Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2059898 C 2004/10/19

(11)(21) 2 059 898

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(22) Date de dépôt/Filing Date: 1992/01/23

(41) Mise à la disp. pub./Open to Public Insp.: 1992/08/22

(45) Date de délivrance/Issue Date: 2004/10/19

(30) Priorités/Priorities: 1991/02/21 (07/658,935) US; 1991/02/21 (07/658,934) US; 1991/06/06 (07/710,890) US; 1991/06/12 (07/715,184) US

(51) Cl.Int.^{5}/Int.Cl.^{5} C12N 15/31, A01N 63/00, A01H 5/00, C12N 1/21, C12N 7/01

(72) Inventeurs/Inventors:
BRADFISCH, GREGORY A., US;
NARVA, KENNETH E., US;
MICHAELS, TRACY, US;
PAYNE, JEWEL M., US;
SCHWAB, GEORGE E., US

(73) Propriétaire/Owner:
MYCOGEN CORPORATION, US

(74) Agent: MACRAE & CO.

(54) Titre : ISOLATS DE BACILLUS THURINGIENSIS A ACTIVITE MICROBIOLOGIQUE ET GENE CODANT POUR UNE TOXINE ACTIVE CONTRE LES COLEOPTERES

(54) Title: BIOLOGICALLY ACTIVE BACILLUS THURINGIENSIS ISOLATES AND GENE ENCODING A COLEOPTERAN-ACTIVE TOXIN

(57) Abrégé/Abstract:
Disclosed and claimed are Bacillus thuringiensis isolates designated B.t. PS86A1 and B.t. PS86Q3, which are active against corn rootworm larvae and the alfalfa weevil. Thus, these isolates, or mutants thereof, can be used to control such pests. Further, genes encoding novel δ-endotoxins can be removed from these isolates and transferred to other host microbes, or plants. Expression of the δ-endotoxins in such hosts results in the control of susceptible coleopteran insects.

Abstract of the Disclosure

Disclosed and claimed are Bacillus thuringiensis isolates designated B.t. PS86A1 and B.t. PS86Q3, which are active against corn rootworm larvae and the alfalfa weevil. Thus, these isolates, or mutants thereof, can be used to control such pests. Further, genes encoding novel δ-endotoxins can be removed from these isolates and transferred to other host microbes, or plants. Expression of the δ-endotoxins in such hosts results in the control of susceptible coleopteran insects.

DESCRIPTION

BIOLOGICALLY ACTIVE *BACILLUS THURINGIENSIS* ISOLATES AND GENE ENCODING A COLEOPTERAN-ACTIVE TOXIN

Background of the Invention

Many hundreds of strains of Bacillus thuringiensis (B.t.) produce insecticidal toxins designated as delta endotoxins. They are synthesized by sporulating B.t. cells. When toxin is ingested by a susceptible insect, the cells of the gut epithelium are destroyed.

The reported activity spectrum of B.t. covers insect species within the orders Lepidoptera and Coleoptera, many of which are major pests in agriculture and forestry. The activity spectrum also includes the insect order Diptera, which includes mosquitoes and black flies. See 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. Dipteran insects are serious nuisances as well as being vectors of many serious human and animal diseases such as malaria, onchocerciasis, equine encephalitis, and dog heartworm.

Approximately 9.3 million acres of U.S. corn is infested with the corn rootworm species complex, which includes the northern corn rootworm, Diabrotica barberi, the southern corn rootworm, D. undecimpunctata howardi, and the western corn rootworm, D. virgifera virgifera. The soil-dwelling larvae of these Diabrotica species feed on corn root, causing lodging of the corn plant. This eventually results in yield reduction or death of the plant. By feeding on cornsilks, the adults reduce pollination and, therefore, the yield of corn per plant. In addition, adults and larvae of the southern corn rootworm, also known as the spotted cucumber beetle, attack cucurbit crops (cucumbers, melons, squash, etc.)

and many vegetable and field crops in commercial production as well as in home gardens.

Control of the corn rootworm has been partially addressed by cultural methods, such as crop rotation and application of high nitrogen levels to stimulate the growth of adventitious root systems. However, chemical insecticides are relied upon heavily to guarantee the desired level of control. Insecticides are banded onto the soil or incorporated into the soil. The major problem associated with the use of these chemicals is the development of resistance among the treated insect populations.

U.S. Patent No. 4,849,217 discloses Bacillus thuringiensis isolates active against the alfalfa weevil. One of the isolates disclosed is B. thuringiensis PS86A1 (NRRL B-18400).

The alfalfa weevil, Hypera postica, and the closely related Egyptian alfalfa weevil, Hypera brunneipennis, are the most important insect pests of alfalfa grown in the United States, with 2.9 million acres infested in 1984. An annual sum of 20 million dollars is spent to control these pests. The Egyptian alfalfa weevil is the predominant species in the southwestern U.S., where it undergoes aestivation (i.e., hibernation) during the hot summer months. In all other respects, it is identical to the alfalfa weevil, which predominates throughout the rest of the U.S.

The larval stage is the most damaging in the weevil life cycle. By feeding at the alfalfa plant's growing tips, the larvae cause skeletonization of leaves, stunting, reduced plant growth, and, ultimately, reductions in yield. Severe infestations can ruin an entire cutting of hay. The adults, also foliar feeders, cause additional, but less significant, damage.

Chemical insecticides play a major role in effective alfalfa weevil control. However, there are several problems associated with their use including:

1. acute mammalian toxicity: several of the most effective insecticides used for weevil control are highly toxic to humans and other mammals, and are sold on a restricted basis in many states. Toxic residues are an additional problem for hay sold as feed for livestock.

2. honeybee toxicity: the honeybee is sensitive to some of the insecticides used for alfalfa weevil control. Because alfalfa is the major source of nectar for commercial honeybee colonies in the U.S., the use of insecticides with honeybee toxicity is incompatible with the needs of the honey producers.

3. toxicity to natural enemies: the insect parasites and predators which normally help control populations of minor alfalfa pests (aphids, spider mites, leafhoppers, caterpillars) are highly susceptible to all insecticides presently used for alfalfa weevil control. Reductions in the numbers of beneficial insects can result in increased populations of these once minor pests (secondary pests outbreaks), and in the consequent application of additional insecticides. Secondary pest outbreaks of aphids and mites often lead to serious yield reductions.

At present there is a need for more effective control agents, particularly efficacious agents that act selectively and do not cause the secondary outbreaks of mites and aphids that can be so devastating to alfalfa.

The cloning and expression of the B.t. crystal protein gene in Escherichia coli has been described in the published literature (Schnepf, H.E. and Whitely, H.R. [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. European Patent Application, Publication No. 0 202 739, discloses a novel B. thuringiensis microbe which can be used to control coleopteran pests in various environments.

Brief Summary of the Invention

The subject invention concerns novel Bacillus thuringiensis isolates which have activity against corn rootworm larvae (Diabrotica undecimpunctata undecimpunctata) and the Egyptian Alfalfa weevil (Hypera brunneipennis). Also disclosed and claimed is a novel toxin gene which expresses a novel toxin toxic to

coleopteran insects. This toxin gene can be transferred to suitable hosts via plasmid vector.

Specifically, the invention comprises B.t. isolates designated B.t. PS86A1 and B.t. PS86Q3, and mutants thereof, and novel delta endotoxin genes obtainable from these B.t. isolates which encode proteins which are active against corn rootworm larvae and the Egyptian alfalfa weevil. The B.t. microbes and the transformed microbes, disclosed herein, can be used alone or in mixtures thereof. The invention also comprises a novel delta endotoxin gene which encodes an approximately 58 kDa protein, as determined by SDS-PAGE analysis, which has the DNA sequence shown in Sequence ID No. 1. Also embodied within the invention is the novel toxin having the amino acid sequence shown in Sequence ID No. 2

Brief Description of the Drawings

Figure 1 is a photograph of a 9% SDS polyacrylamide gel showing alkali-soluble proteins of B.t. PS86Q3 and B.t. PS86A1.

Figure 2 is a photograph of a standard SDS polyacrylamide gel showing alkali-soluble proteins of alfalfa weevil-active B.t. strains. All the B. thuringiensis strains are disclosed in U.S. 4,849,217, except B. thuringiensis PS86Q3 which is disclosed herein.

Figure 3 – Restriction map of plasmid pMYC2320.

Brief Description of the Sequences

Sequence ID No. 1 - Gene 86A1-A probe

Sequence ID No. 2 - N-terminal amino acid sequence

Sequence ID No. 3 - DNA sequence of novel gene.

Sequence ID No. 4 - Amino acid sequence of novel toxin.

Detailed Disclosure of the Invention

The Bacillus thuringiensis isolates of the subject invention have the following characteristics:

B.t. PS86A1

Colony morphology—large colony, dull surface, typical B.t.

Vegetative cell morphology—typical B.t.

Culture methods—typical for B.t.

Activity—inclusions kill corn rootworm larvae and alfalfa weevil larvae

Inclusion type—multiple attached

Molecular weight of proteins (kDa)—58, 45

B.t. PS86Q3

Colony morphology—large colony, dull surface, typical B.t.

Vegetative cell morphology—typical B.t.

Culture methods—typical for B.t.

Activity—inclusions kill corn rootworm larvae and alfalfa weevil larvae

Inclusion type—long attached

Molecular weight of proteins (kDa)—155, 135, 98, 62, 58

The B.t. isolates of the invention, and mutants thereof, can be cultured using standard known 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, a 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. The formulation and application procedures are all well known in the art and are used with commercial strains. The novel B.t. isolates, and mutants thereof, can be used to control pests as disclosed herein.

The novel toxin gene of B.t. PS86A1 was obtained as disclosed herein.

The cultures of the subject invention were deposited in the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 North University Street, Peoria, Illinois 61604 USA.

Culture	Accession No.	Deposit date
Bacillus thuringiensis PS86A1	NRRL B-18400	August 16, 1988
Bacillus thuringiensis PS86Q3	NRRL B-18765	February 6, 1991
E. coli NM522(pMYC2320)	NRRL B-18769	February 14, 1991

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. These 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 a deposit, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of any patent which may issue disclosing a culture. The depositor acknowledges the duty to replace a deposit should the depository be unable to furnish a sample when requested, due to the condition of a deposit. 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.

The subject cultures were deposited in an acknowledged highly qualified culture repository. The invention also includes deposits of the same cultures in

other culture repositories. Thus, the disclosure and claims are not limited to the specific culture accession number(s) disclosed herein. Also, within this invention are deposits at other repositories which can be shown to have the same biological activity characteristics of the culture(s) disclosed herein.

The toxin genes harbored by the novel isolates of the subject invention can be introduced into a wide variety of microbial hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. With suitable hosts, e.g., Pseudomonas, the microbes can be applied to the situs of corn rootworm larvae or the alfalfa weevil where they will proliferate and be ingested by the larvae or weevil. The result is a control of these pests. 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 expressing a toxin into the microorganism host under conditions which allow for stable maintenance and expression of the gene. One can provide for DNA constructs which include the transcriptional and translational regulatory signals for expression of the toxin gene, the toxin gene under their regulatory control and a DNA sequence homologous with a sequence in the host organism, whereby integration will occur, and/or a replication system which is functional in the host, whereby integration or stable maintenance will occur.

The transcriptional initiation signals will include a promoter and a transcriptional initiation start site. In some instances, it may be desirable to provide for regulative expression of the toxin, where expression of the toxin will only occur after release into the environment. This can be achieved with operators or a region binding to an activator or enhancers, which are capable of induction upon a change in the physical or chemical environment of the microorganisms. For example, a temperature sensitive regulatory region may be employed, where the organisms may be grown up in the laboratory without expression of a toxin, but upon release into the environment, expression would begin. Other techniques may employ a specific nutrient medium in the laboratory,

which inhibits the expression of the toxin, where the nutrient medium in the environment would allow for expression of the toxin. For translational initiation, a ribosomal binding site and an initiation codon will be present.

Various manipulations may be employed for enhancing the expression of the messenger RNA, particularly by using an active promoter, as well as by employing sequences, which enhance the stability of the messenger RNA. The transcriptional and translational termination region will involve stop codon(s), a terminator region, and optionally, a polyadenylation signal. A hydrophobic "leader" sequence may be employed at the amino terminus of the translated polypeptide sequence in order to promote secretion of the protein across the inner membrane.

In the direction of transcription, namely in the 5′ to 3′ direction of the coding or sense sequence, the construct will involve the transcriptional regulatory region, if any, and the promoter, where the regulatory region may be either 5′ or 3′ of the promoter, the ribosomal binding site, the initiation codon, the structural gene having an open reading frame in phase with the initiation codon, the stop codon(s), the polyadenylation signal sequence, if any, and the terminator region. This sequence as a double strand may be used by itself for transformation of a microorganism host, but will usually be included with a DNA sequence involving a marker, where the second DNA sequence may be joined to the toxin expression construct during introduction of the DNA into the host.

By a marker is intended a structural gene which provides for selection of those hosts which have been modified or transformed. The marker will normally provide for selective advantage, for example, providing for biocide resistance, e.g., resistance to antibiotics or heavy metals; complementation, so as to provide prototropy to an auxotrophic host, or the like. Preferably, complementation is employed, so that the modified host may not only be selected, but may also be competitive in the field. One or more markers may be employed in the development of the constructs, as well as for modifying the host. The organisms may be further modified by providing for a competitive advantage against other

wild-type microorganisms in the field. For example, genes expressing metal chelating agents, e.g., siderophores, may be introduced into the host along with the structural gene expressing the toxin. In this manner, the enhanced expression of a siderophore may provide for a competitive advantage for the toxin-producing host, so that it may effectively compete with the wild-type microorganisms and stably occupy a niche in the environment.

Where no functional replication system is present, the construct will also include a sequence of at least 50 basepairs (bp), preferably at least about 100 bp, and usually not more than about 5000 bp of a sequence homologous with a sequence in the host. In this way, the probability of legitimate recombination is enhanced, so that the gene will be integrated into the host and stably maintained by the host. Desirably, the toxin gene will be in close proximity to the gene providing for complementation as well as the gene providing for the competitive advantage. Therefore, in the event that a toxin gene is lost, the resulting organism will be likely to also lose the complementing gene and/or the gene providing for the competitive advantage, so that it will be unable to compete in the environment with the gene retaining the intact construct.

A large number of transcriptional regulatory regions are available from a wide variety of microorganism hosts, such as bacteria, bacteriophage, cyanobacteria, algae, fungi, and the like. Various transcriptional regulatory regions include the regions associated with the trp gene, lac gene, gal gene, the lambda left and right promoters, the tac promoter, the naturally-occurring promoters associated with the toxin gene, where functional in the host. See for example, U.S. Patent Nos. 4,332,898, 4,342,832 and 4,356,270. The termination region may be the termination region normally associated with the transcriptional initiation region or a different transcriptional initiation region, so long as the two regions are compatible and functional in the host.

Where stable episomal maintenance or integration is desired, a plasmid will be employed which has a replication system which is functional in the host. The replication system may be derived from the chromosome, an episomal element

normally present in the host or a different host, or a replication system from a virus which is stable in the host. A large number of plasmids are available, such as pBR322, pACYC184, RSF1010, pRO1614, and the like. See for example, Olson et al., (1982) J. Bacteriol. 150:6069, and Bagdasarian et al., (1981) Gene 16:237, and U.S. Patent Nos. 4,356,270, 4,362,817, and 4,371,625.

The B.t. gene can be introduced between the transcriptional and translational initiation region and the transcriptional and translational termination region, so as to be under the regulatory control of the initiation region. This construct will be included in a plasmid, which will include at least one replication system, but may include more than one, where one replication system is employed for cloning during the development of the plasmid and the second replication system is necessary for functioning in the ultimate host. In addition, one or more markers may be present, which have been described previously. Where integration is desired, the plasmid will desirably include a sequence homologous with the host genome.

The transformants can be isolated in accordance with conventional ways, usually employing a selection technique, which allows for selection of the desired organism as against unmodified organisms or transferring organisms, when present. The transformants then can be tested for pesticidal activity.

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. Illustrative prokaryotes, both Gram-negative and -positive, include Enterobacteriaceae, such as Escherichia, Erwinia, Shigella, Salmonella, and Proteus; Bacillaceae; Rhizobiceae, such as Rhizobium;

Spirillaceae, such as photobacterium, Zymomonas, Serratia, Aeromonas, Vibrio, Desulfovibrio, Spirillum; Lactobacillaceae; Pseudomonadaceae, such as Pseudomonas and Acetobacter; Azotobacteraceae and Nitrobacteraceae. Among eukaryotes are fungi, such as Phycomycetes and Ascomycetes, which includes yeast, such as Saccharomyces and Schizosaccharomyces; and Basidiomycetes yeast, such as Rhodotorula, Aureobasidium, Sporobolomyces, and the like.

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.

Host organisms of particular interest include yeast, such as Rhodotorula sp., Aureobasidium sp., Saccharomyces sp., and Sporobolomyces sp.; phylloplane organisms such as Pseudomonas sp., Erwinia sp. and Flavobacterium sp.; or such other organisms as Escherichia, Lactobacillus sp., Bacillus sp., and the like. Specific organisms include Pseudomonas aeruginosa, Pseudomonas fluorescens, Saccharomyces cerevisiae, Bacillus thuringiensis, Escherichia coli, Bacillus subtilis, and the like.

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 inactivation 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. The method of inactivation or killing retains at least a substantial portion of the bio-availability or bioactivity of the toxin.

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.

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.

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 alfalfa weevil or the corn rootworm larvae, e.g., soil, by spraying, dusting, sprinkling, or the like.

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 30°C. 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 30°C. 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 30°C 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 of the *B.t.* Isolates

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_2PO_4	3.4 g/l
K_2HPO_4	4.35 g/l
Salt Solution	5.0 ml/l
$CaCl_2$ Solution	5.0 ml/l

pH 7.2

Salts Solution (100 ml)

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

$CaCl_2$ Solution (100 ml)

$CaCl_2.2H_2O$	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 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.

With regard to the purification of coleopteran-active toxin from B.t. PS86A1, the parasporal inclusion bodies, spores, and cellular debris were collected by centrifugation (7.14k*g*20 min.). The parasporal inclusion bodies were partially purified by sodium bromide (28-38%) isopycnic gradient centrifugation (M.A. Pfannenstiel et al. [1984] FEMS Microbiol. Lett. 21:39). The partially purified protein toxic to the Egyptian alfalfa weevil, Hypera brunneipennis, was bound to the Immobilon*-P, PVDF membrane (Millipore, Bedford, MA) by western blotting techniques (H. Towbin et al. [1979] Proc. Natl. Acad. Sci. USA 76:4350). The N-terminal amino acid sequence was determined by the standard Edman reaction with an automated gas-phase sequenator (M.W. Hunkapiller et al. [1983] Meth. Enzymol. 91:399). The sequence obtained was as follows:

$$NH_2 - M\ I\ I\ D\ S\ K\ T\ T\ L\ P\ R\ H\ S\ L\ I\ H\ T\ I\ K\ L - CO_2H$$

*Trade-mark

From this sequence, the following oligonucleotide probe was designed:

5' ATG ATT GAT TCT AAA ACA ACA TTA CCA AGA CAT TCT/A
TTA ATT/A CAT ACT/A ATT/A AA 3'

Example 2 – Activity of *B.t.* Isolates Against Corn Rootworm Larvae

B.t. isolates PS86Q3 and PS86A1 can be grown using known media and culturing techniques. Spore/crystal preparations are obtained by centrifuging broths and reconstituting pellets with a 0.05% aqueous solution of SILWET® (Union Carbide Corp.) surfactant (L-77) at a 20-fold concentration of the original broth. Fifty μl of this solution is then pipetted onto 1 ml of artificial diet in wells of a standard 24 well assay plate. One first instar D. undecimpunctata undecimpunctata larva was added to each well.

Growth was measured by weighing larvae at the end of a 5-8 day assay period. Growth reduction (G.R.) was determined according to the formula:

$$G.R. = (1–T/C)^* 100$$

where,

C = mass of control larvae (mg) and,

T = mass of treated larvae (mg).

Both B.t. PS86A1 and PS86Q3 decreased the rate of growth of D.u. undecimpunctata (Table 1).

Table 1. Decreased rate of growth of Diabrotica undecimpunctata undecimpunctata fed diet treated with Bacillus thuringiensis isolates

Isolate	Inclusion	Major Proteins (kDa)	Growth Reduction (%)
PS86A1	Attached multiple	58, 45	86
PS86Q3	Attached long	155, 135, 98, 62, 58	83

Example 3 - Activity of B.t. PS86Q3 Against the Egyptian alfalfa weevil

The B. thuringiensis isolate PS86Q3 was tested as a spray-dried powder of a fermentation broth which was concentrated by centrifugation. Pellets, which consist of water and biomass (spores, crystalline δ-endotoxins, cellular debris and growth media) were mixed with a standard carrier, preservative and surfactant. The powders, which consisted of 25% biomass, were made using a Yamato spray drier. (Sold by Yamato Scientific Co., Ltd. Tokoyo, Japan).

Approximately two ml of a 1.5% agar diet is added to each well of a Corning Cell Wells™ 24 well assay plate (Corning Glass Works, Corning, New York). The trays containing diet are dried under an air hood. Spray dried powder of PS86Q3 is suspended in water at 100 mg substance/ml. 50 µl of the suspension is pipetted onto the diet. The trays are then placed in a clean air hood until completely dry. One second instar larvae of Alfalfa weevil, Hypera brunneipennis was placed in each well. The infested trays are covered with a sheet of polyolen-treated Mylar* and heat sealed with a tacking iron. The Mylar covering is pierced carefully with minute pins (four holes per well) and the tray is held in an incubator at 25°C. Evaluation for mortality is determined at six days.

Toxicity of B.t. sprayed-dried powder PS86Q3 to second instar alfalfa weevil, *Hyper brunneipennis*

B.t.	Percent Mortality
PS86Q3	79%
Control	8%

Example 4 – Molecular Cloning of Gene Encoding a Novel 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 37°C 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

*Trade-mark

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 37°C 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:

5′ ATG ATT GAT TCT AAA ACA ACA TTA CCA AGA CAT TCT/A
TTA ATT/A CAT ACT/A ATT/A AA 3′

The probe was mixed at four positions, as shown. Hybridizing bands included an approximately 3.6 kbp HindIII fragment and an approximately 9.3 kbp EcoRV fragment.

A gene library was constructed from PS86A1 DNA partially digested with Sau3A. 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 Sau3A inserts were ligated into BamHI-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 EcoRI and SalI, and electrophoresed on an agarose gel. The approximately

*Trade-mark

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 EcoRI + SalI-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 EcoRI and SalI digests on agarose gels. The desired plasmid construct, pMYC2320, contains the novel toxin gene of the invention. See Figure 1. The DNA sequence of this gene is shown in Sequence ID No. 1. The novel toxin expressed by this gene is shown in Sequence 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 and activity against the alfalfa weevil.

The restriction enzymes disclosed herein can be purchased from Bethesda Research Laboratories, Gaithersburg, MD, or New England Biolabs, Beverly, MA. The enzymes are used according to the instructions provided by the supplier.

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.

It is well known in the art that the amino acid sequence of a protein is determined by the nucleotide sequence of the DNA. Because of the redundancy of the genetic code, i.e., more than one coding nucleotide triplet (codon) can be used for most of the amino acids used to make proteins, different nucleotide

*Trade-mark

sequences can code for a particular amino acid. Thus, the genetic code can be depicted as follows:

Phenylalanine (Phe)	TTK	Histidine (His)	CAK
Leucine (Leu)	XTY	Glutamine (Gln)	CAJ
Isoleucine (Ile)	ATM	Asparagine (Asn)	AAK
Methionine (Met)	ATG	Lysine (Lys)	AAJ
Valine (Val)	GTL	Aspartic acid (Asp)	GAK
Serine (Ser)	QRS	Glutamic acid (Glu)	GAJ
Proline (Pro)	CCL	Cysteine (Cys)	TGK
Threonine (Thr)	ACL	Tryptophan (Trp)	TGG
Alanine (Ala)	GCL	Arginine (Arg)	WGZ
Tyrosine (Tyr)	TAK	Glycine (Gly)	GGL
Termination signal	TAJ		

Key: Each 3-letter deoxynucleotide triplet corresponds to a trinucleotide of mRNA, having a 5′-end on the left and a 3′-end on the right. All DNA sequences given herein are those of the strand whose sequence correspond to the mRNA sequence, with thymine substituted for uracil. The letters stand for the purine or pyrimidine bases forming the deoxynucleotide sequence.

A = adenine

G = guanine

C = cytosine

T = thymine

X = T or C if Y is A or G

X = C if Y is C or T

Y = A, G, C or T if X is C

Y = A or G if X is T

W = C or A if Z is A or G

W - C if Z is C or T

Z = A, G, C or T if W is C

Z = A or G if W is A

QR = TC if S is A, G, C or T; alternatively

QR = AG if S is T or C

J = A or G

K = T or C

L = A, T, C or G

M = A, C or T

The above shows that the novel amino acid sequences of the B.t. toxins can be prepared by equivalent nucleotide sequences encoding the same amino acid sequence of the protein. Accordingly, the subject invention includes such equivalent nucleotide sequences. In addition it has been shown that proteins of identified structure and function may be constructed by changing the amino acid sequence if such changes do not alter the protein secondary structure (Kaiser, E.T. and Kezdy, F.J. [1984] Science 223:249-255). Thus, the subject invention includes mutants of the amino acid sequence depicted herein which do not alter the protein secondary structure, or if the structure is altered, the biological activity is retained to some degree.

Example 5 – Insertion of Toxin Genes Into Plants

The novel genes, obtainable from the B.t. isolates of the invention, coding for the novel toxin, as disclosed herein, can be inserted into plant cells using the Ti plasmid from Agrobacter tumefaciens. Plant cells can then be caused to regenerate into plants (Zambryski, P., Joos, H., Gentello, C., Leemans, J., Van Montague, M. and Schell, J [1983] Cell 32:1033-1043). A particularly useful vector in this regard is pEND4K (Klee, H.J., Yanofsky, M.F. and Nester, E.W. [1985] Bio/Technology 3:637-642). This plasmid can replicate both in plant cells and in bacteria and has multiple cloning sites for passenger genes. The toxin gene, for example, can be inserted into the BamHI site of pEND4K, propagated in E. coli, and transformed into appropriate plant cells.

Example 6 – Cloning of Novel *B. thuringiensis* Genes Into Baculoviruses

The novel genes, obtainable from the B.t. isolates of the invention, can be cloned into baculoviruses such as Autographa californica nuclear polyhedrosis virus (AcNPV). Plasmids can be constructed that contain the AcNPV genome cloned into a commercial cloning vector such as pUC8. The AcNPV genome is modified so that the coding region of the polyhedrin gene is removed and a unique cloning site for a passenger gene is placed directly behind the polyhedrin promoter. Examples of such vectors are pGP-B6874, described by Pennock et al. (Pennock, G.D., Shoemaker, C. and Miller, L.K. [1984] Mol. Cell. Biol. 4:399-406), and pAC380, described by Smith et al. (Smith, G.E., Summers, M.D. and Fraser, M.J. [1983] Mol Cell. Biol. 3:2156-2165). The gene coding for the novel protein toxin can be modified with BamHI linkers at appropriate regions both upstream and downstream from the coding region and inserted into the passenger site of one of the AcNPV vectors.

Example 7 – Toxicity of Purified Protein from the Isolate PS86A1 and Purified Toxin Expressed in *B.t.* HD-1 Cry B Host to Egyptian Alfalfa Weevil, *Hypera brunneipennis*

Fifty microliters of 2 mg/ml purified protein was pipetted onto 1 ml of artificial diet in wells of a standard 24-well bioassay plate. One first or second instar larva was added to each well.

Assays were graded for mortality 6 days post-treatment. Larvae which did not respond to prodding with a dull probe were considered dead. Experiments were replicated on successive days. Toxicity is shown below (Table 1)

Table 1. Toxicity of purified protein to the Egyptian alfalfa weevil, Hypera brunneipennis

Source of protein	Mean Percent Mortality
PS86A1	46.8
Expression in B.t. HD-1 Cry B	61.5
Untreated	2.0

Example 8 – Growth Inhibition by Purified Toxin Expressed in *B.t.* HD-1 Cry B Host to Western Spotted Cucumber Beetle, *Diabrotica undecimpunctata undecimpunctata*

One hundred microliters of a 2 mg/ml aqueous suspension of purified toxin was pipetted onto 1 ml of an artificial diet in wells of a standard 24-well assay plate. One first instar D.u. undecimpunctata larva was added to each well.

Growth was measured by weighing larva at the end of an 8-day assay period. Growth reduction (G.R.) was determined according to the formula:

$$G.R. = (1-T/C) * 100$$

where, C = average mass of control larvae (mg) and, T = average mass of treated larvae (mg).

Experiments were replicated on separated days. Purified toxin reduced the rate of growth of D.u. undecimpunctata by 36.6 percent relative to untreated controls.

Example 9

The novel toxin of the invention when expressed in a B.t. HD-1 Cry B host is also active against other Hypera spp., for example, H. meles (clover head weevil), H. nigrirostris (lesser clover leaf weevil), H. postica (alfalfa weevil), and H. punctata (clover leaf weevil).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Narva, Kenneth E.
Schwab, George E.
Bradfisch, Gregory A.
Tracy Michaels
Jewel M. Payne

(ii) TITLE OF INVENTION: Novel Bacillus Thuringiensis Gene Encoding a Coleopteran-active Toxin

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Roman Saliwanchik
(B) STREET: 2421 N.W. 41st Street suite A-1
(C) CITY: Gainesville
(D) STATE: Florida
(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: Saliwanchik, Roman
(C) REFERENCE/DOCKET NUMBER: MA58.C1

(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: 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:1:

ATGATTGATT CTAAAACAAC ATTACCAAGA CATTCWTTAA TWCATACWAT WAA 53

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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

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

Met Ile Ile Asp Ser Lys Thr Thr Leu Pro Arg His Ser Leu Ile His
1 5 10 15

Thr Ile Lys Leu
20

(4) INFORMATION FOR SEQ ID NO:3:

(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:
(A) LIBRARY: LAMBDAGEM (TM) - 11 LIBRARY OF KENNETH NARVA
(B) CLONE: PS86A1-A

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

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

ATGATTATTG ATAGTAAAAC GACTTTACCT AGACATTCAC TTATTCATAC AATTAAATTA 60

AATTCTAATA AGAAATATGG TCCTGGTGAT ATGACTAATG GAAATCAATT TATTATTTCA 120

AAACAAGAAT GGGCTACGAT TGGAGCATAT ATTCAGACTG GATTAGGTTT ACCAGTAAAT 180

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

ATGACATCAA ATCAATATAT GATTTCACAT GAATATACAA GTTTACCAAA TAATTTTATG 1320

TTATCAAGAA ATAGTAATTT AGAATATAAA TGTCCTGAAA ATAATTTTAT GATATATTGG 1380

TATAATAATT CGGATTGGTA TAATAATTCG GATTGGTATA ATAAT 1425

(5) INFORMATION FOR SEQ ID NO:4:

(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

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

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

Met Ile Ile Asp Ser Lys Thr Thr Leu Pro Arg His Ser Leu Ile His
1 5 10 15

Thr Ile Lys Leu Asn Ser Asn Lys Lys Tyr Gly Pro Gly Asp Met Thr
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
275 280 285

Ser Ala Gln His Asp Leu Asp Arg Asp Val Lys Ile Ile Gly Met Leu
290 295 300

Asn Ser Ile Asn Thr Asp Ile Asp Asn Leu Tyr Ser Gln Gly Gln Glu
305 310 315 320

Ala Ile Lys Val Phe Gln Lys Leu Gln Gly Ile Trp Ala Thr Ile Gly
325 330 335

Ala Gln Ile Glu Asn Leu Arg Thr Thr Ser Leu Gln Glu Val Gln Asp
340 345 350

Ser Asp Asp Ala Asp Glu Ile Gln Ile Glu Leu Glu Asp Ala Ser Asp
355 360 365

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
450 455 460

Asp Trp Tyr Asn Asn Ser Asp Trp Tyr Asn Asn
465 470 475

Claims

1. A process for controlling corn rootworm larvae which comprises incorporating in the soil habitat of said corn rootworm larvae a corn rootworm larvae-controlling effective amount of Bacillus thuringiensis PS86Q3, NRRL B-18765 toxic crystals or spores therof.

2. A composition of matter comprising an inert carrier and a stable lysis resistant, asporogenus Bacillus thuringiensis having insecticidal activity towards corn rootworm larvae which is prepared from Bacillus thuringiensis PS86Q3, NRRL B-18765.

3. A composition of matter comprising Bacillus thuringiensis PS86Q3, NRRL B-18765, and an inert carrier.

4. A pesticidal composition comprising a suitable carrier and substantially intact, treated cells having prolonged pesticidal activity and greater persistence in the feeding zone when applied to the environment of a target pest, wherein said treated cells are Bacillus thuringiensis PS86Q3, NRRL B-18765.

5. A method for controlling corn rootworm larvae which comprises contacting said larvae with a corn rootworm larvae-controlling effective amount of a pesticidal composition comprising a suitable carrier and substantially intact, treated cells having prolonged pesticidal activity when applied to the environment of corn rootworm larvae, wherein said treated cells are Bacillus thuringiensis PS86Q3, NRRL B-18765.

6. A culture of plant or bacterial cells transformed by a gene wherein said gene encodes a toxin comprising SEQ ID NO. 4.

7. A biologically pure culture of Bacillus thuringiensis PS86Q3 having the identifying characteristic activity against corn rootworm larvae and the alfalfa weevil of deposit NRRL B-18765.

8. A process for controlling insect infestation of alfalfa, said infestation by an alfalfa weevil, which comprises contacting said infesting insect, or treating the environment of said infesting insect, with an insect-controlling effective amount of Bacillus thuringiensis PS86Q3, NRRL B-18765, which retain substantially the activity against the alfalfa weevil, and toxins, crystals or spores thereof.

9. The process, according to claim 8, wherein said alfalfa weevil is the Egyptian alfalfa weevil (EAW).

10. A process, according to claim 8, wherein substantially intact B.t. cells of the isolate, are treated to prolong the pesticidal activity when the substantially intact B.t. cells are applied to the environment of said alfalfa weevil.

11. DNA encoding an ≈ 58 kDa Bacillus thuringiensis toxin active against coleopteran pests, said DNA having the DNA sequence shown in Sequence ID No. 3.

12. Toxin having insecticidal activity against coleopteran pests, said toxin having the amino acid sequence shown in Sequence ID No. 4.

13. A recombinant DNA transfer vector comprising DNA which codes for a toxin having insecticidal activity against coleopteran pests, said toxin having the amino acid sequence shown in Sequence ID No. 4.

14. The DNA transfer vector, according to claim 13, transferred to and replicated in a prokaryotic or eukaryotic host.

15. A bacterial host transformed to express a Bacillus thuringiensis toxin as defined in claim 12.

16. Escherichia coli NM522(pMYC2320), having the identifying characteristics of NRRL B-18769, a host according to claim 15.

17. A microorganism transformed to express a Bacillus thuringiensis toxin as defined in Claim 12, wherein said microorganism is a species of Pseudomonas, Bacillus, Azotobacter, Erwinia, Serratia, Klebsiella, Rhizobium, Rhodopseudomonas, Methylophilius, Agrobacterium, Acetobacter or Alcaligenes.

18. A method for controlling an insect from the genus Hypera which comprises administering to said pest or to the environment of said pest a microorganism according to claim 17.

19. The method, according to claim 18, wherein said Hypera spp. is Hypera brunneipennis (Egyptian alfalfa weevil).

20. The method, according to claim 18, wherein said Hypera spp. is selected from the group consisting of H. meles (clover head weevil), H. nigrirostris (lesser clover leaf weevil), H. postica (alfalfa weevil), and H. punctata (clover leaf weevil).

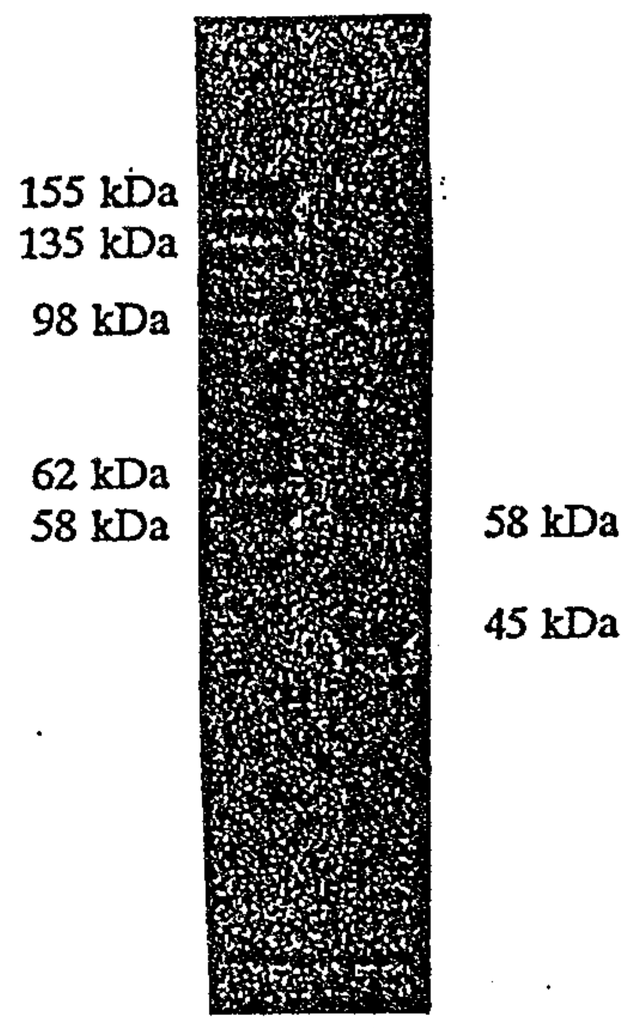
21. An insecticidal composition comprising a suitable carrier and an insecticide comprising substantially intact, treated cells having prolonged pesticidal activity when applied to the environment of a target pest, wherein said

cells are transformed microbes capable of expressing the Bacillus thuringiensis toxin as defined in claim 12.

22. Treated, substantially intact unicellular microorganism cells containing an intracellular toxin, which toxin is a result of expression of a Bacillus thuringiensis toxin gene toxic to coleopeteran insects which codes for a polypeptide toxin as defined in claim 12, wherein said cells are treated under conditions which prolong the insecticidal activity when said cell is applied to the environment of a target insect.

23. Plasmid denoted pMYC2320.

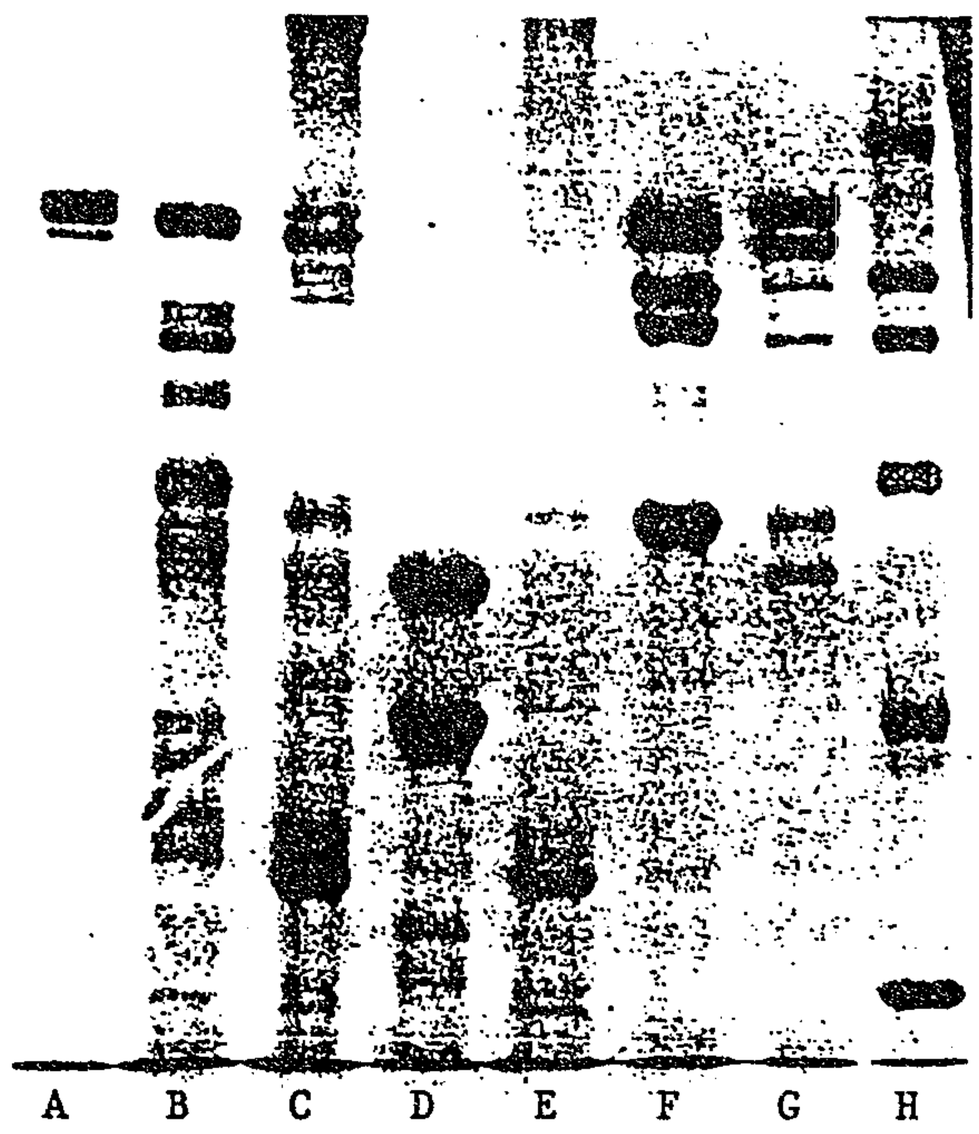
Figure 1



A. B.t. PS86Q3

B. B.t. PS86A1

Figure 2



KDa

205
116
97.4
66
45
29
205
116
97.4
66
45
29

A PS17
B PS33F2
C PS45B1
D PS52A1
E PS62B1
F PS74G1
G PS86Q3
H Mol. Wt. Standard
I PS84C3
J PS86A1
K PS98A3
L PS80JJ1
M PS80PP3
N PS80PP4
O PS86Q3
P Mol. Wt. Standard

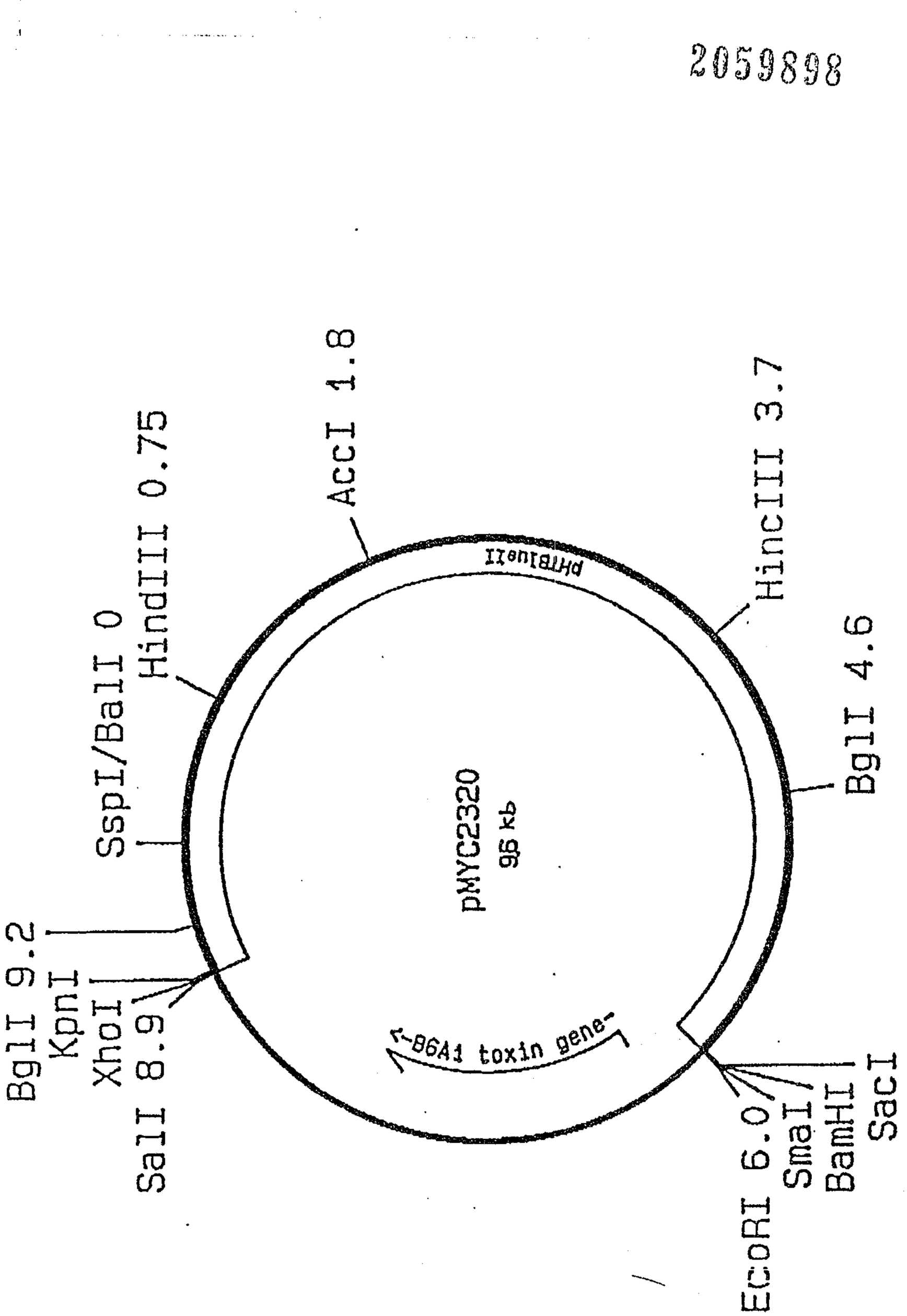

pMYC2320
96 kb
pHTBluell
r-B6A1 toxin gene-
EcoRI
HincII 3.7
BglII 4.6
6.0
SmaI
BamHI
SacI
SalI 8.9
XhoI
KpnI
BglII 9.2
SspI/BalI 0
HindIII
AccI 1.8
0.75

FIGURE 3