGC
Gly His Ile Arg Val Asn Ile Asn Pro Pro Phe Ala Gln Arg Tyr Arg>

1690      1700      1710      1720
*      *      *      *
GTG AGG ATG TCC TAT GCT TCT ACT ACA GAT TTA CAA TTC CAT ACG TCA
Val Arg Met Ser Tyr Ala Ser Thr Thr Asp Leu Gln Phe His Thr Ser>

1730      1740      1750      1760      1770
*      *      *      *      *
ATT AAC GGT AAA GCT ATT AAT CAA GGT AAT TTT TCA GCA ACT ATG AAT
Ile Asn Gly Lys Ala Ile Asn Gln Gly Asn Phe Ser Ala Thr Met Asn>

1780      1790      1800      1810      1820
*      *      *      *      *
AGA GGA GAG GAC TTA GAC TAT AAA ACC TTT AGA ACT GTA GGC TTT ACC
Arg Gly Glu Asp Leu Asp Tyr Lys Thr Phe Arg Thr Val Gly Phe Thr>

1830      1840      1850      1860      1870
*      *      *      *      *
ACT CCA TTT AGC TTT TCA GAT GTA CAA AGT ACA TTC ACA ATA GGT GCT
Thr Pro Phe Ser Phe Ser Asp Val Gln Ser Thr Phe Thr Ile Gly Ala>

1880      1890      1900      1910      1920
*      *      *      *      *
TGG AAC TTC TCT TCA GGT AAC GAA GTT TAT ATA GGT CGA ATT GAA TTT
Trp Asn Phe Ser Ser Gly Asn Glu Val Tyr Ile Gly Arg Ile Glu Phe>

1930      1940      1950      1960
*      *      *      *
GTT CCG GTA GAA GTA ACA TAT GAG GCA GAA TAT GAT TTT GAA AAA GCG
Val Pro Val Glu Val Thr Tyr Glu Ala Glu Tyr Asp Phe Glu Lys Ala>

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FIGURE 5 (cont.)

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1970      1980      1990      2000      2010
*          *          *          *          *
CAA GAG AAG GTT ACT GCA CTG TTT ACA TCT ACG AAT CCA AGA GGA TTA
Gln Glu Lys Val Thr Ala Leu Phe Thr Ser Thr Asn Pro Arg Gly Leu>

2020      2030      2040      2050      2060
*          *          *          *          *
AAA ACA GAT GTA AAG GAT TAT CAT ATT GAC CAG GTA TCA AAT TTA GTA
Lys Thr Asp Val Lys Asp Tyr His Ile Asp Gln Val Ser Asn Leu Val>

2070      2080      2090      2100      2110
*          *          *          *          *
GAG TCT CTA TCA GAT GAA CTC TAT CTT GAT GAA AAG AGA GAA TTA TTC
Glu Ser Leu Ser Asp Glu Leu Tyr Leu Asp Glu Lys Arg Glu Leu Phe>

2120      2130      2140      2150      2160
*          *          *          *          *
GAG ATA GTT AAA TAC GCG AAG CAA ATC CAT ATT GAG CGT AAC ATG TAG
Glu Ile Val Lys Tyr Ala Lys Gln Ile His Ile Glu Arg Asn Met * >

2170      2180
*          *          *
AGC TCT AGA GGA TCC CGG CA
Ser Ser Arg Gly Ser Arg Xxx>
TRANSLATION OF CRYV A >

```

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 98/00357

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/32 C07K14/325 A01H5/00 C12Q1/68 C12N1/20 //(C12N1/20,C12R1:07)				
According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07K A01H C12Q C12R				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	WO 95 02693 A (MYCOGEN CORP) 26 January 1995 see page 6, line 12 - page 7, line 5 see examples	1,2,8-12		
X,P	WO 97 12980 A (MYCOGEN CORP) 10 April 1997 see page 4, line 1 - page 5, line 16 see table 2 see examples 6-8	1,8-12		
L	shows an additional nematocidal activity for strain PS201T6.			
X	EP 0 537 105 A (SANDOZ AG ;SANDOZ LTD (CH); SANDOZ AG (DE)) 14 April 1993 see example 2	1-4		
--- -/--				
☒ Further documents are listed in the continuation of box C. ☒ Patent family members are listed in annex.				
* Special categories of cited documents :				
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Date of the actual completion of the international search 24 April 1998		Date of mailing of the international search report 18/05/1998		
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Andres, S		

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/00357

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHIN, B.-S. ET AL.: "Distribution of CryV-type insecticidal protein genes in <i>Bacillus thuringiensis</i> and cloning of cryV-type genes from <i>Bacillus thuringiensis</i> subsp. <i>kurstaki</i> and <i>Bacillus thuringiensis</i> subsp. <i>entomocidus</i> " APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 61, June 1995, pages 2402-2407, XP002063408 see figure 3	1,16
X	CHAMBERS J A ET AL: "ISOLATION AND CHARACTERIZATION OF A NOVEL INSECTICIDAL CRYSTAL PROTEIN GENE FROM <i>BACILLUS THURINGIENSIS</i> SUBSP. <i>AIZAWAI</i> " JOURNAL OF BACTERIOLOGY, vol. 173, no. 13, 1 July 1991, pages 3966-3976, XP000567681 see ORF2	16
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/US 98/00357

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