

(12) PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. **AU 199867040 B2**
(10) Patent No. **740906**

(54) Title
Pesticidal bacillus thuringiensis strains

(51)⁶ International Patent Classification(s)
C12N 015/32 C12N 001/20
A01N 063/00 C12N 015/82
C07K 014/325

(21) Application No: **199867040**

(22) Application Date: **1998.03.13**

(87) WIPO No: **WO98/40491**

(30) Priority Data

(31) Number (32) Date
60/040416 1997.03.13

(33) Country
US

(43) Publication Date : **1998.09.29**

(43) Publication Journal Date : **1998.11.12**

(44) Accepted Journal Date : **2001.11.15**

(71) Applicant(s)
Mycogen Corporation

(72) Inventor(s)
Gregory A. Bradfisch; Brian Stockhoff; Judy Muller-Cohn

(74) Agent/Attorney
SPRUSON and FERGUSON, GPO Box 3898, SYDNEY NSW 2001

(56) Related Art
EP 462721
US 4849217
US 5262158

OPI DATE 29/09/98 APPLN. ID 67040/98
AOJP DATE 12/11/98 PCT NUMBER PCT/US98/05185



AU9867040

ST)

(51) International Patent Classification 6: C12N 15/32, 15/82, C07K 14/325, A01N 63/00, C12N 1/20		A2	(11) International Publication Number: WO 98/40491
			(43) International Publication Date: 17 September 1998 (17.09.98)
(21) International Application Number: PCT/US98/05185 (22) International Filing Date: 13 March 1998 (13.03.98) (30) Priority Data: 60/040,416 13 March 1997 (13.03.97) US (71) Applicant: MYCOGEN CORPORATION [US/US]; 5501 Oberlin Drive, San Diego, CA 92121 (US). (72) Inventors: BRADFISCH, Gregory, A.; 17046 Palacia Place, San Diego, CA 92127 (US). STOCKHOFF, Brian; 11771 Ramsdel Court, San Diego, CA 92131 (US). MULLER-COHN, Judy; 12744 Via Donada, Del Mar, CA 92014 (US). (74) Agents: SANDERS, Jay, M. et al.; Saliwanchik, Lloyd & Saliwanchik, Suite A-1, 2421 N.W. 41st Street, Gainesville, FL 32606 (US).			(81) Designated States: AU, BR, CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published Without international search report and to be republished upon receipt of that report.
(54) Title: PESTICIDAL BACILLUS THURINGIENSIS STRAINS			
(57) Abstract This application pertains to <i>Bacillus thuringiensis</i> isolates and their toxins active against various lepidopteran, coleopteran or aphid pests. The isolates are PS17, PS28Q2, PS33F2, PS32B, PS54G2, PS50C, PS71M3, PS86BB1, PS86Q3, PS14OE2, PS169E, PS62B, PS158C2, PS167P, PS196S1, PS201T6, HD511, PS18, PS28K1, PS43A2, PS19E6, PS164H2, PS186EE, PS196Q3, PS198A2, PS225K1, KB6, KB19, HD977 and HD541.			

DESCRIPTION

PESTICIDAL BACILLUS THURINGIENSIS STRAINS

5

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 inclusions often appear microscopically as distinctively shaped crystals. The proteins can be highly toxic to pests and specific in their toxic activity. Certain *B.t.* toxin genes have been isolated and sequenced, and recombinant DNA-based *B.t.* products have been produced and approved for use. In addition, with the use of genetic engineering techniques, new approaches for delivering these *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.

Until the last fifteen 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 crystalline δ -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 *morrisoni* (a.k.a. *tenebrionis*, a.k.a. *B.t.* M-7, a.k.a. *B.t. san diego*), 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; and 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 *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*.

More 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 four 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). CryV has been proposed to designate a class of toxin genes that are nematode-specific. Lambert *et al.* (Lambert, B., L. Buysse, C. Decock, S. Jansens, C. Piens, B. Saey, J. Seurinck, K. van Audenhove, J. Van Rie, A. Van Vliet, M. Peferoen [1996] *Appl. Environ. Microbiol.* 62(1):80-86) describe the characterization of a Cry9 toxin active against lepidopterans. Published PCT applications WO 94/05771 and WO 94/24264 also describe *B.t.* isolates active against lepidopteran pests. Gleave *et al.* ([1991] *JGM* 138:55-62), Shevelev *et al.* ([1993] *FEBS Lett.* 336:79-82; and Smulevitch *et al.* ([1991] *FEBS Lett.* 293:25-26) also describe *B.t.* toxins. Many other classes of *B.t.* genes have now been identified.

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. Whiteley [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 4,990,332; 5,039,523; 5,126,133; 5,164,180; and 5,169,629 are among those which disclose *B.t.* toxins having activity against lepidopterans. PCT application WO96/05314 discloses PS86W1, PS86V1, and other *B.t.* isolates active against lepidopteran pests. The PCT patent applications published as WO94/24264 and WO94/05771 describe *B.t.* isolates and toxins active against lepidopteran pests. *B.t.* proteins with activity against members of the family Noctuidae are described by Lambert *et al., supra*. U.S. Patents 4,797,276 and 4,853,331 disclose *B. thuringiensis* strain *tenebrionis* which can be used to control coleopteran pests in various environments. U.S. Patent No. 4,918,006 discloses *B.t.* toxins having activity against dipterans. U.S. Patent No. 5,151,363 and U.S. Patent No. 4,948,734 disclose certain isolates of *B.t.* which have activity against nematodes. Other U.S. patents which disclose activity against nematodes include 5,093,120; 5,236,843; 5,262,399; 5,270,448; 5,281,530; 5,322,932; 5,350,577; 5,426,049; and 5,439,881. U.S. Patent Nos. 5,262,159 and 5,468,636 disclose *B.t.* isolates PS157C1, PS86A1, and PS75J1 as having activity against aphids.

As a result of extensive research and investment of resources, other patents have issued for new *B.t.* isolates and new uses of *B.t.* isolates. See Feitelson *et al., supra*, for a review. However, the discovery of new *B.t.* isolates and new uses of known *B.t.* isolates remains an

empirical, unpredictable art. U.S. Patent No. 5,506,099 describes methods for identifying unknown *B.t.* isolates. Also, U.S. Patent No. 5,204,237 describes specific and universal probes for the isolation of *B.t.* toxin genes.

Brief Summary of the Invention

5 The subject invention concerns materials and methods useful in the control of non-mammalian pests and particularly, plant pests.

According to one embodiment of the invention, there is provided a method for controlling a lepidopteran pest, wherein said method comprises contacting said pest with a lepidopteran-active *Cry5A* toxin obtainable from a *Bacillus thuringiensis* isolate
10 selected from the group consisting of PS17 and PS86Q3.

According to another embodiment of the invention, there is provided the use of a transformed cell which expresses a lepidopteran-active *Cry5A* toxin obtainable from a *Bacillus thuringiensis* isolate selected from the group consisting of PS17 and PS86Q3 for controlling a lepidopteran pest.

15 According to a preferred aspect of these embodiments, the toxin is obtainable from *Bacillus thuringiensis* isolate PS17, and even more preferably, the toxin is a *Cry5Aa* toxin.

According to another preferred aspect of these embodiments, the toxin is obtainable from *Bacillus thuringiensis* isolate PS86Q3, and even more preferably, the toxin is a
20 *Cry5Ac* toxin.

In preferred aspects, the pests are selected from the group consisting of diamondback moth, Bertha armyworm, tobacco budworm, and band sunflower moth.

Also disclosed herein are new isolates and toxins useful for the control of lepidopterans.

25 Nucleotide sequences useful according to the subject invention encode pesticidal toxins. Thus, the subject invention also concerns plants cells transformed with at least one polynucleotide sequence encoding pesticidal toxins such that the transformed plant cells express pesticidal toxins in tissues consumed by target pests. Such transformation of plants can be accomplished using techniques well known to those skilled in the art and
30 would typically involve modification of the gene to optimize expression of the toxin in plants.

As described herein, the toxins useful according to the subject invention may be chimeric toxins produced by combining portions of multiple toxins. Also, toxins of the subject invention may be used in combination to achieve enhanced pest control.



Alternatively, the *B.t.* isolates described herein, or recombinant microbes expressing the toxins described herein, can be used to control pests. In this regard, the invention includes the treatment of substantially intact *B.t.* cells, and/or recombinant cells containing the expressed toxins of the invention, treated to prolong the pesticidal activity
5 when the substantially intact cells are applied to the environment of a target pest. The treated cell acts as a protective coating for the pesticidal toxin. The toxin becomes active upon ingestion by a target insect.

Detailed Disclosure of the Invention

10 Herein disclosed are *B.t.* isolates and toxins active against diamondback moth, Bertha armyworm, tobacco budworm, band sunflower moth, sunflower beetle, red sunflower seed weevil, canola flea beetle, sunflower stem weevil, and/or greenbug.

Also disclosed herein are novel *B.t.* isolates and the toxins and genes obtainable from these isolates. The novel *B.t.* isolates described herein have been

3
2
1
0

3
2
1
0



designated PS18, PS28K1, PS43A2, PS159E6, PS164H2, PS186EE, PS196Q3, PS198A2, and PS225K1.

Microorganisms useful according to the subject invention have been deposited in the permanent collection of the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 North University Street, Peoria, Illinois 61604, USA. The culture repository numbers of the deposited strains are shown in Table 1.

Table 1.			
	Culture	Repository No.	Deposit Date
10	PS18	NRRL B-21954	March 12, 1998
	PS28K1	NRRL B-21955	March 12, 1998
	PS43A2	NRRL B-21956	March 12, 1998
	PS159E6	NRRL B-21958	March 12, 1998
	PS164H2	NRRL B-21959	March 12, 1998
15	PS186EE	NRRL B-21960	March 12, 1998
	PS196Q3	NRRL B-21961	March 12, 1998
	PS198A2	NRRL B-21962	March 12, 1998
	PS225K1	NRRL B-21963	March 12, 1998
	KB6	NRRL B-18873	August 27, 1991
20	KB19	NRRL B-21964	March 12, 1998

The isolates of the subject invention 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 U.S.C. 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 isolates of the subject invention 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 a

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 deposit(s) 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.

Certain isolates which are useful according to the subject invention are available to the public by virtue of the issuance of U.S. patents. These isolates, their deposit accession number, and their date of deposit are shown in Table 2(a). The corresponding U.S. patents that disclose these isolates are shown in Table 2(b).

Table 2(a). Pesticidal *B.t.* strains of the subject invention

Culture	NRRL Deposit	Deposit Date
PS17	B-18243	10 AUG 87
PS28Q2	B-18888	25 SEP 91
PS32B	B-21531	1 APR 96
PS33F2	B-18244	10 AUG 87
PS50C	B-18746	23 JAN 91
PS54G2	B-21543	1 APR 96
PS62B	B-18398	7 SEP 88
PS71M3	B-18930	8 JAN 92
PS80JJ1	B-18679	1 AUG 90
PS86BB1	B-21557	2 MAY 96
PS86Q3	B-18765	13 FEB 91
PS140E2	B-18812	10 APR 91
PS158C2	B-18872	17 SEP 91
PS167P	B-18681	1 AUG 90
PS169E	B-18682	1 AUG 90
PS196S1	B-18748	23 JAN 91
PS201T6	B-18750	23 JAN 91

The isolates HD511, HD541, and HD977 are available from the USDA-ARS NRRL Culture Collection, Peoria, Illinois.

Table 2(b)

	U.S. Patent No. or Publication No.	Isolate Disclosed	Sequences (of Toxin(s) and Gene(s)) Disclosed	Pesticidal Activity Disclosed
5	4,849,217	PS17		alfalfa weevils
	5,281,530	PS17	17(a) and 17(b)	nematodes
	5,427,786	PS28Q2		certain coleopteran genera, including <i>Hypera</i> , <i>Diabrotica</i> , and <i>Phyllotreta</i>
10	4,849,217	PS33F2		alfalfa weevils
	5,439,881	PS33F2	33F2	nematodes
	5,707,619	PS33F2		various weevils
	5,670,365	PS32B		nematodes
	5,277,905	PS50C		coleopterans
	5,366,892	PS50C	50C	coleopterans
	5,185,148	PS50C		scarabs
	5,457,179	PS50C		coleopterans
15	5,554,534	PS50C	50C(a) and 50C(b)	scarabs
	5,667,993	PS54G2		nematodes
	5,670,365	PS54G2		nematodes
	4,849,217	PS62B		alfalfa weevils
	5,723,440	PS71M3		hemipterans
20	4,849,217	PS80JJ1		alfalfa weevils
	WO94/23036	PS80JJ1		wireworms (click beetles)
	5,632,987	PS80JJ1	80JJ1 (130kda)	corn rootworm
	5,589,382	PS80JJ1	80JJ1 (130kda)	nematodes
25	5,670,365	PS80JJ1	80JJ1 (130kda)	nematodes
	WO 97/40162	PS80JJ1	80JJ1 (14 and 44kda)	coleopterans
	WO 98/00546	PS86BB1	86BB1(a), 86BB1(b), and 86BB1(c)	lepidopterans
	5,596,071	PS86Q3	86Q3(a) and 86Q3(c)	ants

Table 2(b)			
U.S. Patent No. or Publication No.	Isolate Disclosed	Sequences (of Toxin(s) and Gene(s)) Disclosed	Pesticidal Activity Disclosed
5,427,786	PS140E2		certain coleopteran genera, including <i>Hypera</i> , <i>Diabrotica</i> , and <i>Phyllotreta</i>
5,596,071	PS140E2		ants
5,268,172	PS158C2		lepidopterans
5,723,758	PS158C2	158C2(a), 158c2(b), 158C2(c), and 158C2(d)	lepidopterans
5	5,632,987	PS167P	corn rootworms
	5,707,619	PS167P	various weevils
	5,589,382	PS167P 167P	nematodes
	5,670,365	PS167P 167P	nematodes
	5,632,987	PS169E	corn rootworm
10	5,707,619	PS169E	various weevils
	5,436,002	PS196S1	dipterans and corn rootworm
	5,707,619	PS196S1	various weevils
	5,635,480	PS196S1	dipterans and corn rootworm
	5,436,002	PS201T6 201T6 (30 & 25 kda)	dipterans and corn rootworm
15	5,707,619	PS201T6	various weevils
	5,635,480	PS201T6 201T6 (30 & 25 kda)	dipterans and corn rootworm
	5,723,440	PS201T6	hemipterans
	5,262,324	HD511	coleopterans
	5,286,486	HD511 HD511	coleopterans
20	5,306,494	HD511	coleopterans

These patents, with their disclosure of the indicated isolates as well as their toxins and genes, are incorporated herein by reference.

Genes and toxins. The genes and toxins useful according to the subject invention include not only the full length sequences disclosed but also fragments of these sequences, variants, mutants, and fusion proteins which retain the characteristic pesticidal activity of the toxins specifically exemplified herein. As used herein, the terms "purified toxin" and "isolated toxin" refer to toxins that have been affected by "the hand of man" and that are substantially free from naturally associated impurities, as the toxins would be found in their natural state. As used herein, the terms "variants" or "variations" of genes refer to nucleotide sequences which encode the same toxins or which encode equivalent toxins having pesticidal activity. As used herein, the term "equivalent toxins" refers to toxins having the same or essentially the same biological activity against the target pests as the exemplified toxins.

It should be apparent to a person skilled in this art that genes encoding 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 synthesizer. Variations of 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 *Bal31* or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes which encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these toxins.

Equivalent toxins and/or genes encoding these equivalent toxins can be derived 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.

Fragments and equivalents 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 sequences 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 pesticidal activity are also included in this definition.

A further method for identifying the toxins and genes of the subject invention is through the use of oligonucleotide probes. These probes are detectable nucleotide sequences. These sequences may be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO93/16094. 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. Preferably, hybridization is conducted under stringent conditions by techniques well-known in the art, as described, for example, in Keller, G.H., M.M. Manak (1987) *DNA Probes*, Stockton Press, New York, NY., pp. 169-170. Detection of the probe 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 using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

Certain toxins of the subject invention have been specifically exemplified herein. Since these toxins are merely exemplary of the toxins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent toxins (and nucleotide sequences coding for equivalent toxins) having the same or similar pesticidal activity of the exemplified toxin. Equivalent toxins will have amino acid homology with an exemplified toxin. This amino acid identity will typically be greater than 60%, preferably be greater than 75%, more preferably greater than 80%, more preferably greater than 90%, and can be greater than 95%. The amino acid homology will be highest in 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, 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 3 provides a listing of examples of amino acids belonging to each class.

TABLE 3

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 the pest, where they will proliferate and be ingested. The result is a control of the pest. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin and stabilize the cell. The treated cell, which retains the toxic activity, then can be applied to the environment of the target pest.

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.

Alternatively, a plant transformed to express a toxin of the subject invention can be used to contact the target pest with the toxin. Synthetic genes which are functionally equivalent to the toxins of the subject invention can also be used to transform hosts. Methods for the production of synthetic genes can be found in, for example, U.S. Patent No. 5,380,831.

Treatment of cells. As mentioned above, *B.t.* or recombinant cells expressing a *B.t.* toxin can be treated to prolong the toxin activity and stabilize the cell. The pesticide microcapsule that is formed comprises the *B.t.* toxin within a cellular structure that has been stabilized and will protect the toxin when the microcapsule is applied to the environment of the

target pest. Methods for treatment of microbial cells are disclosed in United States Patent Nos. 4,695,455 and 4,695,462, which are incorporated herein by reference.

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.

Methods and formulations for pest control. Control of pests using the isolates, toxins, and genes of the subject invention can be accomplished by a variety of methods known to those skilled in the art. These methods include, for example, the application of *B.t.* isolates to the pests (or their location), the application of recombinant microbes to the pests (or their locations), and the transformation of plants with genes which encode the pesticidal toxins of the subject invention. Recombinant microbes may be, for example, a *B.t.*, *E. coli*, or *Pseudomonas*. Transformations can be made by those skilled in the art using standard techniques. Materials necessary for these transformations are disclosed herein or are otherwise readily available to the skilled artisan.

Formulated bait granules containing an attractant and spores and crystals of the *B.t.* isolates, or recombinant microbes comprising the genes 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. Plant and soil treatments of *B.t.* cells may be employed as liquids, wettable powders, granules or dusts, by mixing with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, and the like).

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 about 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 pest, *e.g.*, soil and foliage, by spraying, dusting, sprinkling, or the like.

Mutants. Mutants of the 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 an isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

All of the U.S. patents cited herein are hereby incorporated by reference.

Following are examples which illustrate procedures 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 of *B.t.* Isolates of the Invention

A subculture of the *B.t.* isolates, or mutants thereof, can be used to inoculate the following medium, a peptone, glucose, salts medium.

	Bacto Peptone	7.5 g/l
	Glucose	1.0 g/l
	KH ₂ PO ₄	3.4 g/l
25	K ₂ HPO ₄	4.35 g/l
	Salt Solution	5.0 ml/l
	CaCl ₂ Solution	5.0 ml/l
	pH 7.2	
30	Salts Solution (100 ml)	
	MgSO ₄ ·7H ₂ O	2.46 g
	MnSO ₄ ·H ₂ O	0.04 g
	ZnSO ₄ ·7H ₂ O	0.28 g
	FeSO ₄ ·7H ₂ O	0.40 g

CaCl₂ Solution (100 ml)

CaCl₂·2H₂O 3.66 g

The salts solution and CaCl₂ solution are filter-sterilized and added to the autoclaved and cooked broth at the time of inoculation. Flasks are incubated at 30°C on a rotary shaker at 200 rpm for 64 hr.

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

The *B.t.* spores and/or 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.

The supernatant from these cultures can be used to obtain toxins according to the subject invention. Thus, the subject invention is not limited to crystal proteins; useful soluble proteins are also contemplated.

Example 2 – Activity Against Diamond Back Moth (*Plutella xylostella*)

Certain *B.t.* isolates and toxins were found to be active against diamond back moth. These results are shown in Table 4.

Table 4.	
Strain	Toxin
PS86Q3	Cry5Ac
PS17	Cry5Aa
HD511	Cry7Ab2

Example 3 – Activity Against Bertha Armyworm (*Mamestra configurata*)

Certain *B.t.* isolates and toxins were found to be active against Bertha armyworm. These results are shown in Table 5.

Table 5.

Strain	Toxin
HD511	Cry7Ab
PS86Q3	Cry5AC

Example 4 – Activity Against Tobacco Budworm (*Heliothis virescens*)

Certain *B.t.* isolates and toxins were found to be active against tobacco budworm. These results are shown in Table 6.

Table 6.

Strain	Toxin
PS86Q3	Cry5Ac
PS17	Cry5Aa

Example 5 – Activity Against Band Sunflower Moth (*Cochylis hospes*)

Certain *B.t.* isolates and toxins have been found to be active against banded sunflower moth. The results are shown in Table 7.

Table 7.

Strain	Gene
PS17	Cry5Aa

Example 6 – Activity Against Sunflower Beetle (*Zygogramma exclamationis*)

Certain *B.t.* isolates and toxins have been found to be active against sunflower beetle. These results are shown in Table 8.

Table 8.

Strain	Toxin
PS80JJ1	80JJ1
PS50C	Cry8B
PS158C2	158C2(c)

The sequence of a 1227 amino acid 158C2(c) toxin is disclosed as SEQ ID NO. 8 in U.S. Patent No. 5,723,758. A 45 kda toxin from PS80JJ1 is an example of a preferred toxin.

Example 7 -- Activity Against Red Sunflower Seed Weevil (*Smicronyx fulvus*)

The *B.t.* isolates designated PS28Q2, PS32B, PS54G2, and PS43A2 have been found to be active against red sunflower seed weevil.

Example 8 -- Activity Against Canola Flea Beetle (*Phyllotreta cruciferae*)

The *B.t.* isolates designated PS201T6, PS33F2, HD977, PS196Q3, PS225K1, PS164H2, KB19, PS86BB1, PS186EE, KB6, and PS198A2 have been found to be active against canola flea beetle. A cyt1B toxin and a 130 kda toxin obtainable from PS201T6 are examples of preferred toxins for controlling *Phyllotreta*.

Example 9 -- Activity Against Greenbug (*Schizaphis graminum*)

The *B.t.* isolates designated PS159E6, PS167P, PS71M3, PS201T6, PS196SI, HD541, PS28K1, PS18, and PS50C have been found to be active against greenbug.

Example 10 -- Activity Against Sunflower Spotted Stem Weevils (*Cylindrocopturus adspersus*)

The *B.t.* isolates designated PS28Q2, PS43A2, PS62B, PS86B1, PS140E2, PS169E, and PS201T6 have been found to be active against sunflower stem weevils.

Example 10 -- Insertion of Toxin Genes Into Plants

One aspect of the subject invention is the transformation of plants with genes encoding insecticidal toxins. The transformed plants are resistant to attack by the target pest.

Genes encoding pesticidal 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*.

5 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
10 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
15 and sufficiently described in EP 120 516; Hoekema (1985) In: *The Binary Plant Vector System*, Offset-drukkerij Kanters B.V., Alblasterdam, 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
20 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.
25 Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), 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
30 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.

In a preferred embodiment of the subject invention, plants will be transformed with genes wherein the codon usage has been optimized for plants. See, for example, U.S. Patent No. 5,380,831. Also, advantageously, plants encoding a truncated toxin will be used. The truncated toxin typically will encode about 55% to about 80% of the full length toxin. Methods for creating synthetic *B.t.* genes for use in plants are known in the art.

Example 11 — Cloning of *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, genes encoding the insecticidal toxins, as described herein, can be placed within 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.

The claims defining the invention are as follows:

1. A method for controlling a lepidopteran pest, wherein said method comprises contacting said pest with a lepidopteran-active *Cry5A* toxin obtainable from a *Bacillus thuringiensis* isolate selected from the group consisting of PS17 and PS86Q3.
2. The method according to claim 1, wherein said toxin is obtainable from *Bacillus thuringiensis* isolate PS17.
3. The method according to claim 2, wherein said toxin is a *Cry5Aa* toxin.
4. The method according to claim 2 or claim 3, wherein said toxin is expressed by a transformed plant.
5. The method according to any one of claims 2 to 4, wherein said lepidopteran pest is *Plutella xylostella*.
6. The method according to any one of claims 2 to 4, wherein said lepidopteran pest is *Heliothis virescens*.
7. The method according to any one of claims 2 to 4, wherein said lepidopteran pest is *Cochylis hospes*.
8. The method according to claim 1, wherein said toxin is obtainable from *Bacillus thuringiensis* isolate PS86Q3.
9. The method according to claim 8, wherein said toxin is a *Cry5Ac* toxin.
10. The method according to claim 8 or claim 9, wherein said toxin is expressed by a transformed plant.
11. The method according to any one of claims 8 to 10, wherein said lepidopteran pest is *Plutella xylostella*.
12. The method according to any one of claims 8 to 10, wherein said lepidopteran pest is *Heliothis virescens*.
13. The method according to any one of claims 8 to 10, wherein said lepidopteran pest is *Mamestra configurata*.
14. A method for controlling a lepidopteran pest, wherein said method comprises contacting said pest with a lepidopteran-active *Cry5A* toxin obtainable from a *Bacillus thuringiensis* isolate selected from the group consisting of PS17 and PS86Q3, substantially as hereinbefore described.
15. Use of a transformed cell which expresses a lepidopteran-active *Cry5A* toxin obtainable from a *Bacillus thuringiensis* isolate selected from the group consisting of PS17 and PS86Q3 for controlling a lepidopteran pest.
16. The use according to claim 15, wherein said toxin is obtainable from *Bacillus thuringiensis* isolate PS17.



17. The use according to claim 16, wherein said toxin is a cry5Aa toxin.
18. The use according to claim 16 or claim 17, wherein said cell is a plant cell.
19. The use according to any one of claims 16 to 18, wherein said lepidopteran pest is *Plutella xylostella*.
- 5 20. The use according to any one of claims 16 to 18, wherein said lepidopteran pest is *Heliothis virescens*.
21. The use according to any one of claims 16 to 18, wherein said lepidopteran pest is *Cochylis hospes*.
22. The use according to claim 15, wherein said toxin is obtainable from *Bacillus*
- 10 *thuringiensis* isolate PS86Q3.
23. The use according to claim 22, wherein said toxin is a Cry5Ac toxin.
24. The use according to claim 22 or claim 23, wherein said toxin is expressed by a transformed plant.
25. The use according to any one of claims 22 to 24, wherein said lepidopteran
- 15 pest is *Plutella xylostella*.
26. The use according to any one of claims 22 to 24, wherein said lepidopteran pest is *Heliothis virescens*.
27. The use according to any one of claims 22 to 24, wherein said lepidopteran pest is *Mamestra configurata*.
- 20 28. Use of a transformed cell which expresses a lepidopteran-active Cry5A toxin obtainable from a *Bacillus thuringiensis* isolate selected from the group consisting of PS17 and PS86Q3 for controlling a lepidopteran pest, substantially as hereinbefore described.

Dated 12 September, 2001

Mycogen Corporation

Patent Attorneys for the Applicant/Nominated Person

SPRUSON & FERGUSON

