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(57) Abstract

Disclosed and claimed is the use of *Bacillus thuringiensis* strains designated *B.t.* PS211B2, *B.t.* PS86A1, and *B.t.* PS80JJ1, and variants thereof, to control wireworms. Further, variants which retain the activity of the parent can be used to control wireworms. Still further, genes encoding δ-endotoxins can be removed from these strains using standard well-known techniques, and transferred to other host microbes or to plants. Expression of the δ-endotoxin in such hosts results in control of wireworms.

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DESCRIPTION

MATERIALS AND METHODS FOR THE CONTROL OF WIREWORMS

Background of the Invention

The soil microbe *Bacillus thuringiensis* (*B.t.*) is a Gram-positive, spore-forming bacterium characterized by parasporal crystalline protein inclusions. These often appear microscopically as distinctively shaped crystals. The proteins can be highly toxic to pests and specific in their activity. Certain *B.t.* toxin genes have been isolated and sequenced, and recombinant DNA-based *B.t.* products produced and approved. In addition, with the use of genetic engineering techniques, new approaches for delivering *B.t.* endotoxins to agricultural environments are under development, including the use of plants genetically engineered with endotoxin genes for insect resistance and the use of stabilized intact microbial cells as *B.t.* endotoxin delivery vehicles (Gaertner, F.H., L. Kim [1988] *TIBTECH* 6:S4-S7). Thus, isolated *B.t.* endotoxin genes are becoming commercially valuable.

Over the past 30 years, commercial use of *B.t.* pesticides has been largely restricted to a narrow range of lepidopteran (caterpillar) pests. Preparations of the spores and crystals of *B. thuringiensis* subsp. *kurstaki* have been used for many years as commercial insecticides for lepidopteran pests. For example, *B. thuringiensis* var. *kurstaki* HD-1 produces a crystal called a delta endotoxin which is toxic to the larvae of a number of lepidopteran insects.

In recent years, however, investigators have discovered *B.t.* pesticides with specificities for a much broader range of pests. For example, other species of *B.t.*, namely *israelensis* and *san diego* (a.k.a. *B.t. tenebrionis*, a.k.a. M-7), have been used commercially to control insects of the orders Diptera and Coleoptera, respectively (Gaertner, F.H. [1989] "Cellular Delivery Systems for Insecticidal Proteins: Living and Non-Living Microorganisms," in *Controlled Delivery of Crop Protection Agents*, R.M. Wilkins, ed., Taylor and Francis, New York and London, 1990, pp. 245-255). See also Couch, T.L. (1980) "Mosquito Pathogenicity of *Bacillus thuringiensis* var. *israelensis*," *Developments in Industrial Microbiology* 22:61-76; Beegle, C.C., (1978) "Use of Entomogenous Bacteria in Agroecosystems," *Developments in Industrial Microbiology* 20:97-104. Krieg, A., A.M. Huger, G.A. Langenbruch, W. Schnetter (1983) *Z. ang. Ent.* 96:500-508, describe a *B.t.* isolate named *Bacillus thuringiensis* var. *tenebrionis*, which is reportedly active against two beetles

in the order Coleoptera. These are the Colorado potato beetle, *Leptinotarsa decemlineata*, and *Agelastica alni*.

Recently, new subspecies of *B.t.* have been identified, and genes responsible for active δ-endotoxin proteins have been isolated (Höfte, H., H.R. Whiteley [1989] *Microbiological Reviews* 52(2):242-255). Höfte and Whiteley classified *B.t.* crystal protein genes into 4 major classes. The classes were CryI (Lepidoptera-specific), CryII (Lepidoptera- and Diptera-specific), CryIII (Coleoptera-specific), and CryIV (Diptera-specific). The discovery of strains specifically toxic to other pests has been reported. (Feitelson, J.S., J. Payne, L. Kim [1992] *Bio/Technology* 10:271-275).

The cloning and expression of a *B.t.* crystal protein gene in *Escherichia coli* has been described in the published literature (Schnepf, H.E., H.R. Whitely [1981] *Proc. Natl. Acad. Sci. USA* 78:2893-2897). U.S. Patent 4,448,885 and U.S. Patent 4,467,036 both disclose the expression of *B.t.* crystal protein in *E. coli*. U.S. Patents 4,797,276 and 4,853,331 disclose *B. thuringiensis* strain *san diego* (a.k.a. *B.t. tenebrionis*, a.k.a. M-7) which can be used to control coleopteran pests in various environments. U.S. Patent No. 5,151,363 discloses certain isolates of *B.t.* which have activity against nematodes. Many other patents have issued for new *B.t.* isolates and new uses of *B.t.* isolates. The discovery of new *B.t.* isolates and new uses of known *B.t.* isolates remains an empirical, unpredictable art.

Wireworms are important pests causing enormous damage on crops throughout the world. Adult wireworms are known as "click beetles." The larvae are polyphagous, damaging sugar cane, sugar beet, and other root crops, as well as corn and other field corps. Damage is caused by larval feeding on roots. Damaged plants fail to grow and are more susceptible to drought and disease. Wireworms are serious pests in both the New and Old World. Damage can be quite serious in newly ploughed grasslands. United States Patent No. 4,849,217, which issued on July 18, 1989, discloses two of the isolates described in the subject application. The '217 patent does not describe or suggest the new use of these isolates which is described and claimed herein.

Brief Summary of the Invention

The subject invention concerns novel materials and methods for controlling wireworms. The materials and methods of the subject invention result from the unexpected discovery that certain *B.t.* isolates have activity against these pests.

More specifically, the methods of the subject invention use *B.t.* microbes, or variants thereof, and/or their toxins, to control wireworms. Specific *B.t.* microbes useful according to

the invention are *B.t.* PS86A1, *B.t.* PS211B2, and *B.t.* PS80JJ1. Further, the subject invention also includes the use of variants of the exemplified *B.t.* isolates which have substantially the same wireworm-active properties as the specifically exemplified *B.t.* isolates. Such variants would include, for example, mutants. Procedures for making mutants are well known in the microbiological art. Ultraviolet light and nitrosoguanidine are used extensively toward this end.

The subject invention also includes the use of genes from the *B.t.* isolates of the invention which genes encode the wireworm-active toxins.

Still further, the invention also includes the treatment of substantially intact *B.t.* cells, or recombinant cells containing the genes of the invention, to prolong the wireworm activity when the substantially intact cells are applied to the environment of a target pest. Such treatment can be by chemical or physical means, or a combination of chemical and physical means, so long as the technique does not deleteriously affect the properties of the pesticide, nor diminish the cellular capability in protecting the pesticide. The treated cell acts as a protective coating for the pesticidal toxin. The toxin becomes available to act as such upon ingestion by a target insect.

Finally, the subject invention further concerns plants which have been transformed with genes encoding wireworm-active toxins.

Brief Description of the Sequences

SEQ ID NO. 1 is the nucleotide sequence (open reading frame only) of the gene designated 86A1.

SEQ ID NO. 2 is the predicted amino acid sequence of the toxin 86A1.

SEQ ID NO. 3 is an oligonucleotide probe designated 86A1-A.

SEQ ID NO. 4 is an oligonucleotide primer used according to the subject invention.

SEQ ID NO. 5 is an oligonucleotide primer used according to the subject invention.

SEQ ID NO. 6 is the combined nucleotide and amino acid sequences of a portion of the gene designated 211B2.

SEQ ID NO. 7 is the predicted amino acid sequence of a portion of the gene designated 211B2.

SEQ ID NO. 8 is a forward oligonucleotide primer used according to the subject invention.

SEQ ID NO. 9 is a reverse oligonucleotide primer used according to the subject invention.

Detailed Disclosure of the Invention

The subject invention concerns the use of selected strains of *Bacillus thuringiensis* for the control of wireworm pests.

Specific *Bacillus thuringiensis* isolates useful according to the subject invention have the following characteristics in their biologically pure form:

Characteristics of *B.t.* PS86A1

Colony morphology--Large colony, dull surface, typical *B.t.*

Vegetative cell morphology--typical *B.t.*

Culture methods--typical for *B.t.*

Flagellar serotype--wuhanensis.

Inclusions--multiple attached.

Alkali-soluble proteins--SDS polyacrylamide gel electrophoresis (SDS-PAGE) shows 58 and 45 kDa proteins.

Characteristics of *B.t.* PS211B2

Colony morphology--large colony, dull surface, typical *B.t.*

Vegetative cell morphology--typical *B.t.*

Culture methods--typical for B.t.

Flagellar serotype--entomocidus.

Inclusions--large round amorphic inclusion with coat, and elliptical inclusion.

Alkali-soluble proteins--SDS-PAGE shows 175, 130, 100, 83, 69, 43, 40, 36, 35, 27 kDa proteins.

Characteristics of B.t. PS80JJ1

Colony morphology--Large colony, dull surface, typical B.t.

Vegetative cell morphology--typical B.t.

Culture methods--typical for B.t.

Flagellar serotype--sotto

Inclusions--multiple small amorphic

Alkali-solubile proteins--SDS-PAGE shows 130, 90, 47, 37 kDa proteins.

A comparison of the characteristics of the *B. thuringiensis* strains of the subject invention to the characteristics of the known *B.t.* strains *B. thuringiensis* var. *tenebrionis* (M-7) and *B. thuringiensis* var. *kurstaki* (HD-1) is shown in Table 1.

Table 1. Comparison of *B.t.* PS211B2, *B.t.* PS86A1, *B.t.* PS80JJ1, *B.t.t.* (M-7), and *B.t.* HD-1

	B.t. PS211B2	*B.t.* PS86A1	*B.t.* PS80JJ1	*B.t.* HD-1	M-7
Inclusions:	Large amorphic and ellipse	Multiple attached	Multiple small amorphic	Bipyramid	Flat square
Approximate molecular mass of proteins by SDS-PAGE (kDa)	175 130 100 83 69 43 40 36 35 34 27	58 45	130 90 47 37	130 68	72 64
Serotype	entomocidus	wuhanensis	sotto	kurstaki	morrisoni

B.t. isolates useful according to the subject invention have been deposited. Also deposited are recombinant microbes comprising the *B.t.* genes of interest.

Culture	Accession Number	Deposit Date
Bacillus thuringiensis PS86A1	NRRL B-18400	August 16, 1988
Bacillus thuringiensis PS80JJ1	NRRL B-18679	July 17, 1990
E. coli NM522(pMYC2320)	NRRL B-18769	February 14, 1991
Bacillus thuringiensis PS211B2	NRRL B-18921	November 15, 1991

The cultures are on deposit in the permanent collection of the Northern Research Laboratory, U.S. Department of Agriculture, Peoria, IL, USA.

The subject cultures have been deposited under conditions that assure that access to the cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposits are available as required

by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposit, and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the cultures. The depositor acknowledges the duty to replace the deposits should the depository be unable to furnish a sample when requested, due to the condition of the deposit(s). All restrictions on the availability to the public of the subject culture deposits will be irrevocably removed upon the granting of a patent disclosing them.

Genes and toxins. The genes and toxins according to the subject invention include not only the full length sequences disclosed herein but also fragments of these sequences, or fusion proteins, which retain the characteristic pesticidal activity of the toxins specifically exemplified herein.

It should be apparent to a person skilled in this art that genes coding for wireworm-active toxins can be identified and obtained through several means. The specific genes exemplified herein may be obtained from the isolates deposited at a culture depository as described above. These genes, or portions or variants thereof, may also be constructed synthetically, for example, by use of a gene machine. As used herein, the terms "variants" or "variations" of genes refer to nucleotide sequences which code for the same toxins or which code for equivalent toxins having wireworm activity. Variations of these genes may be readily constructed using standard techniques for making point mutations. Also, fragments of these genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes

which code for active fragments may be obtained using a variety of other restriction enzymes. Proteases may be used to directly obtain active fragments of these toxins.

Equivalent toxins and/or genes encoding these equivalent toxins can also be located from *B.t.* isolates and/or DNA libraries using the teachings provided herein. There are a number of methods for obtaining the pesticidal toxins of the instant invention. For example, antibodies to the pesticidal toxins disclosed and claimed herein can be used to identify and isolate other toxins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the toxins which are most constant and most distinct from other *B.t.* toxins. These antibodies can then be used to specifically identify equivalent toxins with the characteristic activity by immunoprecipitation, enzyme linked immunoassay (ELISA), or Western blotting. Antibodies to the toxins disclosed herein, or to equivalent toxins, or fragments of these toxins, can readily be prepared using standard procedures in this art. The genes coding for these toxins can then be obtained from the microorganism.

A further method for identifying the toxins and genes of the subject invention is through the use of oligonucleotide probes. These probes are nucleotide sequences having a detectable label. As is well known in the art, if the probe molecule and nucleic acid sample hybridize by forming a strong bond between the two molecules, it can be reasonably assumed that the probe and sample have substantial homology. The probe's detectable label provides a means for determining in a known manner whether hybridization has occurred. Such a probe analysis provides a rapid method for identifying toxin-encoding genes of the subject invention. The nucleotide segments which are used as probes according to the invention can be synthesized by use of DNA synthesizers using standard procedures.

Fragments and variations of the exemplified proteins which retain the pesticidal activity of the exemplified toxins, would be within the scope of the subject invention. Also, because of the redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequence disclosed herein. It is well within the skill of a person trained in the art to create these alternative DNA sequences encoding the same, or essentially the same, toxins. These variant DNA sequences are within the scope of the subject invention. As used herein, reference to "essentially the same" sequence refers to sequences which have amino acid substitutions, deletions, additions,

or insertions which do not materially affect pesticidal activity. Fragments retaining wireworm activity are also included in this definition. As used herein, the phrase "wireworm activity" includes activity against wireworm larvae as well as other stages of development.

Toxins of the subject invention are specifically exemplified herein by the toxin encoded by the gene designated 86A1. Since this toxin is merely exemplary of the toxins of the subject invention, it should be readily apparent that the subject invention further comprises variant toxins (and nucleotide sequences coding for variant toxins) having the same or essentially the same biological activity against wireworms of 86A1. These equivalent toxins will have amino acid homology with 86A1. This amino acid homology will typically be greater than 75%, preferably be greater than 90%, and most preferably be greater than 95%. The amino acid homology will be highest in certain critical regions of the toxin which account for biological activity or are involved in the determination of three-dimensional configuration which ultimately is responsible for the biological activity. In this regard, certain amino acid substitutions are acceptable and can be expected if these substitutions are in regions which are not critical to activity or are conservative amino acid substitutions which do not affect the three-dimensional configuration of the molecule. For example, amino acids may be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution does not materially alter the biological activity of the compound. Table 2 provides a listing of examples of amino acids belonging to each class.

Table 2

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

In some instances, non-conservative substitutions can also be made. The critical factor is that these substitutions must not significantly detract from the biological activity of the toxin.

The toxins of the subject invention can also be characterized in terms of the shape and location of toxin inclusions, which are described above.

Recombinant hosts. The toxin-encoding genes harbored by the isolates of the subject invention can be introduced into a wide variety of microbial or plant hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. With suitable microbial hosts, e.g., *Pseudomonas*, the microbes can be applied to the situs of wireworms where they will proliferate and be ingested by the pest. The result is a control of this pest. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin produced in the cell. The treated cell then can be applied to the environment of the target pest. The resulting product retains the toxicity of the *B.t.* toxin.

Where the *B.t.* toxin gene is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is essential that certain host microbes be used. Microorganism hosts are selected which are known to occupy the soil. These microorganisms are selected so as to be capable of

successfully competing in the soil with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably, provide for improved protection of the pesticide from environmental degradation and inactivation.

A large number of microorganisms are known to inhabit the rhizosphere (the soil surrounding plant roots). These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera *Bacillus, Pseudomonas, Erwinia, Serratia, Klebsiella, Xanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc, Alcaligenes* and *Clostridium*; fungi, particularly yeast, e.g., genera *Saccharomyces, Cryptococcus, Kluyveromyces, Sporobolomyces, Rhodotorula,* and *Aureobasidium*; microalgae, e.g., families *Cyanophyceae, Prochlorophyceae, Rhodophyceae, Dinophyceae, Chrysophyceae, Prymnesiophyceae, Xanthophyceae, Raphidophyceae, Bacillariophyceae, Eustigmatophyceae, Cryptophyceae, Euglenophyceae, Prasinophyceae,* and *Chlorophyceae*. Of particular interest are such phytosphere bacterial species as *Pseudomonas syringae. Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacterium tumefaciens, Rhodopseudomonas spheroides, Xanthomonas campestris, Rhizobium melioti, Alcaligenes entrophus,* and *Azotobacter vinlandii*; and phytosphere yeast species such as *Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus, C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces roseus, S. odorus, Kluyveromyces veronae,* and *Aureobasidium pollulans.* Of particular interest are the pigmented microorganisms.

A wide variety of ways are available for introducing a *B.t.* gene encoding a toxin into a microorganism host under conditions which allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in United States Patent No. 5,135,867, which is incorporated herein by reference.

Treatment of cells. As mentioned above, *B.t.* or recombinant cells expressing a *B.t.* toxin can be treated to prolong activity in the environment. Suitable host cells, where the pesticide-containing cells will be treated to prolong the activity of the toxin

in the cell when the then treated cell is applied to the environment of target pest(s), may include either prokaryotes or eukaryotes, normally being limited to those cells which do not produce substances toxic to higher organisms, such as mammals. However, organisms which produce substances toxic to higher organisms could be used, where the toxin is unstable or the level of application sufficiently low as to avoid any possibility of toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi.

The cell will usually be intact and be substantially in the proliferative form when treated, rather than in a spore form, although in some instances spores may be employed.

Treatment of the microbial cell, e.g., a microbe containing the *B.t.* toxin gene, can be by chemical or physical means, or by a combination of chemical and/or physical means, so long as the technique does not deleteriously affect the properties of the toxin, nor diminish the cellular capability in protecting the toxin. Examples of chemical reagents are halogenating agents, particularly halogens of atomic no. 17-80. More particularly, iodine can be used under mild conditions and for sufficient time to achieve the desired results. Other suitable techniques include treatment with aldehydes, such as formaldehyde and glutaraldehyde; anti-infectives, such as zephiran chloride and cetylpyridinium chloride; alcohols, such as isopropyl and ethanol; various histologic fixatives, such as Lugol iodine, Bouin's fixative, and Helly's fixative (See: Humason, Gretchen L., *Animal Tissue Techniques*, W.H. Freeman and Company, 1967); or a combination of physical (heat) and chemical agents that preserve and prolong the activity of the toxin produced in the cell when the cell is administered to the host animal. Examples of physical means are short wavelength radiation such as gamma-radiation and X-radiation, freezing, UV irradiation, lyophilization, and the like.

The cells generally will have enhanced structural stability which will enhance resistance to environmental conditions. Where the pesticide is in a proform, the method of cell stabilization should be selected so as not to inhibit processing of the proform to the mature form of the pesticide by the target pest pathogen. For example, formaldehyde will crosslink proteins and could inhibit processing of the proform of a polypeptide pesticide. Preferably, the method of stabilization results in the cell retaining at least a substantial portion of the bio-availability or bioactivity of the toxin.

Characteristics of particular interest in selecting a host cell for purposes of production include ease of introducing the *B.t.* gene into the host, availability of expression systems, efficiency of expression, stability of the pesticide in the host, and the presence of auxiliary genetic capabilities. Characteristics of interest for use as a pesticide microcapsule include protective qualities for the pesticide, such as thick cell walls, pigmentation, and intracellular packaging or formation of inclusion bodies; survival in aqueous environments; lack of mammalian toxicity; attractiveness to pests for ingestion; ease of killing and fixing without damage to the toxin; and the like. Other considerations include ease of formulation and handling, economics, storage stability, and the like.

Growth of cells. The cellular host containing the *B.t.* insecticidal gene may be grown in any convenient nutrient medium, where the DNA construct provides a selective advantage, providing for a selective medium so that substantially all or all of the cells retain the *B.t.* gene. These cells may then be harvested in accordance with conventional ways. Alternatively, the cells can be treated prior to harvesting.

The *B.t.* cells of the invention can be cultured using standard art media and fermentation techniques. Upon completion of the fermentation cycle the bacteria can be harvested by first separating the *B.t.* spores and crystals from the fermentation broth by means well known in the art. The recovered *B.t.* spores and crystals can be formulated into a wettable powder, liquid concentrate, granules or other formulations by the addition of surfactants, dispersants, inert carriers, and other components to facilitate handling and application for particular target pests. These formulations and application procedures are all well known in the art.

Formulations. Formulated bait granules containing an attractant and spores and crystals of the *B.t.* isolates, or recombinant microbes comprising the gene(s) obtainable from the *B.t.* isolates disclosed herein, can be applied to the soil. Formulated product can also be applied as a seed-coating or root treatment or total plant treatment at later stages of the crop cycle.

As would be appreciated by a person skilled in the art, the pesticidal concentration will vary widely depending upon the nature of the particular formulation, particularly whether it is a concentrate or to be used directly. The pesticide will be present in at least 1% by weight and may be 100% by weight. The dry formulations

will have from about 1-95% by weight of the pesticide while the liquid formulations will generally be from about 1-60% by weight of the solids in the liquid phase. The formulations will generally have from about 10^2 to about 10^4 cells/mg. These formulations will be administered at about 50 mg (liquid or dry) to 1 kg or more per hectare.

The formulations can be applied to the environment of the wireworm, e.g., soil, by spraying, dusting, sprinkling, or the like.

Mutants. Mutants of the novel isolates of the invention can be made by procedures well known in the art. For example, an asporogenous mutant can be obtained through ethylmethane sulfonate (EMS) mutagenesis of a novel isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

A smaller percentage of the asporogenous mutants will remain intact and not lyse for extended fermentation periods; these strains are designated lysis minus (−). Lysis minus strains can be identified by screening asporogenous mutants in shake flask media and selecting those mutants that are still intact and contain toxin crystals at the end of the fermentation. Lysis minus strains are suitable for a cell fixation process that will yield a protected, encapsulated toxin protein.

To prepare a phage resistant variant of said asporogenous mutant, an aliquot of the phage lysate is spread onto nutrient agar and allowed to dry. An aliquot of the phage sensitive bacterial strain is then plated directly over the dried lysate and allowed to dry. The plates are incubated at 30RC. The plates are incubated for 2 days and, at that time, numerous colonies could be seen growing on the agar. Some of these colonies are picked and subcultured onto nutrient agar plates. These apparent resistant cultures are tested for resistance by cross streaking with the phage lysate. A line of the phage lysate is streaked on the plate and allowed to dry. The presumptive resistant cultures are then streaked across the phage line. Resistant bacterial cultures show no lysis anywhere in the streak across the phage line after overnight incubation at 30RC. The resistance to phage is then reconfirmed by plating a lawn of the resistant culture onto a nutrient agar plate. The sensitive strain is also plated in the same manner to serve as the positive control. After drying, a drop of the phage lysate is plated in the center of the plate and allowed to dry. Resistant cultures showed no

lysis in the area where the phage lysate has been placed after incubation at 30RC for 24 hours.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Culturing *Bacillus thuringiensis* Isolates

A subculture of a *B.t.* isolate of the invention can be used to inoculate the following medium, a peptone, glucose, salts medium.

Bacto Peptone	7.50 g/l
Glucose	1.00 g/l
KH_2PO_4	3.40 g/l
K_2HPO_4	4.35 g/l
Salt Solution	5.00 ml/l
$CaCl_2$ Solution	5.00 ml/l

pH 7.2

Salts Solution (100 ml)

$MgSO_4 \cdot 7H_2O$	2.46 g
$MnSO_4 \cdot H_2O$	0.04 g
$ZnSO_4 \cdot 7H_2O$	0.28 g
$FeSO_4 \cdot 7H_2O$	0.40 g

$CaCl_2$ Solution (100 ml)

$CaCl_2 \cdot 2H_2O$	3.66 g

The salts solution and $CaCl_2$ solution are filter-sterilized and added to the autoclaved and cooked broth at the time of inoculation. Flasks are incubated at 30RC on a rotary shaker at 200 rpm for 64 hours.

The above procedure can be readily scaled up to large fermentors by procedures well known in the art.

The *B.t.* spores and crystals, obtained in the above fermentation, can be isolated by procedures well known in the art. A frequently-used procedure is to subject the harvested fermentation broth to separation techniques, e.g., centrifugation.

Example 2 – Purification and Amino Acid Sequencing

A *Bacillus thuringiensis* (*B.t.*) can be cultured as described in Example 1 or by using other standard media and fermentation techniques well known in the art. The toxin protein inclusions can be harvested by standard sedimentation centrifugation. The recovered protein inclusions can be partially purified by sodium bromide (28-38%) isopycnic gradient centrifugation (Pfannenstiel, M.A., E.J. Ross, V.C. Kramer, K.W. Nickerson [1984] *FEMS Microbiol. Lett.* 21:39). Thereafter the individual toxin proteins can be resolved by solubilizing the crystalline protein complex in an alkali buffer and fractionating the individual proteins by DEAE-sepharose CL-6B (Sigma Chem. Co., St. Louis, MO) chromatography by step-wise increments of increasing concentrations of an NaCl-containing buffer (Reichenberg, D., in *Ion Exchangers in Organic and Biochemistry* [C. Calmon and T.R.E. Kressman, eds.], Interscience, New York, 1957).

Fractions containing protein toxic for wireworms can be bound to PVDF membrane (Millipore, Bedford, MA) by western blotting techniques (Towbin, H., T. Staehelin, K. Gordon [1979] *Proc. Natl. Acad. Sci. USA* 76:4350) and the N-terminal amino acids determined by the standard Edman reaction with an automated gas-phase sequenator (Hunkapiller, M.W., R.M. Hewick, W.L. Dreyer, L.E. Hood [1983] *Meth. Enzymol.* 91:399).

From these sequence data oligonucleotide probes can be designed by utilizing a codon frequency table assembled from available sequence data of other *B.t.* toxin genes. Such sequence data is reported in the literature. The probes can be synthesized on an Applied Biosystems, Inc. DNA synthesis machine.

Example 3 – Molecular Cloning of Gene Encoding a Toxin from *Bacillus thuringiensis* Strain PS86A1

Total cellular DNA was prepared from PS86A1 cells grown to an optical density, at 600 nm, of 1.0. Cells were pelleted by centrifugation and resuspended in

protoplast buffer (20 mg/ml lysozyme in 0.3 M sucrose, 25 mM Tris-Cl, pH 8.0, 25 mM EDTA). After incubation at 37RC for 1 hour, protoplasts were lysed by two cycles of freezing and thawing. Nine volumes of a solution of 0.1 M NaCl, 0.1% SDS, 0.1 M Tris-Cl were added to complete lysis. The cleared lysate was extracted twice with phenol:chloroform (1:1). Nucleic acids were precipitated with two volumes of ethanol and pelleted by centrifugation. The pellet was resuspended in 10 mM Tris-Cl, 1 mM EDTA (TE), pH 8.0, and RNAse was added to a final concentration of 50 μg/ml. After incubation at 37RC for 1 hour, the solution was extracted once each with phenol:chloroform (1:1) and TE-saturated chloroform. DNA was precipitated from the aqueous phase by the addition of one-tenth volume of 3 M NaOAc and two volumes of ethanol. DNA was pelleted by centrifugation, washed with 70% ethanol, dried, and resuspended in TE.

Restriction fragment length polymorphism (RFLP) analyses were performed by standard hybridization of southern blots of PS86A1 DNA with a ^{32}P-labeled oligonucleotide probe designated as 86A1-A. The sequence of the 86A1-A probe was:

5Q ATG ATT GAT TCT AAA ACA ACA TTA CCA AGA CAT TCT/A TTA ATT/A CAT ACT/A ATT/A AA 3Q (SEQ ID NO. 3)

The probe was mixed at four positions, as shown. Hybridizing bands included an approximately 3.6 kbp *Hin*dIII fragment and an approximately 9.3 kbp *Eco*RV fragment.

A gene library was constructed from PS86A1 DNA partially digested with *Sau*3A. Partial restriction digests were fractionated by agarose gel electrophoresis. DNA fragments 6.6 to 23 kbp in size were excised from the gel, electroeluted from the gel slice, and recovered by ethanol precipitation after purification on an Elutip-D ion exchange column. The *Sau*3A inserts were ligated into *Bam*HI-digested LambdaGem-11 (Promega, Madison, WI). Recombinant phage were packaged and plated on *E. coli* KW251 cells (Promega). Plaques were screened by hybridization with the radiolabeled 86A1-A oligonucleotide probe. Hybridizing phage were plaque-purified and used to infect liquid cultures of *E. coli* KW251 cells for isolation of phage DNA by standard procedures (Maniatis *et al.* [1982] *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). For subcloning, preparative amounts of DNA were digested with *Eco*RI and *Sal*I, and

electrophoresed on an agarose gel. The approximately 2.9 kbp band containing the toxin gene was excised from the gel, electroeluted from the gel slice, and purified by ion exchange chromatography as above. The purified DNA insert was ligated into *Eco*RI + *Sal*I-digested pHTBlueII (an *E. coli/B.t.* shuttle vector comprised of pBlueScript S/K, Stratagene, San Diego, CA) and the replication origin from a resident *B.t.* plasmid (D. Lereclus *et al.* [1989] *FEMS Microbiol. Lett.* 60:211-218). The ligation mix was used to transform frozen, competent *E. coli* NM522 cells (ATCC 47000). Transformants were plated on LB agar (Maniatis *et al.*, *supra*) containing ampicillin, isopropyl-(β)-D-thiogalactoside (IPTG), and 5-bromo-4-chloro-3-indolyl-(β)-D-galactoside (XGAL). Plasmids were purified from putative recombinants by alkaline lysis (Maniatis *et al.*, *supra*) and analyzed by electrophoresis of *Eco*RI and *Sal*I digests on agarose gels. The desired plasmid construct, pMYC2320, contains the toxin gene of the invention. The DNA sequence of this gene is shown in SEQ ID NO. 1. The toxin expressed by this gene is shown in SEQ ID NO. 2.

Plasmid pMYC2320 was introduced into an acrystalliferous (Cry^-) *B.t.* host (*B.t.* HD-1 Cry B, A.I. Aronson, Purdue University, West Lafayatte, IN) by electroporation. Expression of an approximately 58 kDa protein is verified by SDS-PAGE analysis.

Plasmid pMYC2320 containing the *B.t.* toxin gene, can be removed from the transformed host microbe by use of standard well-known procedures. For example, *E. coli* NM522(pMYC2320) can be subjected to cleared lysate isopycnic density gradient procedures, and the like, to recover pMYC2320.

Example 4 — Restriction Fragment Length Polymorphism (RFLP) Analysis of δ-endotoxin Genes From *Bacillus thuringiensis* strain PS80JJ1

Total cellular DNA was prepared from *Bacillus thuringiensis* (*B.t.*) cells grown to an optical density, at 600 nm, of 1.0. Cells were pelleted by centrifugation and resuspended in protoplast buffer (20 mg/ml lysozyme in 0.3 M sucrose, 25 mM Tris-Cl (pH 8.0), 25 mM EDTA). After incubation at 37RC for 1 hour, protoplasts were lysed by two cycles of freezing and thawing. Nine volumes of a solution of 0.1 M NaCl, 0.1% SDS, 0.1 M Tris-Cl were added to complete lysis. The cleared lysate was extracted twice with phenol:chloroform (1:1). Nucleic acids were precipitated with

two volumes of ethanol and pelleted by centrifugation. The pellet was resuspended in TE buffer and RNase was added to a final concentration of 50 μg/ml. After incubation at 37RC for 1 hour, the solution was extracted once each with phenol:chloroform (1:1) and TE-saturated chloroform. DNA was precipitated from the aqueous phase by the addition of one-tenth volume of 3 M NaOAc and two volumes of ethanol. DNA was pelleted by centrifugation, washed with 70% ethanol, dried, and resuspended in TE buffer.

An approximately 700-800 bp DNA fragment from a novel PS80JJ1 130 kDa toxin gene was obtained by polymerase chain reaction (PCR) amplification using PS80JJ1 cellular DNA and the following primers:

5Q GGACCAGGATTTACAGG(TA)GG(AG)(AG)A 3Q (SEQ ID NO. 4)

5Q TAACGTGTAT(AT)CG(CG)TTTTAATTT(TA)GA(CT)TC 3Q

(SEQ ID NO. 5)

This DNA fragment was cloned into pBluescript S/K (Stratagene, LaJolla, CA) and partially sequenced by dideoxynucleotide DNA sequencing methodology (Sanger *et al.* [1977] *Proc. Natl. Acad. Sci. USA* 74:5463-5467) using Sequenase (USBiochemical, Cleveland, OH). DNA sequences unique to at least one PS80JJ1 toxin gene were identified by computer comparison with other known δ-endotoxin genes.

The 700-800 bp DNA fragment was radiolabled with ^{32}P and used in standard hybridizations of Southern blots of PS80JJ1 total cellular DNA. Hybridizing bands included an approximately 1.8 kbp *Eco*RI fragment and an approximately 9.5 kbp *Hin*dIII fragment. These hybridizing DNA bands contain toxin genes or restriction fragments of toxin genes from PS80JJ1.

Example 5 — Molecular Cloning and Expression of a Novel Toxin Gene from *Bacillus thuringiensis* Strain PS211B2

Total cellular DNA was prepared from *Bacillus thuringiensis* (*B.t.*) cells grown to an optical density of 1.0 at 600 nm. Cells were pelleted by centrifugation and resuspended in protoplast buffer (20 mg/ml lysozyme in 0.3 M sucrose, 35 mM Tris-Cl (pH 8.0), 25 mM EDTA). After incubation at 37RC for 1 hour, protoplasts were lysed by two cycles of freezing and thawing. Nine volumes of a solution of 0.1 M

NaCl, 0.1% SDS, 0.1 M Tris-Cl were added to complete lysis. The cleared lysate was extracted twice with phenol:chloroform (1:1). Nucleic acids were precipitated with two volumes of ethanol and pelleted by centrifugation. The pellet was resuspended in TE buffer and RNase was added to a final concentration of 30 μg/ml. After incubation at 37RC for 1 hour, the solution was extracted once each with phenol:chloroform (1:1) and TE-saturated chloroform. From the aqueous phase, DNA was precipitated by the addition of one-tenth volume of 3 M NaOAc and two volumes of ethanol. DNA was pelleted by centrifugation, washed with 70% ethanol, dried, and resuspended in TE buffer.

An approximately 300-bp-sized fragment of the novel 70 kDa toxin gene was obtained (SEQ ID NOS. 6 and 7) by polymerase chain reaction (PCR) amplification from PS211B2 cellular DNA using the following primers: "Forward": 5Q-GCAGGATCCGATTATATT(T or A)GATAT(T or A)A(C or G or A)TCC-3Q (SEQ ID NO. 8) and "Reverse": 5Q-GCGGCCGCACTTCATCTTC(T or A)GG(T or A)GCATT(T or A)GCATA(T or A)GTATC-3Q (SEQ ID NO. 9). This DNA fragment was cloned into pBLuescript II SK− (Stratagene, La Jolla, CA) and the DNA sequence determined by dideoxynucleotide sequencing methodology (Sanger *et al., supra*) using Sequenase (U.S. Biochemicals, Cleveland, OH). This fragment was subsequently radiolabelled with ^{32}P and used as a probe in standard hybridizations of Southern blots of PS211B2 total cellular DNA.

A gene library was constructed from PS211B2 DNA partially digested with *Nde*II. Partial restriction digests were fractionated by agarose gel electrophoresis. DNA fragments 9.3 to 23 kbp in size were excised from the gel, electroeluted from the gel slice, purified on an Elutip-D ion exchange column (Schleicher and Schuell, Keene, NH), and recovered by ethanol precipitation. The *Nde*II inserts were ligated into *Bam*HI-digested LambdaGem 11 (Promega, Madison, WI) cells. Plaques were screened by hybridization with the probe described above. Hybridizing phage were plaque-purified and used to infect liquid cultures of *E. coli* KW251 cells for isolation of DNA by standard procedures (Maniatis *et al., supra*).

For subcloning the gene encoding the 70 kDa toxin, preparative amounts of phage DNA were digested with *Sal*I. The approximately 16 kbp band was ligated into *Xho*I-digested pHTBlueII (an *E. coli/B. thuringiensis* shuttle vector comprised of

pBluescript II SK− (Stratagene) and the replication origin from a resident *B.t.* plasmid (D. Lereclus *et al., supra*). The ligation mix was used to transform frozen, competent *E. coli* NM522 cells (ATCC 47000). β-galactosidase transformants were screened by restriction digestion of alkaline lysate plasmid minipreps as above. The desired plasmid construct, pMYC2371, contains a toxin gene that is novel compared to other toxin genes containing insecticidal proteins.

pMYC2371 was introduced into the acrystalliferous (Cry$^-$) *B.t.* host, CryB (A. Aronson, Purdue University, West Lafayatte, IN), by electroporation. Expression of the toxin was demonstrated by SDS-PAGE analysis.

Example 6 – Soil Wireworm Bioassay

Pioneer 3475 field corn was used for the bioassay. A spray-dried powder of each *B. thuringiensis* strain was resuspended in water for treatment of the corn seeds using a Hege seed treater to ensure uniform coverage. The seeds were allowed to dry.

A sand and loam soil mixture was placed in the base of a 4P x 5P seed tray. Seeds were dispersed evenly over the soil surface, and wireworms were placed over the soil and seed surface. Another layer of the sand and loam soil mixture was spread over the top, and water was sprinkled over the soil surface.

Trays were placed in a 25RC growth chamber. After 7 days, the contents of each tray were washed out onto a 10 mesh sieve. Wireworms were assessed as (1) alive, (2) moribund, or (3) dead.

An untreated control and a chemical control (Lindane 30) were used as comparative standards.

Table 3

Strain	% live wireworms	% moribund wireworms	% dead wireworms
PS86A1	33.3	25	41.7
PS211B2	50	25	25
PS80JJ1	25	25	50
Untreated control	100	0	0
Lindane	0	25	75

Example 7 – Insertion of Toxin Genes Into Plants

One aspect of the subject invention is the transformation of plants with genes encoding a wireworm toxin. The transformed plants are resistant to attack by wireworms.

Genes encoding wireworm-active toxins, as disclosed herein, can be inserted into plant cells using a variety of techniques which are well known in the art. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, etc. Accordingly, the sequence encoding the *B.t.* toxin can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be cleaved and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted.

The use of T-DNA for the transformation of plant cells has been intensively researched and sufficiently described in EP 120 516; Hoekema (1985) In: *The Binary Plant Vector System*, Offset-durkkerij Kanters B.V., Alblasserdam, Chapter 5; Fraley *et al.*, *Crit. Rev. Plant Sci.* 4:1-46; and An *et al.* (1985) *EMBO J.* 4:277-287.

Once the inserted DNA has been integrated in the genome, it is relatively stable there and, as a rule, does not come out again. It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G 418, bleomycin, hygromycin, or chloramphenicol, *inter alia*. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA.

A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, or electroporation as well as other possible methods. If agrobacteria are used for the

transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the *vir* region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in agrobacteria. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in agrobacteria. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into agrobacteria (Holsters *et al.* [1978] *Mol. Gen. Genet.* 163:181-187). The agrobacterium used as host cell is to comprise a plasmid carrying a *vir* region. The *vir* region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can advantageously be cultivated with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

Example 8 – Cloning of Novel *B.t.* Genes Into Insect Viruses

A number of viruses are known to infect insects. These viruses include, for example, baculoviruses and entomopoxviruses. In one embodiment of the subject invention, lepidopteran-active genes, as described herein, can be placed with the

genome of the insect virus, thus enhancing the pathogenicity of the virus. Methods for constructing insect viruses which comprise *B.t.* toxin genes are well known and readily practiced by those skilled in the art. These procedures are described, for example, in Merryweather *et al.* (Merryweather, A.T., U. Weyer, M.P.G. Harris, M. Hirst, T. Booth, R.D. Possee [1990] *J. Gen. Virol. 71*:1535-1544) and Martens *et al.* (Martens, J.W.M., G. Honee, D. Zuidema, J.W.M. van Lent, B. Visser, J.M. Vlak [1990] *Appl. Environmental Microbiol. 56*(9):2764-2770).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: MYCOGEN CORPORATION

(ii) TITLE OF INVENTION: Materials and Methods for the Control of Wireworms

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: KYLE, JEAN
(B) STREET: 2421 N.W. 41st Street, Suite A-1
(C) CITY: Gainesville
(D) STATE: FL
(E) COUNTRY: USA
(F) ZIP: 32606

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: KYLE, JEAN
(B) REGISTRATION NUMBER: 36,987
(C) REFERENCE/DOCKET NUMBER: MA80

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 904-375-8100
(B) TELEFAX: 904-372-5800

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1425 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: BACILLUS THURINGIENSIS
(C) INDIVIDUAL ISOLATE: PS86A1

(vii) IMMEDIATE SOURCE:
(B) CLONE: E. coli NM522(pMYC2320) NRRL B-18769

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 1..1425

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GAACAACAAT TAAGAACACA TGTTAATTTA AGTCAGGATA TATCAATACC TAGTGATTTT     240

TCTCAATTAT ATGATGTTTA TTGTTCTGAT AAAACTTCAG CAGAATGGTG GAATAAAAAT     300
TTATATCCTT TAATTATTAA ATCTGCTAAT GATATTGCTT CATATGGTTT TAAAGTTGCT     360

GGTGATCCTT CTATTAAGAA AGATGGATAT TTTAAAAAAT TGCAAGATGA ATTAGATAAT     420

ATTGTTGATA ATAATTCCGA TGATGATGCA ATAGCTAAAG CTATTAAAGA TTTTAAAGCG     480

CGATGTGGTA TTTTAATTAA AGAAGCTAAA CAATATGAAG AAGCTGCAAA AAATATTGTA     540

ACATCTTTAG ATCAATTTTT ACATGGTGAT CAGAAAAAAT TAGAAGGTGT TATCAATATT     600

CAAAAACGTT TAAAAGAAGT TCAAACAGCT CTTAATCAAG CCCATGGGGA AAGTAGTCCA     660

GCTCATAAAG AGTTATTAGA AAAAGTAAAA AATTTAAAAA CAACATTAGA AAGGACTATT     720

AAAGCTGAAC AAGATTTAGA GAAAAAAGTA GAATATAGTT TTCTATTAGG ACCATTGTTA     780

GGATTTGTTG TTTATGAAAT TCTTGAAAAT ACTGCTGTTC AGCATATAAA AAATCAAATT      840

GATGAGATAA AGAAACAATT AGATTCTGCT CAGCATGATT TGGATAGAGA TGTTAAAATT     900

ATAGGAATGT TAAATAGTAT TAATACAGAT ATTGATAATT TATATAGTCA AGGACAAGAA     960

GCAATTAAAG TTTTCCAAAA GTTACAAGGT ATTTGGGCTA CTATTGGAGC TCAAATAGAA    1020

AATCTTAGAA CAACGTCGTT ACAAGAAGTT CAAGATTCTG ATGATGCTGA TGAGATACAA    1080

ATTGAACTTG AGGACGCTTC TGATGCTTGG TTAGTTGTGG CTCAAGAAGC TCGTGATTTT    1140

ACACTAAATG CTTATTCAAC TAATAGTAGA CAAAATTTAC CGATTAATGT TATATCAGAT    1200

TCATGTAATT GTTCAACAAC AAATATGACA TCAAATCAAT ACAGTAATCC AACAACAAAT    1260

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 475 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: BACILLUS THURINGIENSIS
(C) INDIVIDUAL ISOLATE: PS86A1

(vii) IMMEDIATE SOURCE:
(B) CLONE: E. coli NM522(pMYC2320) NRRL B-18769

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..475

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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20 25 30

Asn Gly Asn Gln Phe Ile Ile Ser Lys Gln Glu Trp Ala Thr Ile Gly
35 40 45

Ala Tyr Ile Gln Thr Gly Leu Gly Leu Pro Val Asn Glu Gln Gln Leu
50 55 60
Arg Thr His Val Asn Leu Ser Gln Asp Ile Ser Ile Pro Ser Asp Phe
65 70 75 80

Ser Gln Leu Tyr Asp Val Tyr Cys Ser Asp Lys Thr Ser Ala Glu Trp
85 90 95

Trp Asn Lys Asn Leu Tyr Pro Leu Ile Ile Lys Ser Ala Asn Asp Ile
100 105 110

Ala Ser Tyr Gly Phe Lys Val Ala Gly Asp Pro Ser Ile Lys Lys Asp
115 120 125

Gly Tyr Phe Lys Lys Leu Gln Asp Glu Leu Asp Asn Ile Val Asp Asn
130 135 140

Asn Ser Asp Asp Asp Ala Ile Ala Lys Ala Ile Lys Asp Phe Lys Ala
145 150 155 160

Arg Cys Gly Ile Leu Ile Lys Glu Ala Lys Gln Tyr Glu Glu Ala Ala
165 170 175

Lys Asn Ile Val Thr Ser Leu Asp Gln Phe Leu His Gly Asp Gln Lys
180 185 190

Lys Leu Glu Gly Val Ile Asn Ile Gln Lys Arg Leu Lys Glu Val Gln
195 200 205

Thr Ala Leu Asn Gln Ala His Gly Glu Ser Ser Pro Ala His Lys Glu
210 215 220

Leu Leu Glu Lys Val Lys Asn Leu Lys Thr Thr Leu Glu Arg Thr Ile
225 230 235 240

Lys Ala Glu Gln Asp Leu Glu Lys Lys Val Glu Tyr Ser Phe Leu Leu
245 250 255

Gly Pro Leu Leu Gly Phe Val Val Tyr Glu Ile Leu Glu Asn Thr Ala
260 265 270

Val Gln His Ile Lys Asn Gln Ile Asp Glu Ile Lys Lys Gln Leu Asp
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Ser Ala Gln His Asp Leu Asp Arg Asp Val Lys Ile Ile Gly Met Leu
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Asn Ser Ile Asn Thr Asp Ile Asp Asn Leu Tyr Ser Gln Gly Gln Glu
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Ala Ile Lys Val Phe Gln Lys Leu Gln Gly Ile Trp Ala Thr Ile Gly
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Ala Trp Leu Val Val Ala Gln Glu Ala Arg Asp Phe Thr Leu Asn Ala
    370                 375                 380

Tyr Ser Thr Asn Ser Arg Gln Asn Leu Pro Ile Asn Val Ile Ser Asp
385                 390                 395                 400

Ser Cys Asn Cys Ser Thr Thr Asn Met Thr Ser Asn Gln Tyr Ser Asn
                405                 410                 415

Pro Thr Thr Asn Met Thr Ser Asn Gln Tyr Met Ile Ser His Glu Tyr
            420                 425                 430

Thr Ser Leu Pro Asn Asn Phe Met Leu Ser Arg Asn Ser Asn Leu Glu
        435                 440                 445

Tyr Lys Cys Pro Glu Asn Asn Phe Met Ile Tyr Trp Tyr Asn Asn Ser
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus thuringiensis
(B) STRAIN: PS86A1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGATTGATT CTAAAACAAC ATTACCAAGA CATTCWTTAA TWCATACWAT WAA 53

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGACCAGGAT TTACAGGWGG RRA 23

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 309 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..307

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
G ATT TTT CAA ACA AAT GGA TCT GCT ACA TTT AAT TCT AAT ACC AAT          46
  Ile Phe Gln Thr Asn Gly Ser Ala Thr Phe Asn Ser Asn Thr Asn
    1               5                  10                  15

ATT ACA ACT TTA ACA CAA GCT ATA AAT AGT CAA GCA GGA GCA ATT GCA        94
Ile Thr Thr Leu Thr Gln Ala Ile Asn Ser Gln Ala Gly Ala Ile Ala
                20                  25                  30

GGA AAG ACT GCT CTA GAT ATG AGA CAT GAC TTT ACT TTT AGA GCA GAT       142
Gly Lys Thr Ala Leu Asp Met Arg His Asp Phe Thr Phe Arg Ala Asp
            35                  40                  45

ATT TTT CTT GGA ACT AAA AGT AAC GGA GCA GAC GGT ATT GCA ATC GCA       190
Ile Phe Leu Gly Thr Lys Ser Asn Gly Ala Asp Gly Ile Ala Ile Ala
        50                  55                  60

TTT CAT AGA GGA TCA ATT GGG TTT GTT GGA ACA AAA GGC GGA GGA CTT       238
Phe His Arg Gly Ser Ile Gly Phe Val Gly Thr Lys Gly Gly Gly Leu
    65                  70                  75

GGA ATA TTA GGT GCA CCT AAA GGG ATA GGG TTT GAA TTA GAT ACT TAT       286
Gly Ile Leu Gly Ala Pro Lys Gly Ile Gly Phe Glu Leu Asp Thr Tyr
80                  85                  90                  95

GCA AAT GCA CCT GAA GAT GAA GT                                        309
Ala Asn Ala Pro Glu Asp Glu
                100
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 102 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Ile Phe Gln Thr Asn Gly Ser Ala Thr Phe Asn Ser Asn Thr Asn Ile
  1               5                  10                  15

Thr Thr Leu Thr Gln Ala Ile Asn Ser Gln Ala Gly Ala Ile Ala Gly
            20                  25                  30
```

Lys Thr Ala Leu Asp Met Arg His Asp Phe Thr Phe Arg Ala Asp Ile
35 40 45

Phe Leu Gly Thr Lys Ser Asn Gly Ala Asp Gly Ile Ala Ile Ala Phe
50 55 60

His Arg Gly Ser Ile Gly Phe Val Gly Thr Lys Gly Gly Gly Leu Gly
65 70 75 80

Ile Leu Gly Ala Pro Lys Gly Ile Gly Phe Glu Leu Asp Thr Tyr Ala
85 90 95

Asn Ala Pro Glu Asp Glu
100

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCAGGATCCG ATTATATTWG ATATWAVTCC 30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCGGCCGCAC TTCATCTTCW GGWGCATTWG CATAWGTATC 40

Claims

1. A method for controlling wireworms which comprises contacting said wireworms with a wireworm-controlling amount of a *Bacillus thuringiensis* strain selected from the group consisting of *B.t.* PS211B2, *B.t.* PS86A1, and *B.t.* PS80JJ1, and variants or toxins of said microbes which have activity against wireworms.

2. The method, according to claim 1, wherein said microbe is *Bacillus thuringiensis* PS211B2, having an accession number NRRL B-18921.

3. The method, according to claim 1, wherein said microbe is *Bacillus thuringiensis* PS86A1, having an accession number NRRL B-18400.

4. The method, according to claim 1, wherein said microbe is *Bacillus thuringiensis* PS80JJ1, having an accession number NRRL B-18679.

5. A composition of matter for controlling wireworms comprising a *Bacillus thuringiensis* strain selected from the group consisting of *B.t.* PS211B2, *B.t.* PS86A1, and *B.t.* PS80JJ1, and variants or toxins of said microbes which have activity against wireworms, in association with an inert carrier.

6. A method for controlling wireworms which comprises contacting said wireworms with a wireworm-controlling amount of a pesticidal composition comprising intact treated cells having prolonged pesticidal activity when applied to the environment of wireworm larvae, wherein said insecticide is produced by a *Bacillus thuringiensis* gene obtainable from a *B.t.* selected from the group consisting of *B.t.* PS211B2, *B.t.* PS86A1, and *B.t.* PS80JJ1, and variants or toxins of said microbes which have activity against wireworms.

7. A polynucleotide sequence comprising DNA wherein said DNA encodes a toxin which is active against wireworms and wherein said toxin, or a variant thereof, is obtainable from a *Bacillus thuringiensis* strain selected from the group consisting of *B.t.* PS211B2, *B.t.* PS86A1, *B.t.* PS80JJ1, and variants thereof.

8. The polynucleotide sequence, according to claim 7, comprising DNA obtainable from *Bacillus thuringiensis* PS80JJ1 having a fragment selected from the group consisting of a *Hin*dIII fragment of approximately 9.5 kbp and an *Eco*RI fragment of approximately 1.8 kbp wherein said fragment hybridizes with a 700 to 800 bp DNA sequence produced by PCR amplification of PS80JJ1 DNA utilizing SEQ ID NO. 4 as a forward primer and SEQ ID NO. 5 as a reverse primer.

9. The polynucleotide sequence, according to claim 7, comprising DNA which encodes all or part of SEQ ID NO. 7.

10. The polynucleotide sequence, accoding to claim 9, comprising all or part of the nucleotide sequence shown in SEQ ID NO. 6.

11. The polynucleotide sequence, according to claim 7, comprising DNA which encodes all or part of SEQ ID NO. 2.

12. The polynucleotide sequence, according to claim 11, comprising DNA comprising all or part of SEQ ID NO. 1.

13. A toxin which is active against wireworms and which is encoded by a polynucleotide sequence of claim 7.

14. The toxin, according to claim 13, wherein said toxin is encoded by a polynucleotide sequence of claim 8.

15. The toxin, according to claim 13, wherein said toxin comprises all or part of the amino acid sequence of SEQ ID NO. 2.

16. The toxin, according to claim 13, wherein said toxin comprises all or part of the amino acid sequence shown in SEQ ID NO. 7.

17. A plant cell transformed by a polynucleotide sequence of claim 7.

18. A microbe transformed by a polynucleotide sequence of claim 7.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 94/03308

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/32 A01N63/00 C12N15/82

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 5 C07K C12N A01N

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	EP,A,0 500 311 (MYCOGEN CORPORATION) 26 August 1992 see page 3, line 35 - line 42 see examples 4,5 see SEQ IDs 1, 3 and 4, and figure 3 ---	5,11,12, 15
X	WO,A,93 04587 (MYCOGEN CORPORATION) 18 March 1993 see page 4, line 26 - line 27; table 1 see examples 4,8 see SEQ IDs 5, 6 and 7 ---	5,11,12, 15
X	EP,A,0 516 306 (MYCOGEN CORPORATION) 2 December 1992 see page 3, line 15 - line 23; table 2 see examples 2,3 --- -/--	5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 July 1994	22. 07. 94

Name and mailing address of the ISA	Authorized officer
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	Andres, S

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 462 721 (MYCOGEN CORPORATION) 27 December 1991 see page 12, line 45 - page 13, line 35 see examples 6,10 see SEQ IDs 7 and 8 see claims	5,11,12, 15

INTERNATIONAL SEARCH REPORT

...information on patent family members

Inter ...nal Application No
PCT/US 94/03308

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		NZ-A-	242560	26-01-94
		WO-A-	9219739	12-11-92