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(54) Title: MUTANTS OR VARIANTS OF *BACILLUS THURINGIENSIS* PRODUCING HIGH YIELDS OF DELTA ENDOTOXIN

(57) Abstract

This invention relates to mutants or variants of *Bacillus thuringiensis* producing high amounts of active delta-endotoxins. The delta-endotoxins produced by the mutant or variant *B. thuringiensis* will have an activity directed towards the same pest insects as its parent *B. thuringiensis* delta-endotoxins, such as against lipidopterans (mutants from *B. thuringiensis subsp. kurstaki* or *subsp. aizawai*), dipterans (mutants from *B. thuringiensis subsp. israelensis*) or coleopterans (mutants from *B. thuringiensis subsp. tenebrionis*).

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Title: Mutants or variants of Bacillus thuringiensis Producing High Yields of Delta Endotoxin

BACKGROUND OF THE INVENTION

Commercial preparations of Bacillus thuringiensis are used worldwide for biological control of pest insects. The advantages of these bacterial insecticides are that they are highly selective for a very limited range of target insects and are biodegradable.

Commercial preparations of Bacillus thuringiensis can be applied right up to the time of harvest with no adverse effects.

Bacillus thuringiensis is a rodshaped, aerobic, spore forming bacterium uniquely characterized by the production during the sporulation process of one or more inclusions, referred to as parasporal crystals. These crystals are composed of high molecular weight proteins, referred to as delta-endotoxins. The delta-endotoxins are the active ingredient in available commercial preparations of Bacillus thuringiensis.

Many B. thuringiensis strains with different insect host spectra have been identified. They are classified into different subspecies based on their flagellar antigens. Of particular interest is Bacillus thuringiensis subspecies kurstaki and subspecies aizawai used for the control of lepidopteran pest insects, Bacillus thuringiensis subspecies israelensis used for the control of dipteran pest insects and Bacillus thuringiensis subspecies tenebrionis used for the control of coleopteran pest insects.

The first isolation of a coleopteran toxic Bacillus thuringiensis was reported in 1983 (A. Krieg et al., Z.and.Ent. 96, 500-508, European Patent Publication EP 0149162 A2).

The isolate, which was designated Bacillus thuringiensis subsp. tenebrionis, has been deposited with the German Collection of Microorganisms under accession number DSM 2803. Bacillus thuringiensis subsp. tenebrionis was isolated in

1982 from a dead pupa of the mealworm, Tenebrio molitor (Tenebrionidae, Coleoptera). The strain produces within each cell one spore and one or more insecticidal parasporal crystals which are of flat platelike form with an edge length of about 0.8 µm to 1.5 µm. It belongs to serotype H8a,8b and pathotype C of Bacillus thuringiensis (Krieg et al., System.Appl.Microbiol. 9, 138-141, 1987, US patent 4,766,203, 1988).

It is only toxic against certain leaf eating beetle larvae (Chrysomelidae), but ineffective against caterpillars (Lepidoptera), mosquitoes (Diptera) or other insects.

Bacillus thuringiensis subsp. tenebrionis has been shown to be an effective control agent for the colorado potato beetle larvae. After uptake of crystals and spores from Bacillus thuringiensis subsp. tenebrionis or isolated crystals larvae, and to a certain extent adults, of the colorado potato beetle (Leptinotarsa decemlineata) stop feeding. Larval stages L1-L3 die within 1-3 days (Schnetter et al., in "Fundamental & applied aspects of invertebrate pathology", eds. R.A. Samson et al., Proceedings of the 4th Int. colloquium of Invertebrate Pathology, p. 555, 1986).

It has recently been shown that Bacillus thuringiensis subsp. tenebrionis in addition to the coleopteran active crystal also produces another parasporal crystal that is spindle-, speroidal or plateshaped (A.M. Huger & A. Krieg, J.Appl.Ent. 108, 490-497, 1989). The activity of the second crystal is not yet known.

Four commercial products of Bacillus thuringiensis subsp. tenebrionis have been developed for the control of coleopteran pests. NOVODOR® from Novo Nordisk A/S, TRIDENT® from Sandoz, and DiTerra® from Abbott Laboratories Inc., and Foil® from Ecogen.

The isolation of another coleopteran toxic Bacillus thuringiensis strain was reported in 1986 (Hernnstadt et al. Bio/Technology vol. 4, 305-308, 1986, US patent 4,764,372, 1988). This strain, designated "Bacillus thuringiensis subsp. san diego", M7, has been deposited at the Northern Regional

Research Laboratory, USA under accession number NRRL B-15939. A commercial product based on "Bacillus thuringiensis subsp. san diego" has been developed by Mycogen Corp.

Comparative studies of Bacillus thuringiensis subsp. tenebrionis, DSM 2803 and "Bacillus thuringiensis subsp. san diego", NRRL-B 15939 including phenotypic characterization of the vegetative cells, characterization of the toxic parasporal crystal and analysis of plasmid DNA have, however, shown that "Bacillus thuringiensis subsp. san diego" apparently is identical to the formerly isolated strain DSM 2803, Bacillus thuringiensis subsp. tenebrionis (Krieg et al.: J.Appl.Ent. 104, 417-424, 1987). Furthermore, the nucleotide sequences and deduced amino acid sequences of the coleopteran active delta endotoxin genes from Bacillus thuringiensis subsp. tenebrionis and "Bacillus thuringiensis subsp. san diego" are identical.

Under the same culture conditions the above-mentioned second type of crystals are also synthesized by "Bacillus thuringiensis subsp. san diego" (A.M. Huger & A. Krieg, J.Appl.Ent. 108, 490-497, 1989).

According to H. de Barjac & E. Frachon (Entomophaga 35(2), 233-240, 1990) the "san diego" isolate is similar to "tenebrionis" and it cannot be justified to regard it as a different subspecies.

The utility of Bacillus thuringiensis strains for the control of coleopteran pests is dependent upon efficient and economical production of the coleopteran active toxins and the potency of the product produced. This in turn is dependent upon the amount of delta endotoxins which can be produced by fermentation of the coleopteran active Bacillus thuringiensis strains.

B. thuringiensis has been used for many years for the production of insecticides, but although mutants of B. thuringiensis with increased delta-endotoxins yield would be advantageous, no such mutants have previously been described. Mutants producing higher yields of delta-endotoxins would give a more efficient and economical production of B. thuringiensis toxins and a possibility for manufacture of B. thuringiensis

products with increased potency at equal cost. This in turn would be an advantage for the user as reduced volumes of pesticide formulation have to be stored and handled for a given acreage. In addition, the users will have less container material to dispose of, thereby reducing the impact on the environment

Improvements of the production of delta endotoxin by Bacillus thuringiensis subsp. tenebrionis through mutation have not previously been reported.

One problem associated with the use of especially B. thuringiensis subspecies tenebrionis in controlling beetle larvae has been the relatively low potency or strength of the preparations requiring the application of relatively large amounts of preparation to the areas to be treated, such as 5 to 10 liter/ha compared to 1 to 2 liter/ha of most other B. thuringiensis products and most other insecticides.

Consequently a recognized need for products of improved strength exists.

One way to overcome this problem would be to concentrate the preparations. However, this would add considerably to the production cost in comparison to the savings obtained in storage and transportation.

A much more elegant solution would be to create mutants of existing B. thuringiensis strains capable of producing substantially larger amounts of delta endotoxins per cell.

SUMMARY OF THE INVENTION

The present invention consequently in one aspect relates to variant or mutant Bacillus thuringiensis strains capable of producing substantially larger amounts of toxins than their parent strain.

In another aspect the invention relates to such high producing variants or mutants of B. thuringiensis strains belonging to the subspecies tenebrionis.

Further aspects of the invention relate to the use of such variant or mutant Bacillus thuringiensis strains for the production of pesticidal products, and also to such pesticidal compositions comprising as the active ingredient delta-endotoxins produced by the variant or mutant Bacillus thuringiensis strains of the invention.

Also, the invention in one of its aspects relate to a method of controlling pests by applying a composition according to the invention to an area where pests susceptible to the activity of the delta-endotoxins in question are to be controlled.

In a still further aspect this invention relates to methods of selecting, or mutating and selecting B. thuringiensis strains in order to obtain such variated or mutated B. thuringiensis strains capable of producing substantially larger amounts of delta endotoxins than their parent strain.

DEPOSITION OF MICROORGANISMS

For the purpose of describing this invention in detail a mutant of Bacillus thuringiensis subsp. tenebrionis which produces high amounts of delta-endotoxin has been deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroderweg 1b, D-3300 Braunschweig, Federal Republic of Germany, for the purposes of patent procedure on the date indicated below. DSM being an international depositary under the Budapest Treaty affords permanence of the deposit in accordance with rule 9 of said treaty.

Deposit date	10 August 1989
Depositors ref.	NB 176-1
DSM designation	DSM 5480

The mutant DSM 5480 was obtained by mutation of Bacillus thuringiensis subsp. tenebrionis, strain DSM 5526, that has also been deposited under the Budapest Treaty as indicated below

Deposit date	14 September 1989
Depositor's ref.	NB 125
DSM designation	DSM 5526

DETAILED DESCRIPTION OF THE INVENTION

This invention relates in its general aspect to variants or mutants of Bacillus thuringiensis producing high amounts of active delta-endotoxins as compared to its parent strain.

In this context the expression "high amounts" preferably means at least twice as much or more.

Both in this general aspect and the more specific aspects of the invention indicated below, the delta-endotoxins produced by a mutant B. thuringiensis will have an activity directed towards the same pest insects as its parent B. thuringiensis delta-endotoxins, such as against lepidopterans (mutants from B. thuringiensis subsp. kurstaki and subsp. aizawai), dipterans (mutants from B. thuringiensis subsp. israelensis) or coleopterans (mutants from B. thuringiensis subsp. tenebrionis).

In one specific embodiment of this aspect of the invention the B. thuringiensis belongs to the subspecies tenebrionis, and the delta-endotoxin produced is active against coleopterans.

Under this aspect a preferred embodiment of the invention the variated or mutated B. thuringiensis subsp. tenbrionis is capable of producing more than three times as much delta endotoxin as the strain DSM 2803.

Further embodiments of the invention comprise variants or mutated B. thuringiensis subsp. tenebrionis strains that are capable of producing a parasporal crystal having a mean edge length of 2 μm or more.

A still further embodiment of the invention comprises varianted or mutated B. thuringiensis subsp. tenebrionis strains showing a sporulation frequency 10 to 100 or even 10^6 times lower than the sporulation frequency of the parent strain or the strain DSM 2803.

An even more specific embodiment comprises the deposited mutant B. thuringiensis subsp. tenebrionis, DSM 5480.

While working on this invention a mutant of B. thuringiensis subsp. tenebrionis (DSM 5480) with more than a twofold increase in delta-endotoxin production as compared to its parent strain (DSM 5526) has been isolated. Phase contrast microscopy, scanning electron microscopy and transmission electron microscopy of this mutant indicate that the high productivity of this mutant is due to changes in the regulation of delta endotoxin production relative to sporulation resulting in the production of protein crystals which are up to more than five times bigger than the crystals produced by the existing coleopteran active Bacillus thuringiensis strains. The close correlation between crystal formation and sporulation seems to have been removed and the mutant produces high amounts of delta endotoxin prior to sporulation.

In one of its aspects the invention relates to the use of the variated or mutated strains of the invention in a method for the production of insecticidal B. thuringiensis products, by which method the variant or mutant B. thuringiensis strain is cultivated in a suitable culture medium comprising sources for carbon, nitrogen, and other components known to the skilled person for a suitable period of time, whereafter the delta endotoxins are recovered.

In a further aspect of the invention the B. thuringiensis delta endotoxins product obtained as above is used in pesticidal compositions as an active component.

In such compositions the delta endotoxins of the invention may be utilized either alone or in combination with other biocidally active products.

The invention also relates to such pesticidal compositions or preparations comprising the B. thuringiensis delta endotoxin product of the invention in admixture with agriculturally acceptable diluents or carriers.

The invention also relates to such pesticidal compositions or preparations comprising a B. thuringiensis delta endotoxin product, which pesticidal composition in a liquid form has a potency of at least 15,000 BTTU/g, corresponding to at least 3% w/w coleopteran insecticidal crystal protein, or which pesticidal composition in a dry form has a potency of at least 50,000 BTTU/g, corresponding to at least 10% w/w coleopteran insecticidal crystal protein.

In a specific embodiment the invention relates to pesticidal compositions produced from DSM 5480 having at least twice the potency of pesticidal compositions produced from DSM 2803 or other coleopteran active Btt strains.

The compositions of the invention can take any form known in the art for the formulation of agrochemicals, for example, a suspension, a dispersion, an aqueous emulsion, a dusting powder, a dispersible powder, an emulsifiable concentrate or granules. Moreover it can be in a suitable form for direct application or as a concentrate or primary composition which requires dilution with a suitable quantity of water or other diluent before application.

The concentration of the insecticidally active B. thuringiensis delta endotoxins in the compositions of the present invention when used alone or in combination with

another pesticide, as applied to plants is preferably within the range from about 0.5 to about 25 per cent by weight, especially 1 to 15 per cent by weight.

In a still further aspect the invention relates to a method of controlling pests, wherein a pesticidal composition according to the preceding aspect is applied to an area infected with said pest.

In a specific embodiment the pest to be controlled belongs to the group comprising lepidopterans, dipterans, and coleopterans, especially a coleopteran, such as the colorado potato beetle.

In specific embodiments the pest can be controlled by the application of 1 quart per acre of a liquid pesticidal composition, or by the application of 0.5 lb. per acre of a dry pesticidal composition.

The active B. thuringiensis preparation or the compositions of the invention can be applied directly to the plant by, for example, spraying or dusting at the time when the pest has begun to appear on the plant. The preferred mode of application is by spraying. It is generally important to obtain good control of pests in the early stages of larval development as this is the time when the plant has suffered least damage.

In one method of mutating Bacillus thuringiensis strains and selecting such mutants that are capable of producing substantially larger amounts of delta endotoxins than their parent strains, the parent strain is:

i) treated with a mutagen,

ii) the thus treated mutants are grown on a medium suitable for the selection of asporogenous and/or oligosporogenous strains,

iii) translucent colonies are selected and grown in a medium that does not fluidize on heating, and

iv) truly asporogenous strains are deselected by subjecting the colonies to a heat treatment.

According to a preferred embodiment of this method the thus selected colonies are grown in a normal production medium, and a final selection for strains capable of increasing the delta endotoxin production is performed.

In step (i) of the above method the mutagen may be any suitable conventional chemical mutagen, such as N-methyl-N'-nitro-N-nitrosoguanidine, or ethyl methanesulfonate, ,or the parent strain may be treated with electromagnetic radiation, such as γ-, X-ray-, or UV-radiation.

In step (ii) a suitable medium could be a modified nutrient sporulation medium including phosphate (NSMP medium) as described by Johnson et al., In "Spores VI": eds. P. Gerhardt et al., pp. 248-254, 1975.

In step (iv) of the method of the invention a suitable medium could be a NSMP medium supplemented with $MgCl_2$ and Gelrite, Kelco.

Another method of obtaining the high producing variants or mutants of the invention may be contemplated such as growing the parent strain in a liquid medium and selecting spontaneous mutants or variants after spreading the culture broth on an agar medium suitable for selection of asporogeneous and/or oligosporogenous mutants.

Other methods of screening for the high producing variants or mutants of the invention may be contemplated such as using the mass of these mutants directly through centrifugation or other means of separating for mass.

Example 1

A mutant of B. thuringiensis subsp. tenebrionis with more than a twofold increase in delta-endotoxin production has

been isolated. Phase contrast microscopy, scanning electron microscopy and transmission electron microscopy of this mutant indicate that the high productivity of this mutant is due to changes in the regulation of delta endotoxin production relative to sporulation resulting in the production of protein crystals which are up to more than five times bigger than the crystals produced by the existing coleopteran active Bacillus thuringiensis strains. The close correlation between crystal formation and sporulation seems to have been removed and the mutant produces high amounts of delta-endotoxin prior to sporulation.

Production of High Yield Mutant

Spores of B. thuringiensis subsp. tenebrionis, strain DSM5526 were γ-irradiated to give a dosis of 7 kGy. The irradiated spores were spread onto NSMP agar plates (modified nutrient sporulation medium including phosphate as described by Johnson et al., In "Spores VI": eds. P. Gerhardt et al., pp. 248-254, 1975). A medium suitable for selection of asporogenous and/or oligosporogenous mutants.

The NSMP-agar plates were incubated at 30°C for 2-3 days. Translucent colonies were picked out and transferred to NSMP gelrite plates (NSMP medium supplemented with $MgCl_2$ (0.57 g/l) and Gelrite, Kelco (20 g/l)).

The NSMP gelrite plates were incubated for one hour at 90°C and then further incubated for 1-2 days at 30°C.

Mutants that grew well on the NSMP gelrite plates were selected. In this way all asporogenous mutants were deselected as they fail to grow after the heat treatment.

The selected mutants were grown in shakeflasks containing a commercial medium. The amounts of delta-endotoxin produced were determined by immunological methods described below.

Only mutants producing significantly higher amounts of delta-endotoxin than the parent strain were selected.

The morphology of the selected mutants on solid medium and in liquid media were studied by phase contrast microscopy (x 2500) and by scanning and transmission electron microscopy. The number of spores and crystals were counted and the size of the protein crystals were determined.

Among the mutants obtained, one (DSM 5480) was selected for its outstanding ability to produce delta-endotoxin.

The amount of delta endotoxin produced by mutant DSM 5480 was compared with that of DSM 2803 the original isolate of Bacillus thuringiensis subsp. tenebrionis, Bacillus thuringiensis subsp. tenebrionis, strain DSM 5526 used for the production of NOVODOR®, Bacillus thuringiensis subsp. tenebrionis, strain NB178, isolated from Sandoz' Bacillus thuringiensis tenebrionis product TRIDENT® from 1989, strain NB 198, isolated from Sandoz' B. thuringiensis subs. tenebrionis product TRIDENT® from 1990, "Bacillus thuringiensis subsp. san diego", strain NRRL-B-15939, and strain NB 197 isolated from Mycogen's B. thuringiensis subs. san diego" product M-ONE® from 1990. As shown in Table I of Example 2 the yield improved mutant of the invention produces 2-3.5 times as much delta endotoxin as the coleopteran active strains of Bacillus thuringiensis available today.

Example 2

In this example the delta endotoxin yield of Bacillus thuringiensis subspecies tenebrionis, mutant DSM 5480 was compared with the delta endotoxin yields of Bacillus thuringiensis subspecies tenebrionis strains DSM 2803 (the original isolate of Bacillus thuringiensis subsp. tenebrionis), DSM 5526 (production strain of Novo-Nordisk), and NB 178 and NB 198 (production strains of Sandoz), and Bacillus thuringiensis subsp. san diego, strain NRRL-B 15939 and NB 197 (production strains of Mycogen) in a commercial medium. Each of the strains was grown for 17 hours at 30°C on agar slants of the following composition expressed as gram per litre of distilled water

Peptone, Difco	5 g
Beef extract, Difco	3 g
Agar, Difco	20 g
pH	7.0

5 ml of a suspension of cells from each strain were then transferred to 100 ml of production medium in 500 ml baffle-bottom Erlenmeyer flasks. The production medium consisted of the following components in the quantities indicated (expressed as grams per litre of tap water).

Soy bean meal	50 g
Hydrolyzed starch	40 g
KH_2PO_4	1.77 g
K_2HPO_4	4.53 g
pH	7.0

The inoculated flasks were incubated at 30°C with shaking (250 rpm). After 96 hours of incubation the culture broths were assayed for delta endotoxin yields by immunological methods.

The amounts of delta endotoxin produced by the individual strains were determined by rocket immuno electrophoresis (RIE) and a photometric immuno assay (PIA) using antibodies raised against purified protein crystals from Bacillus thuringiensis subsp. tenebrionis.

400 mg of each culture broth were weighed. 7 ml trisodium phosphate buffer (0.125 M, pH 12) was added to each sample. The suspensions were shaken for 1 hour in order to solubilize the delta endotoxin proteins.

The samples were then centrifuged at 3.500 rpm for 15 minutes and the supernatants were tested for delta-endotoxin by rocket immuno electrophoresis against antiserum raised against purified protein crystals from B. thuringiensis subsp.

tenebrionis. The amounts of delta endotoxin were determined relatively to a standard with known content of crystal protein.

The concentration of crystal protein was also determined by a photometric immuno assay. The crystal proteins were dissolved in an alkaline solution. The dissolved proteins were precipitated by their antibodies. The rate of this reaction was determined turbidimetrically. The amounts of delta-endotoxin were determined relatively to a standard with known content of crystal protein.

Crystal antigens for production of the antibodies used in the assays were obtained from crystals isolated from B. thuringiensis subsp. tenebrionis.

Polyclonal antibodies were raised by injecting rabbits subcutaneously every fortnight with 0.25 mg of crystal antigen.

The results obtained are shown in the following Tables Ia and Ib. Delta endotoxin yields are expressed as BTTU/g (units per g culture broth, determined by rocket immuno electrophoresis, RIE, or by a photometric immuno assay, PIA). The value used for pure B. thuringiensis subsp. tenebrionis crystal protein is 500,000 BTTU/g. The values indicated in table Ia below being averages of 6-7 independent fermentations, and those in Table Ib being averages of 3 independent fermentations.

Table Ia

Delta endotoxin production by strains of Bacillus thuringiensis subsp. tenebrionis in shakeflasks

	Delta endotoxin yield	
	RIE	PIA
Strain	BTTU/g	BTTU/g
DSM 2803	676	1293
NRRL-B 15939	747	1126
NB 178	986	1728
DSM 5526	1097	1860
DSM 5480	2382	4169

Table Ib

Delta endotoxin production by strains of Bacillus thuringiensis subsp. tenebrionis in shakeflasks

	Delta endotoxin yield
	RIE
Strain	BTTU/g
NB 197	1103
NB 198	1237
DSM 5480	2867

From Tables Ia and Ib it appears that DSM 5480 produces more than three times as much delta endotoxin as the original strain of Bacillus thuringiensis subsp. tenebrionis, DSM 2803 and "Bacillus thuringiensis subsp. san diego", strain NRRL-B15939 and more than twice the amount of delta endotoxin as the strains used today for the manufacture of commercial products of Bacillus thuringiensis subsp. tenebrionis.

Phase contrast microscopy, scanning electron microscopy and transmission electron microscopy of Bacillus thuringiensis subsp. tenebrionis, mutant DSM 5480 have revealed that the protein crystals produced by this mutant are much bigger than the corresponding protein crystals produced by Bacillus thuringiensis subsp. tenebrionis, strains DSM 2803,

DSM 5526, NB178 and NB 198, and "Bacillus thuringiensis subsp. san diego", strains NRRL-B 15939 and NB 197.

Culture broth of Bacillus thuringiensis subsp. tenebrionis, mutant DSM 5480 was tested for activity against colerado potato beetle larvae. The increased amount of delta-endotoxin produced by mutant DSM 5480 as determined by the immunological methods was reflected in the biological activity against colerado potato beetle larvae.

Example 3

In this example sporulation and parasporal crystal formation in B. thuringiensis subsp. tenebrionis, strains, DSM 2803, DSM 5526, NB 178 and NB 198, and mutant DSM 5480, and "B. thuringiensis subsp. san diego", strains NRRL-B 15939 and NB 197 were compared on solid medium and in liquid medium.

Each of the strains was grown for 2 days at 30°C on agar plates of the following composition expressed as gram per liter of distilled water

Peptone, Difco	5 g
Beef extract, Difco	3 g
Agar, Difco	20 g
pH	7.0

Each of the strains was also grown in liquid medium. All strains were grown for 17 hours at 30°C on agar slants. 5 ml of a suspension of cells from each strain were then transferred to 500 ml baffle bottom Erlenmeyer flasks each containing 100 ml of medium.

The medium consisted of the following components in the quantities indicated (expressed as grams per liter of tap-water).

Liquid medium:

Yeast extract	5 g
Tryptone	5 g
Glucose	1 g
KH_2PO_4	0.8 g
pH	7.0

The inoculated flasks were incubated at 30°C with shaking (250 rpm) for 96 hours.

The morphology of the strains on the solid medium and in the liquid medium was studied daily by phase contrast microscopy (x 2500). The number of spores and crystals were counted and the size of the parasporal crystals were determined. A few selected samples were also studied by scanning and transmission electron microscopy.

B. thuringiensis subsp. tenebrionis, strains DSM 2803, DSM 5526, NB 178 and NB 198, and "B. thuringiensis subsp. san diego", strains NRRL-B 15939 and NB 197 all sporulated well on both media. Before cell lysis each cell contained a spore and a parasporal crystal. The size of the crystals was from 0.4 to 0.9-1.1 µm in length by the time of cell lysis. The average size of the protein crystals being 0.6-0.7 µm in length.

Mutant DSM 5480 produced only few spores ($<10^6$ spores/ml) on the solid medium and in the defined liquid medium. Before cell lysis most cells contained a huge protein crystal but no spore. The size of the protein crystals was from 0.4-0.7 µm to 5.0 µm, the average size of the protein crystals being 2.2-2.3 µm in length.

Ultrastructural analysis of cells from these media by transmission electron microscopy revealed that the sporulation process in the mutant had been started but had not been completed by the time of cell lysis. The sporulation process had reached different stages in the various cells. In cells where the sporulation had only reached stage II (forespore septum formation) the protein crystals filled up the entire cells.

In the production medium (Example 2) the mutant produced a higher number of spores (10^7-10^8 spores/ml). In this medium the sporulation frequency of the mutant was 10-100 times lower than in the parent strain.

Thus the mutant has retained its ability to produce normal spores. However, the sporulation frequency of the mutant seems to be strongly dependent of the media.

The size of the protein crystals produced by the individual strains are shown in Tables IIa and IIb.

Table IIa

Size of the protein crystals produced by coleopteran active B. thuringiensis strains available today.

	Length of protein crystals in µm		
Strain	Minimum value	Maximum value	Mean value
DSM 2803	0.4	0.9	0.7
NRRL-B-15939	0.4	0.9	0.7
NB 178	0.5	0.9	0.7
DSM 5526	0.4	0.9	0.7
DSM 5480	0.7	5.0	2.3

Table IIb

Size of the protein crystals produced by coleopteran active B. thuringiensis strains available today.

	Length of protein crystals in µm		
Strain	Minimum value	Maximum value	Mean value
NB 197	0.4	0.7	0.6
NB 198	0.4	1.1	0.7
DSM 5480	0.4	4.2	2.0

From Tables IIa and IIb it is clear that mutant DSM 5480 produces much bigger protein crystals than any of the coleopteran active B.t. strains available today.

From the data obtained it appears that the regulation of delta endotoxin production in relation to sporulation has been changed in the mutant.

The mutant seems to produce the protein crystals prior to the development of spores hereby giving the cells a longer period for delta endotoxin production which result in the production of much bigger protein crystals by the time of cell lysis than in the parent strain.

Depending on the available nutrients and the size of the protein crystals in the cells by the time of sporulation a normal spore will be developed before the time of cell lysis.

Example 4

In this example the high yielding Btt mutant DSM 5480 was used to produce high potency products for the control of colorado potato beetle larvae.

DSM 5480 was fermented on the production fermentation medium described in example 2 in an aerated, stirred production fermentation tank. After 96 hours, the broth was recovered by centrifugation on a continuous centrifuge.

The concentrated cream which contains the active protein crystals was stabilized by addition of microbial preservatives and pH was adjusted to 5.0.

One portion of the concentrated cream was spray dried and later used for the formulation of wettable powder. The rest of the concentrated cream was used directly for formulation of two aqueous flowable concentrates (FC).

The wettable powder was formulated as described in Table III. The formulation of the two FC's is described in Table IV.

Table III NOVODOR® wettable powder formulation

Component	% by weight
Spray dried concentrated cream of Btt	40
Detergents	9
Anticaking agent	1
Inert filler	50

Table IV NOVODOR® FC formulations

Component	NOVODOR® FC 1 % by weight	NOVODOR® FC 2 % by weight
Btt concentrated cream	80	55
Preservatives	4	4
Antifreeze agents	9.1	19
Detergents	2.5	2.5
pH regulator	2.85	2.85
water	1.55	16.65
	100.0	100.0

When using a value of 500,000 BTTU/g of pure crystal protein the content of active crystal protein in the formulations are:

		% Btt crystal protein
NOVODOR® WP	70.8 KBTTU/g	14.16
NOVODOR® FC1	24.7 KBTTU/g	4.94
NOVODOR® FC2	14.2 KBTTU/g	2.84

The detergents were chosen among the wide selection of suspension aids and wetting agents normally used in agricultural pesticide products.

The anticaking agent is a hydrophilic silica and the inert filler was chosen from the generally used inert fillers such as bentonites, inorganic salts or clays.

The preservates used in the FC's were chosen from the group of food and cosmetic preservatives. The pH regulator is an inorganic acid.

Example 5

A field trial was conducted to prove the biological effect of the high yielding Btt mutant DSM 5480 on the main target pest, colorado potato beetle larvae. In comparison with the two commercial products Trident® and M-one®. The crop was potatoes.

The crop was sprayed 3 times on July 20th, July 27th and August 3rd (2nd generation larvae).

The products and dosages used were:

Product	volume/acre	Potency KBTTU/g	% Btt crystal protein in the formulation
NOVODOR® FC 2	1 qt/acre	14.2	2.84
	1.5 qts/acre	14.2	2.84
	2.5 qts/acre	14.2	2.84
	3.0 qts/acre	14.2	2.84
TRIDENT®	4 qts/acre	5.5	1.10
M-one®	2 qts/acre	8.9	1.78

The mean % control of CPB larvae compared to the untreated control is given in table V. The colorado potato beetle pressure was very heavy in the untreated control: 370 larvae per 20 plants on August 1st and 904 larvae per 20 plants on August 8th.

Table V

Treatment			% control August 1st	% control August 8th
NOVODOR® FC 2	1	qt	99	99
NOVODOR® FC 2	1.5	qts	95	100
NOVODOR® FC 2	2.5	qts	98	99
NOVODOR® FC 2	3	qts	100	100
TRIDENT®	4	qts	94	98
M-one®	2	qts	98	98

These results clearly show that products made with the high yielding mutant DSM 5480 are effective for the control of colorado potato beetle larvae in the field. The crystal protein produced by the high yielding strain is fully active as 1.5 qts NOVODOR® FC give as good results as Trident at 4 qts and as good as M-one at 2 qts.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 5, line 23 of the description [1]

A. IDENTIFICATION OF DEPOSIT [3]

Further deposits are identified on an additional sheet ☒ [3]

Name of depositary institution [4]

DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH

Address of depositary institution (including postal code and country) [4]

Mascheroder Weg 1b, D-3300 Braunschweig, Federal Republic of Germany

Date of deposit [5]	Accession Number [6]
10 August, 1989	DSM 5480

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC) until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or is deemed to be withdrawn.

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

Ulla Gertsen
Head Clerk

Ulla Gertsen
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 6, line 2 of the description [1]

A. IDENTIFICATION OF DEPOSIT [3]

Further deposits are identified on an additional sheet ☒ [3]

Name of depositary institution [4]

DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH

Address of depositary institution (including postal code and country) [4]

Mascheroder Weg 1b, D-3300 Braunschweig, Federal Republic of Germany

Date of deposit [5]	Accession Number [6]
14 September, 1989	DSM 5526

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC) until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or is deemed to be withdrawn.

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

Ulla Gertsen
Head Clerk
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

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PATENT CLAIMS

1. A mutant or variant of Bacillus thuringiensis capable of producing high amounts of insecticidal delta-endotoxins as compared to its parent strain.

2. The mutant or variant according to claim 1, wherein the mutant or variant B. thuringiensis is capable of producing twice as much or more of insecticidal delta-endotoxins as compared to its parent strain.

3. The mutant or variant according to claim 1 or 2, wherein the mutant or variant B. thuringiensis belongs to the pathotype C of B. thuringiensis

4. The mutant or variant according to claim 1, 2 or 3, wherein the mutant or variant B. thuringiensis will have an activity directed towards the same pest insects as its parent B. thuringiensis delta-endotoxins, such as against lepidopterans (mutants from B. thuringiensis subsp. kurstaki and/or subsp. aizawai), dipterans (mutants from B. thuringiensis subsp. israelensis) or coleopterans (mutants from B. thuringiensis subsp. tenebrionis).

5. The mutant or variant according to claim 1, 2, 3 or 4, wherein the B. thuringiensis belongs to the subspecies tenebrionis, and the delta-endotoxin produced is active against coleopterans.

6. The mutant or variant according to claim 5, wherein the mutated or variated B. thuringiensis subsp. tenebrionis is capable of producing more than three times as much delta endotoxin as the strain DSM 2803.

7. The mutant or variant according to claim 5 or 6, wherein the mutated or variated B. thuringiensis subsp. tenebrionis strain is capable of producing a parasporal crystal

having a mean edge length that is at least twice as long as the mean edge length of the parasporal crystal of its parent strain.

8. The mutant or variant according to claim 7, wherein the mutated or variated B. thuringiensis subsp. tenebrionis strain is capable of producing a parasporal crystal having a mean edge length of 2 μm or more.

9. The mutant or variant according to any of the claims 4 to 8, wherein the mutated or variated B. thuringiensis subsp. tenebrionis strain shows a sporulation frequency 10 to 100 or even 10^6 times lower than the sporulation frequency of the parent strain.

10. The mutant or variant according to claim 9, wherein the mutated B. thuringiensis subsp. tenebrionis strain shows a sporulation frequency 10 to 100 or even 10^6 times lower than the sporulation frequency of the strain DSM 2803.

11. The mutant according to claim 1, wherein the mutant is the deposited mutant B. thuringiensis subsp. tenebrionis, DSM 5480.

12. Use of a mutated or variated strain according to any of the claims 1 to 11 for the production of insecticidal B. thuringiensis products, whereby the mutant or variant B. thuringiensis strain is cultivated in a suitable culture medium comprising sources for carbon, nitrogen, and other components for a suitable period of time, whereafter an insecticidal product comprising the delta endotoxins are recovered alone or in combination with cells and/or spores from the culture medium, and this product optionally is admixed with agriculturally acceptable excipiens.

13. A pesticidal composition comprising the B. thuringiensis delta endotoxin product obtained in accordance with claim 12 as an active component.

14. The composition according to claim 13, wherein the delta endotoxin product is utilized either alone or in combination with other biocidally active products.

15. The pesticidal composition or preparation according to claim 13 or 14, comprising the B. thuringiensis delta endotoxin product in admixture with an agriculturally acceptable diluent or carrier.

16. The pesticidal composition according to claim 13, 14 or 15 having a potency more than twice that of pesticidal compositions produced with the parent strain.

17. A liquid pesticidal composition having a potency of at least 15,000 BTTU/g, corresponding to at least 3% w/w coleopteran insecticidal crystal protein.

18. A dry pesticidal composition having a potency of at least 50,000 BTTU/g, corresponding to at least 10% w/w coleopteran insecticidal crystal protein.

19. A pesticidal composition produced from DSM 5480 having at least twice the potency of pesticidal compositions produced from DSM 2803 or other coleopteran active Btt strains.

20. A composition according to any of claims 13-19, wherein the diluent or carrier in the composition is a solid or a liquid optionally in association with a surface-active agent.

21. A method of controlling pests, wherein a pesticidal composition according to any of the claims 13 to 20 is applied to an area infected with said pest.

22. The method of claim 21, wherein the pest belongs to the group comprising lepidopterans, dipterans, and coleopterans.

23. The method of claim 22, wherein the pest is a coleopteran, especially colorado potato beetle.

24. The method of claim 22 or 23, wherein the pest can be controlled by the application of 1 quart per acre of a liquid pesticidal composition.

25. The method of claim 22 or 23, wherein the pest can be controlled by the application of 0.5 lb. per acre of a dry pesticidal composition.

26. A method of mutating Bacillus thuringiensis strains and selecting such mutants that are capable of producing substantially larger amounts of delta endotoxins than their parent strains, wherein the parent strain is:

i) treated with a mutagen,

ii) the thus treated mutants are grown in a medium suitable for the selection of asporogenous and/or oligosporogenous strains,

iii) translucent colonies are selected and grown in a medium that does not fluidize on heating, and

iv) truly asporogenous strains are deselected by subjecting the colonies to a heat treatment.

27. The method of claim 26, wherein the thus selected colonies are grown in a normal production medium, and a final selection for strains capable of increasing the delta endotoxin production is performed.

28. The method of claim 26 or 27, wherein in step (i) the mutagen is any suitable chemical mutagen, such as N-methyl-N'-nitro-N-nitrosoguanidine, or ethyl methanesulfonate.

29. The method of claim 26 or 27, wherein the parent strain in step (i) is treated with a suitable electromagnetic radiation, such as γ-, X-ray-, or UV-radiation.

30. The method of any one of claims 26 to 29, wherein in step (ii) said medium is a modified nutrient sporulation medium including phosphate (NSMP medium).

31. The method of any one of the claims 26 to 30, wherein in step (iv) said medium is a NSMP medium supplemented with $MgCl_2$ and Gelrite, Kelco.

32. The method of any one of the claims 26 to 31, wherein said parent strain belongs to the group comprising B. thuringiensis subsp. kurstaki, B. thuringiensis subsp. aizawai, B. thuringiensis subsp. israelensis and B. thuringiensis subsp. tenebrionis.

33. The method of claim 32, wherein said parent strain is a B. thuringiensis subsp. tenebrionis strain.

34. The method of claim 33, wherein said parent strain is B. thuringiensis subsp. tenebrionis strain DSM 5526.

35. A method of selecting variants or natural mutants of Bacillus thuringiensis strain capable of producing substantially larger amounts of delta endotoxins than their parent strains, wherein the parent strain is:

i) cultured in a liquid medium,

ii) the culture broth from (i) is spread on a medium suitable for the selection of asporogenous and/or oligosporogenous strains,

iii) translucent colonies are selected and grown in a medium that does not fluidize on heating, and

iv) truly asporogenous strains are deselected by subjecting the colonies to a heat treatment.

36. The method of claim 35, wherein the thus selected colonies are grown in a normal production medium, and a final selection for strains capable of increasing the delta endotoxin production is performed.

37. The method of any one of claims 35 to 36, wherein in step (ii) said medium is a modified nutrient sporulation medium including phosphate (NSMP medium).

38. The method of any one of the claims 35 to 37, wherein in step (iv) said medium is a NSMP medium supplemented with $MgCl_2$ and Gelrite, Kelco.

39. The method of any one of the claims 35 to 38, wherein said parent strain belongs to the group comprising B. thuringiensis subsp. kurstaki, B. thuringiensis subsp. aizawai, B. thuringiensis subsp. israelensis and B. thuringiensis subsp. tenebrionis.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 90/00294

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 1/20, A 01 N 63/00//(C 12 N 1/20, C 12 R 1:07)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N; A 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	EP, A1, 0366398 (MYCOGEN CORPORATION) 2 May 1990, see page 5, line 12- - page 16 --	1-5,26-39
X	EP, A2, 0228228 (MYCOGEN CORPORATION) 8 July 1987, see the whole document --	1-39
X	Chemical Abstracts, volume 80, no. 3, 21 January 1974, (Columbus, Ohio, US), Dulmage, Howard T et al.: "HD-187, a new isolate of Bacillus thuringiensis that produced high yields of alpha-endotoxin ", see page 323, abstract 13629p, & J. Invertebr. Pathol. 1973, 22(2), 273- 277 --	1-39

* Special categories of cited documents:[10]

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

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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

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

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26th February 1991	1991 -02- 28
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Yvonne Siösteen

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	EP, A1, 0325037 (IMPERIAL CHEMICAL INDUSTRIES PLC) 26 July 1989, see the whole document --	1-39
X	Applied and Environmental Microbiology, Vol. 43, No. 6, June 1982 Yoshiharu Wakisaka et al.: "Asporogenous Bacillus thuringiensis Mutant Producing High Yields of alpha-Endotoxin", see o 1498, right column, line 1-4	26-39
A	--	1-25
A	EP, A1, 0278035 (BASF AKTIENGESELLSCHAFT) 17 August 1988, see the whole document --	1-39
A	EP, A1, 0330342 (MYCOGEN CORPORATION) 30 August 1989, see the whole document --	1-39
A	EP, A1, 0328383 (MYCOGEN CORPORATION) 16 August 1989, see the whole document --	1-39
A	DE, A1, 3346138 (BOEHRINGER MANNHEIM GMBH) 11 July 1985, see the whole document --	1-39
A	US, A, 4764372 (CORINNA HERNSTADT ET AL.) 16 August 1988, see the whole document --	1-39

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 90/00294

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-01-31
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A1- 0366398	90-05-02	NONE		
EP-A2- 0228228	87-07-08	JP-A-	62146593	87-06-30
EP-A1- 0325037	89-07-26	AU-D-	2696788	89-06-29
		JP-A-	1273581	89-11-01
EP-A1- 0278035	88-08-17	DE-A-	3541893	87-06-11
EP-A1- 0330342	89-08-30	US-A-	4966765	90-10-30
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DE-A1- 3346138	85-07-11	CA-A-	1268133	90-04-24
		EP-A-	0149162	85-07-24
		JP-A-	60156382	85-08-16
		SU-A-	1448995	88-12-30
		US-A-	4766203	88-08-23
		US-A-	4851340	89-07-25
		US-A-	4889918	89-12-26
		US-A-	4973676	90-11-27
US-A- 4764372	88-08-16	EP-A-	0202739	86-11-26
		US-A-	4797276	89-01-10