638208

FORM 1

SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

APPLICATION FOR A STANDARD PATENT

Ciba-Geigy AG, incorporated in Switzerland, of Klybeckstrasse 141, 4002 Basle, SWITZERLAND, hereby apply for the grant of a standard patent for an invention entitled:

Bacillus Thuringiensis Transformation

which is described in the accompanying complete specification.

Details of basic application(s):-

Basic Applic. No:	Country:	Application Date:
1946/88-5	CH	20 May 1988
3279/88-2	CH	2 September 1988
180/89-8	CH	20 January 1989

The address for service is:-

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DATED this ELEVENTH day of MAY 1989

Ciba-Geigy AG

By:

Registered Patent Attorney

TO: THE COMMISSIONER OF PATENTS
OUR REF: 95078
S&F CODE. 52760

S007586 19/05/89

5845/2

AU Case 5-17038/1-3/= FORM 8

COMMONWEALTH OF AUSTRALIA

Patents Act 1952 - 1969

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION FOR A PATENT

In support of the Convention Application made by CIBA-GEIGY AG for a patent for an invention entitled:

Bacillus thuringiensis transformation

I, Werner Waldegg of CIBA-GEIGY AG, Klybeckstrasse 141, 4002 Basle, Switzerland do solemnly and sincerely declare as follows:

1. I am authorised by the applicant for the patent to make this declaration on its behalf.

2. The basic application(s) as defined by Section 141 on the Act was (were) made in Switzerland
on May 20, 1988,
on September 2, 1988 and
on January 20, 1989

by CIBA-GEIGY AG, 4002 Basle, Switzerland

3. Walter Schurter, Burgfeldermattweg 57, 4123 Allschwil, Switzerland,
Martin Geiser, Hauptstrasse 3A, 4107 Ettingen, Switzerland and
Danièle Mathé, 39, Rue d'Istein, 68870 Bartenheim la Chaussée, France

~~is~~ (are) the actual inventor(s) of the invention and the facts upon which the applicant is entitled to make the application are as follows: The said applicant is the assignee of the actual inventor(s).

4. The basic application(s) referred to in paragraph 2 of this Declaration was (were) the first application(s) made in a Convention country in respect of the invention the subject of the application.

DECLARED at Basle, Switzerland on
April 28, 1989

CIBA-GEIGY AG

W. Waldegg

Werner Waldegg
Single Signature, by special power

To: The Commissioner of Patents

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BACILLUS THURINGIENSIS TRANSFORMATION

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(57) Claim

1. A process for inserting and cloning DNA sequences in gram positive bacteria selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus*, comprising:

(a) isolating the DNA to be introduced;

(b) cloning the thus isolated DNA in a cloning vector that is capable of replicating in a bacterial host cell selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus* cells in a heterologous cloning system;

(c) directly introducing the thus cloned vector DNA into the said bacterial cell via electroporation at a transformation rate sufficient to overcome the restriction barrier present in the said bacterial cells; and

(d) cultivating the thus transformed bacterial cells and isolating the thus cloned vector DNA.

29. A bifunctional vector when used in a process according to any one of claims 1 or 2 that, apart from being capable of replicating in bacterial cells selected from the group consisting of *B. thuringiensis* and *B. cereus* cells, are capable of replicating in at least one other heterologous host organisms and that is identifiable in both the

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homologous and the heterologous host system and that comprises under the control of expression sequences that are capable of functioning in bacterial cells selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus* cells a structural gene encoding a δ-endotoxin polypeptide that occurs naturally in *B. thuringiensis*, or for a polypeptide that has substantial structural homologies therewith and has still substantially the toxicity properties of the said crystalline δ-endotoxin polypeptide.

35. A bacterial host cell selected from the group consisting of *B. thuringiensis* and *B. cereus* cells prepared by a method as described in any one of claims 1 or 2 comprising a bifunctional vector according to any one of claims 29 to 33.

38. A method of controlling insects which comprises treating insects or their habitat

(a) with a bacterial host cell according to claim 35, or with a mixture thereof, or alternatively

(b) with a cell-free crystall-body preparation containing a protoxin that is produced by a bacterial host cell according to claim 35.

41. A composition for controlling insects, comprising

(a) a bacterial host cell according to claim 35, or a mixture thereof, or alternatively

(b) a cell-free crystall-body preparation containing a protoxin that is produced by a bacterial host cell according to claim 35, together with carriers, dispersing agents or carriers and dispersing agents conventionally employed.

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S & F Ref: 95078

FORM 10

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE:

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Related Art:

Name and Address of Applicant: Ciba-Geigy AG
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Complete Specification for the invention entitled:

Bacillus Thuringiensis Transformation

The following statement is a full description of this invention, including the best method of performing it known to me/us

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5-17038/1-3/=

Bacillus thuringiensis transformation

Abstract of the Disclosure

The present invention describes a process that for the first time renders possible a direct and targeted genetic manipulation of Bacillus thuringiensis and the closely related B. cereus using recombinant DNA technology.

The present invention also relates to the construction of plasmids and "shuttle" vectors and to the B. thuringiensis strains that have been transformed therewith.

Also described is a process for the direct cloning, expression and identification of genes in B. thuringiensis and in the closely related B. cereus.

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Bacillus thuringiensis transformation

The present invention describes a process that for the first time renders possible a direct and targeted genetic manipulation of Bacillus thuringiensis and the closely related B. cereus using recombinant DNA technology, based on an efficient transformation process for the said Bacillus species.

The present invention furthermore relates to the construction of plasmids and "shuttle" vectors and to the B. thuringiensis and/or B. cereus strains that have been transformed therewith.

The present invention also relates to a process for inserting and, if desired, expressing genes or other useful DNA sequences in Bacillus thuringiensis and/or Bacillus cereus, but especially to a process for inserting and expressing protoxin genes.

The present invention also includes a process for the direct cloning and, if desired, expression and identification of novel genes or other useful DNA sequences in Bacillus thuringiensis and/or Bacillus cereus, as a result of which it is possible for the first time to establish gene banks directly in Bacillus thuringiensis and/or Bacillus cereus and to express them therein.

Bacillus thuringiensis belongs to the large group of gram-positive, aerobic, endospore-forming bacteria. Unlike the very closely related species of Bacillus, B. cereus and B. anthracis, the majority of the hitherto known B. thuringiensis species produce in the course of their sporulation a parasporal inclusion body which, on account of its crystalline structure, is generally referred to also as a crystalline body. This crystalline body is composed of insecticidally active crystalline protoxin proteins, the so-called δ-endotoxin.

These protein crystals are responsible for the toxicity to insects of B. thuringiensis. The δ-endotoxin does not exhibit its insecticidal activity until after oral intake of the crystalline body, when the latter is dissolved in the alkaline intestinal juice of the target insects and the actual toxic component is released from the protoxin as a result of limited proteolysis caused by the action of proteases from the digestive tract of the insects.

The δ-endotoxins of the various B. thuringiensis strains are distinguished by high specificity with respect to certain target insects, especially with respect to various Lepidoptera, Coleoptera and Diptera larvae, and by their high degree of activity. Further advantages in using δ-endotoxins of B. thuringiensis reside in the obvious difficulty that the target insects have in developing resistance to the crystalline protein and in the fact that the toxins are harmless to humans, other mammals, birds, fish and insects, with the exception of the above-mentioned target insects.

The insecticidal potential of B. thuringiensis protoxins was recognised very early on. Since the end of the twenties B. thuringiensis preparations have been used as bioinsecticides for controlling various diseases caused by insects in cultivated plants. With the discovery of B. thuringiensis var. israelensis by [1] Goldberg and Margalit (1977) and B. thuringiensis var. tenebrionis by [2] Krieg et al. (1983) it was possible for the range of use of B. thuringiensis to be extended even to mosquito and beetle larvae.

With the introduction of genetic engineering and the new possibilities resulting from it, the field of B. thuringiensis toxins has received a fresh impetus.

For example, the cloning of δ-endotoxin genes in foreign host organisms, such as, for example, in E. coli, is already routine. The result of this, meanwhile, has been that the DNA sequences of a whole series of

δ-endotoxin genes are now known (for example [3]Schnepf H.E. and Whiteley H.R., 1981; [4]Klier A. et al., 1982; [5]Geiser M. et al., 1986; [6]Haider M.Z. et al., 1987).

Most of the B. thuringiensis species contain several genes that code for an insecticidally active protein. These genes, which are expressed only during the sporulation phase, are in the majority of cases located on large transferable plasmids (30 - 150 Md) and can therefore very easily be interchanged between the various B. thuringiensis strains and between B. thuringiensis and B. cereus, provided these are compatible ([7]Gonzalez J.M. et al., 1982).

The protoxin genes of B. thuringiensis var. kurstaki belong to a family of related genes, various of which have already been cloned and sequenced. This work has been carried out especially in an E. coli cloning system.

The cloning of B. thuringiensis genes has thus so far essentially been limited to some few and exclusively heterologous host systems, of which the E. coli system is the best researched and understood.

In the meantime, however, reports have also been published on the successful cloning and expression of protoxin genes in other host systems, such as, for example, in B. subtilis ([4]Klier et al., 1982), Pseudomonas fluorescens ([8]Obukowicz M.G. et al., 1986), and Saccharomyces cerevisiae (EP 0 238 441). The insertion and expression of the δ-endotoxin gene in plant host cells has also been successful (EP 0292 435).

In cloning in E. coli, advantage is taken of the fact that some protoxin genes happen to contain, in addition to gram-positive promoters, also an E. coli-like promoter. These promoter-like DNA sequences make it possible for the B. thuringiensis protoxin genes to be expressed also in heterologous host systems, provided these are capable of recognising the above-mentioned control sequences.

After breaking open the host cells, the expressed protoxin proteins can then be isolated and identified using known methods.

It has since been demonstrated, however, that E. coli-like promoters are not present in all protoxin genes ([9] Donovan et al., 1988), and consequently so far only very specific protoxin genes that meet the above-mentioned prerequisites can be expressed and thus identified in heterologous host systems.

The cloning of genes outside the natural host organism and the use of these strains as bioinsecticides in practice is thus associated with a number of disadvantages, some of which are serious:

a) Expression of B. thuringiensis protoxin genes from the native expression sequences is possible only in certain cases.

b) Generally there is no, or only a slight, secretion of expressed foreign proteins.

c) Correct folding of the δ-endotoxins is not always guaranteed in the reducing medium of heterologous host cells, and this could result in an undesirable change in the specific activity or in the host range of the toxins.

d) If expression occurs at all, the expression rates of the cloned foreign genes among the native expression sequences are mostly only low.

[3);10)] Schnepf and Whitley (1981; 1985) estimate that the B. thuringiensis toxin cloned in E. coli constitutes only 0.5 % to 1 % of the total cell protein of E. coli, whereas the crystalline protein in B. thuringiensis amounts to between 30 % and 40 % of the dry weight of sporulating cultures. These considerable discrepancies between the expression rates may possibly be attributed to the lack of sporulation-specific control signals in the heterologous host systems and to difficulties in the recognition of the B. thuringiensis promoters and/or to problems in the post-translational modification of the toxin molecule by the foreign host.

e) Many of the host strains generally used for expression are toxicologically not as harmless as B. thuringiensis and B. cereus.

f) B. thuringiensis and B. cereus form a natural major component of microbial soil flora, which is not true of most of the host strains generally used for expression.

The problems and difficulties mentioned above could be overcome if the said B. thuringiensis genes could be cloned directly in the homologous host system where it is possible to use the natural gram-positive promoters of the protoxin genes for the expression.

As yet, however, there is no process that would make B. thuringiensis, this very important bacterium from the commercial point of view, amenable to direct genetic modification, and that would consequently render possible, for example, efficient reinsertion of a cloned protoxin gene into a B. thuringiensis strain.

The reason for this can be regarded, in particular, as being the fact that the development of an efficient transformation system for B. thuringiensis and the closely related B. cereus that would ensure adequately high transformation rates and consequently render possible the application also to B. thuringiensis of established rDNA techniques has not as yet been successful.

The processes used so far to produce new B. thuringiensis strains having novel insecticidal properties are based chiefly on transfer by conjugation of plasmid-encoded protoxin genes.

Successful reinsertion of a cloned B. thuringiensis crystalline protein gene into B. thuringiensis has to date been described only in one case ([11] Klier A. et al., 1983), but in that case too, owing to the lack of a suitable transformation system for B. thuringiensis, it was necessary to resort to transfer by conjugation between B. subtilis and B. thuringiensis. Furthermore, in this process described by Klier et al. E. coli is used as intermediate host.

The processes of transfer by conjugation, however, have a whole series of serious disadvantages that makes them appear unsuitable for routine use for the genetic modification of B. thuringiensis and/or B. cereus.

a) The transfer of plasmid-encoded protoxin genes by conjugation is possible only between B. thuringiensis strains and between B. cereus and B. thuringiensis strains that are compatible with one another.

b) With transfer of plasmids by conjugation between more distant strains, often only a low transfer frequency is achieved.

c) There is no possible way of regulating or modifying the expression of the protoxin genes.

d) There is no possible way of modifying the gene itself.

e) If several protoxin genes are present in one strain the expression of individual genes may be greatly reduced as a result of the so-called gene-dosage effect.

f) Instabilities may arise as a result of a possible homologous recombination of related protoxin genes.

Alternative transformation processes, which have since been used routinely for many gram-positive organisms, have proved unsuitable both for B. thuringiensis and for B. cereus.

One of the above-mentioned processes is, for example, the direct transformation of bacterial protoplasts by means of polyethylene glycol treatment, which has been used successfully in the case of many Streptomyces strains ([12]Bibb J.J. et al., 1978) and in the case of B. subtilis ([13]Chang S. and Cohen S.N., 1979), B. megaterium ([14]Brown B.J. and Carlton B.C., 1980), Streptococcus lactis ([15]Kondo J.K. and McKay L.L., 1984), S. faecalis ([16]Wirth R. et al.), Corynebacterium glutamicum ([17]Yoshihama M. et al., 1985) and numerous other gram-positive bacteria.

To use this process, the bacterial cells must first of all be converted to protoplasts, that is to say the cell walls are digested using lytic enzymes.

Another prerequisite for the success of this direct transformation process is the expression of the newly introduced genetic information and the regeneration of the transformed protoplasts on complex solid media before successful transformation can be detected, for example using a selectable marker.

This transformation process has proved unsuitable for B. thuringiensis and the closely related B. cereus. As a result of the high resistance of B. thuringiensis cells to lysozyme and the very poor regenerability of the protoplasts to intact cell wall-containing cells, the rates of transformation achievable remain low and difficult to reproduce ([18]Alikhanian S.J. et al., 1981; [19]Martin P.A. et al., 1981; [20]Fischer H-M et al., 1984).

With this process it is possible therefore, at the most, for very simple plasmids, which are unsuitable for work with recombinant DNA, to be inserted at a low frequency into B. thuringiensis or B. cereus cells.

Individual reports on satisfactory rates of transformation that it has been possible to achieve using the afore-described process rely on the formulation of very complex optimising programmes, but these programmes are always applicable specifically to one particular B. thuringiensis strain only and involve high expenditure in terms of time and money ([21]Schall D., 1986). Such processes are therefore unsuitable for routine application on an industrial scale.

As the intensive research work in the field of B. thuringiensis genetics demonstrates, there is substantial interest in developing new processes that would make B. thuringiensis or the closely related B. cereus amenable to direct genetic modification and would thus, for example,

render possible the cloning of protoxin genes in the natural host system. Despite this research there are still no satisfactory solutions to the existing difficulties and problems.

Suitable transformation processes that render possible a rapid, efficient and reproducible transformation of B. thuringiensis and/or B. cereus with an adequately high transformation frequency are not available currently, and neither are suitable cloning vectors that permit the application also to B. thuringiensis of the recombinant DNA techniques already established for other bacterial host systems. The same is true for B. cereus.

This object has now surprisingly been achieved within the scope of the present invention by the use of simple process steps, some of which are known.

The present invention thus relates to a novel process, based on recombinant DNA technology, that for the first time renders possible a direct, specifically controlled and reproducible genetic manipulation of B. thuringiensis and of B. cereus by transforming Bacillus thuringiensis and/or Bacillus cereus with high efficiency by means of a simple transformation process using a recombinant DNA that is suitable for the said genetic manipulation of Bacillus thuringiensis and/or Bacillus cereus.

The present invention furthermore relates to a process for inserting, cloning and expressing genes or other useful DNA sequences, but especially protoxin genes, in B. thuringiensis and/or B. cereus, which comprises:

a) isolating the said genes or DNA;

b) if desired operably joining the isolated genes or DNA to expression sequences that are capable of functioning in Bacillus thuringiensis and/or B. cereus;

c) introducing the genetic constructs from section b) into Bacillus thuringiensis and/or B. cereus cells by transformation using suitable vectors; and

d) if desired expressing a corresponding gene product and, if desired, isolating it.

The present invention also includes a direct process for cloning, expressing and identifying genes or other useful DNA sequences, but especially protoxin genes, in B. thuringiensis and/or B. cereus, which comprises:

a) digesting the total DNA of Bacillus thuringiensis using suitable restriction enzymes;

b) isolating from the resulting restriction fragments those of suitable size;

c) inserting the said fragments into a suitable vector;

d) transforming Bacillus thuringiensis and/or B. cereus cells with the said vector; and

e) locating novel DNA sequences using suitable screening methods and, if desired, isolating them from the transformants.

Apart from structural genes it is obviously also possible for any other useful DNA sequences to be used in the process according to the invention, such as, for example, non-coding DNA sequences that have a regulatory function, such as, for example, "anti-sense DNA".

The process of the invention thus opens up a large number of new possibilities that are of extraordinary interest from both scientific and commercial points of view.

For example, it is now possible for the first time to obtain information on a genetic level about the regulation of δ-endotoxin synthesis, especially in respect of sporulation.

Also, it should now be possible to clarify at which position of the toxin molecule the region(s) responsible for the toxicity to insects is (are) located, and to what extent this (these) is (are) also associated with the host specificity.

Knowledge of the molecular organisation of the various toxin molecules and of the toxin genes coding for these molecules from the various species of B. thuringiensis is of extraordinary practical interest for a controlled genetic manipulation of those genes, which is now possible for the first time using the process of the invention.

In addition to a controlled modification of the δ-endotoxin genes themselves, the novel process of the invention permits also the manipulation of the regulatory DNA sequences controlling the expression of those genes, as a result of which the specific properties of the δ-endotoxins, such as, for example, their host specificity, their resorption behaviour inter alia, can be modified in a specifically controlled manner, and the production rates of the δ-endotoxins can be increased, for example by the insertion of stronger and more efficient promoter sequences.

By specifically controlled mutation of selected genes or subgenes in vitro it is thus possible to obtain new B. thuringiensis and/or B. cereus variants.

Another possible way of constructing novel B. thuringiensis and/or B. cereus variants comprises splicing together genes or portions of genes that originate from different B. thuringiensis sources, resulting in B. thuringiensis and/or B. cereus strains with a broader spectrum of use. It is also possible for synthetically or semi-synthetically produced toxin genes to be used in this manner for constructing new B. thuringiensis and/or B. cereus varieties.

In addition, the process according to the invention renders possible for the first time, as a result of the pronounced increase in the transformation frequency and the simplicity of the process, the establishment of gene banks and the rapid screening of modified and new genes in B. thuringiensis and/or B. cereus.

In particular, the process of the invention now for the first time renders possible direct expression of gene banks in B. thuringiensis and/or B. cereus and the identification of new protoxin genes in B. thuringiensis using known, preferably immunological or biological processes.

The subject of the present invention is accordingly a process, based on a pronounced increase in the efficiency of B. thuringiensis/B. cereus transformation compared with known processes, that for the first time renders possible a direct genetic modification of the B. thuringiensis and/or B. cereus genome.

In particular, the present invention relates to a process for the transformation of B. thuringiensis and/or B. cereus by inserting recombinant DNA, especially plasmid and/or vector DNA, into B. thuringiensis and/or B. cereus cells by means of electroporation.

Preferred is a process for the transformation of B. thuringiensis and/or B. cereus with DNA sequences coding for δ-endotoxin and DNA sequences coding for a protein that has substantially the insect-toxic properties of the said B. thuringiensis toxins.

The present invention also relates to the expression of DNA sequences that code for an δ-endotoxin, or for a protein that at least has substantially the insect-toxic properties of the B. thuringiensis toxin, in transformed B. thuringiensis and/or B. cereus cells.

The present invention also includes a process for the production of bifunctional vectors, so-called "shuttle" vectors, for B. thuringiensis and/or B. cereus, and the use of the said "shuttle" vectors for the transformation of B. thuringiensis and/or B. cereus cells.

Preferred is the construction of bifunctional vectors that in addition to replicating in B. thuringiensis and/or B. cereus also replicate in one or more other heterologous host systems, but especially in E. coli cells.

The present invention relates especially to a process for the production of "shuttle" vectors for B. thuringiensis and/or B. cereus that contain a DNA sequence coding for a δ-endotoxin polypeptide that occurs naturally in B. thuringiensis, or at least a polypeptide that is substantially homologous therewith, that is to say that at least has substantially the insect-toxic properties of the B. thuringiensis toxin. The present invention also includes the use of these "shuttle" vectors for the transformation of B. thuringiensis and/or B. cereus cells and the expression of the DNA sequences present on the said "shuttle" vectors, especially those DNA sequences that code for a δ-endotoxin of B. thuringiensis or at least for a protein that has substantially the insect-toxic properties of the B. thuringiensis toxins.

The present invention also includes the use of B. thuringiensis and/or B. cereus as general host organisms for cloning and expressing homologous and especially also heterologous DNA, or a combination of homologous and heterologous DNA.

This invention also relates to the above more closely characterised plasmids and "shuttle" vectors themselves, to the use thereof for the transformation of B. thuringiensis and/or B. cereus, and to B. thuringiensis and B. cereus cells that have been transformed with them.

Especially preferred within the scope of this invention are the bifunctional ("shuttle") vectors pXI61 (=pK61) and pXI93 (=pK93) which, introduced by transformation into B. thuringiensis var. kurstaki HD1cryB and into B. cereus 569K, have been deposited at the "Deutsche Sammlung von Mikroorganismen" (Braunschweig, Federal Republic of Germany), recognised as an International Depository, in accordance with the Budapest Treaty under the number DSM ~~4573~~ 4572 (pXI61, introduced by transformation into B. thuringiensis var. kurstaki HD1cryB) and DSM 4571 (pXI93, introduced by transformation into B. thuringiensis var. kurstaki HD1cryB) and DSM 4573 (pXI93, introduced by transformation into B. cereus 569K).

The present invention relates especially to novel *B. thuringiensis* and *B. cereus* varieties that have been transformed with a DNA sequence that codes for a delta-endotoxin of *B. thuringiensis* and that can be expressed, or transformed with a DNA sequence coding for at least one protein that has substantially the toxic properties of the B.thuringiensis toxins.

The transformed *B. thuringiensis* and *B. cereus* cells and the toxins produced by them can be used for the preparation of insecticidal compositions, to which the present invention also relates.

The invention also relates to methods of, and to compositions for, controlling insects using the above more closely characterised transformed *B. thuringiensis* and/or *B. cereus* cells or a cell-free crystalline body-(delta-endotoxin) preparation containing protoxins produced by the said transformed *Bacillus* cells.

According to a first embodiment of this invention, there is provided a process for inserting and cloning DNA sequences in gram positive bacteria selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus*, comprising:

(a) isolating the DNA to be introduced;

(b) cloning the thus isolated DNA in a cloning vector that is capable of replicating in a bacterial host cell selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus* cells in a heterologous cloning system;

(c) directly introducing the thus cloned vector DNA into the said bacterial cell via electroporation at a transformation rate sufficient to overcome the restriction barrier present in the said bacterial cells; and

(d) cultivating the thus transformed bacterial cells and isolating the thus cloned vector DNA.

According to a second embodiment of this invention, there is provided a process for inserting, cloning and expressing DNA sequences in gram positive bacteria selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus*, comprising:

(a) isolating the DNA to be introduced and optionally ligating the thus isolated DNA with expression sequences that are capable of functioning in bacterial cells selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus* cells;

(b) cloning the thus isolated DNA in a cloning vector that is capable of replicating in a bacterial host cell selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus* cells in a

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heterologous cloning system;

(c) directly introducing the thus cloned vector DNA into the said bacterial cell via electroporation at a transformation rate sufficient to overcome the restriction barrier present in the said bacterial cells; and

(d) cultivating the thus transformed bacterial cells and isolating the thus cloned vector DNA and the expressed gene product.

According to a third embodiment of this invention, there is provided a bifunctional vector to be used in a process according to the first or second embodiments that, apart from being capable of replicating in bacterial cells selected from the group consisting of *B. thuringiensis* and *B. cereus* cells, are capable of replicating in at least one other heterologous host organism and that is identifiable in both the homologous and the heterologous host system and that comprises under the control of expression sequences that are capable of functioning in bacterial cells selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus* cells a structural gene encoding a delta-endotoxin polypeptide that occurs naturally in *B. thuringiensis*, or for a polypeptide that has substantial structural homologies therewith and has still substantially the toxicity properties of the said crystalline delta-endotoxin polypeptide.

According to a fourth embodiment of this invention, there is provided the bifunctional vector pX193 (pK93) introduced into *B. thuringiensis* var. *kurstaki* HD1cryB (DSM 4571) and B.cereus 569K (DSM 4573).

According to a fifth embodiment of this invention, there is provided a bacterial host cell selected from the group consisting of *B. thuringiensis* and *B. cereus* cells prepared by a method as described in the first or second embodiments comprising a bifunctional vector according to the third embodiment.

According to a sixth embodiment of this invention, there is provided a method of controlling insects which comprises treating insects or their habitat

(a) with a bacterial host cell according to the fifth embodiment, or with a mixture thereof; or alternatively

(b) with a cell-free crystal-body preparation according to a protoxin that is produced by a bacterial host cell according to the fifth embodiment.

According to a seventh embodiment of this invention, there is provided a composition for controlling insects, comprising:

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(a) a bacterial host cell according to the fifth embodiment, or a mixture thereof; or alternatively

(b) a cell-free crystal-body preparation containing a protoxin that is produced by a bacterial host cell according to the fifth embodiment,

together with carriers, dispersing agents or carriers and dispersing agents conventionally employed.

According to an eighth embodiment of this invention, there is provided a process for the identification of new delta-endotoxin encoding genes in *Bacillus thuringiensis*, which process comprises

(a) digesting the total DNA of *Bacillus thuringiensis* using suitable restriction enzymes;

(b) isolating from the resulting restriction fragments those of suitable size;

(c) inserting the said fragments into a suitable vector;

(d) constructing a genomic DNA library by transforming *Bacillus thuringiensis* host cells with the said vector using a process according to the first embodiment;

(e) screening the thus obtainable DNA library for new delta-endotoxin encoding genes.

The following is a brief description of the Figures:

Figure 1: Transformation of *E. coli* HB 101 with pBR322 (o) and **B. thuringiensis* DH1cryB with pBC16(•) (△ number of surviving *HD1cryB cells).

Figure 2: Influence of the age of a **B. thuringiensis* HD1cryB culture on the transformation frequency.

Figure 3: Influence of the pH value of the PBS buffer solution on the transformation frequency.

Figure 4: Influence of the saccharose concentration of the PBS buffer solution on the transformation frequency.

Figure 5: Interdependence of the number of transformants and the amount of DNA used per transformation.

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Figure 6: Simplified restriction map of the "shuttle" vector *pXI61. The shaded region characterises the sequences originating from the gram-positive pBC16, the remainder originating from the gram-negative plasmid pUC8.

Figure 7: Simplified restriction map of *pXI93. The shaded region characterises the protoxin structural gene (arrow, Kurhdl) and the 5' and 3' non-coding sequences. The remaining unshaded part originates from the "shuttle" vector *pXI61.

Figure 8: SDS (sodium dodecyl sulfate)/polyacrylamide gel electrophoresis of extracts of sporulating cultures of *B. thuringiensis HDlcryB, B. cereus 569K and their derivatives. [1: *HDlcryB (pXI93), 2: *HDlcryB (pXI61), 3: *HDlcryB, 4: HDl, LBG B-4449, 5: *B. cereus 569K (pXI93), 6: 569K]

a) Comassie-dyed, M: molecular weight standard, MW: molecular weight (Dalton), arrow: position of the 130,000 Dalton protoxin.
b) Western blot of the same gel, to which there have been added polyclonal antibodies to the K-1 crystalline protein of B. thuringiensis HDl.
Positive bands were found with the aid of labelled anti-goat antibodies. Arrow: position of the 130,000 Dalton protoxin. Other bands: degradation products of the protoxin.

Figure 9: Transformation of B. subtilis LBG B-4468 with pBC16 plasmid DNA using the electroporation process optimised for B. thuringiensis. (o: transformants/µg plasmid DNA; •: number of living bacteria/ml)

* The internal reference pK selected for the nomenclature of the plasmids in the priority document has been replaced for the Auslandsfassung (foreign filing text) by the officially recognised designation pXI.

Also, the names for the asporogenic B. thuringiensis HDl mutants used in the Embodiment Examples have been changed from cryß to cryB.

An essential aspect of the present invention concerns a novel transformation process for B. thuringiensis and B. cereus based on the insertion of plasmid DNA into B. thuringiensis and/or B. cereus cells using electroporation technology, which is known per se.

All attempts up to the time of the present invention to apply the transformation processes already established for other bacterial host systems to B. thuringiensis and the closely related B. cereus having been frustrated, it is now possible within the scope of this invention to achieve surprising success using electroporation technology and accompanying steps.

This success must also be considered surprising and unexpected, especially since electroporation tests with B. thuringiensis protoplasts were carried out at an earlier date by a Soviet group ([22] Shivarova N. et al., 1983), but the transformation frequencies achieved were so low that this process was subsequently regarded as unusable for B. thuringiensis transformation and consequently received no further attention.

Building upon investigations into the process parameters critical for an electroporation of B. thuringiensis and/or B. cereus cells, it has now surprisingly been possible to develop a transformation process that is ideally adapted to the requirements of B. thuringiensis and B. cereus and results in transformation rates ranging from 10^6 to 10^8 cells/µg of plasmid DNA, but especially from 10^6 to 10^7 cells/µg of plasmid DNA.

Roughly equally high transformation rates with values from 10^2 to a maximum of 10^6 transformants/µg of plasmid DNA could hitherto be achieved only with the PEG (polyethylene glycol) transformation process described by [21] Schall (1986). High transformation rates remained restricted, however, to those B. thuringiensis strains for which the PEG process was specifically adapted in very time-consuming optimisation studies, which makes this process appear unsuitable for practical use.

Furthermore, the reproducibility of that process in practice is in many cases non-existent or poor.

In contrast, the process of the present invention is a transformation process that in principle is applicable to all B. thuringiensis and B. cereus strains, and that is less time-consuming, more rational and consequently more efficient than the traditional PEG transformation process.

For example, in the process of the invention it is possible to use. for example, whole intact cells, thus dispensing with the time-consuming production of protoplasts critical for B. thuringiensis and B. cereus and with the subsequent regeneration on complex nutrient media.

Furthermore, when using the PEG process, carrying out the necessary process steps can take up to a week, whereas with the transformation process of the invention the transformed cells can be obtained within a few hours (as a rule overnight).

Another advantage of the process of the invention concerns the number of B. thuringiensis and/or B. cereus cells that can be transformed per unit of time.

Whereas in the traditional PEG process only small aliquots can be plated out simultaneously in order to avoid inhibition of the regeneration as a result of the growth of the cells being too dense, when using the electroporation technique large amounts of B. thuringiensis and/or B. cereus cells can be plated out simultaneously.

This renders possible the detection of transformants even at very low transformation frequencies, which with the afore-described processes is not possible or is possible only with considerable expenditure.

Furthermore, amounts of DNA in the nanogram range are sufficient to obtain at least some transformants.

This is especially important if a very efficient transformation system is necessary, such as, for example, when using DNA material from E. coli, which on account of a strongly pronounced restriction system in B. thuringiensis cells can lead to a reduction of the transformation frequencies by a factor of 10^3 compared with B. thuringiensis DNA.

The transformation process of the invention, which is based essentially on electroporation technology known per se, is characterised by the following specific process steps:

a) Preparation of a cell suspension of suitable cell density in a culture medium suitable for growing B. thuringiensis cells and with aeration adequate for the growth of the cells;

b) separation of the cells from the cell suspension and resuspension in an inoculation buffer suitable for the subsequent electroporation;

c) addition of a DNA sample in a concentration suitable for the electroporation;

d) introduction of the batch described under points b) and c) into an electroporation apparatus;

e) one or more brief discharges of a capacitor across the cell suspension for the short term-production of high electric field strengths for a period that is adequate for transformation of B. thuringiensis and/or B. cereus cells with recombinant DNA;

f) optional reincubation of the electroporated cells;

g) plating out of the electroporated cells onto a suitable selection medium; and

h) selection of the transformed cells.

In a specific embodiment of the process of the invention that is preferred within the scope of the invention, the B. thuringiensis cells are first of all incubated in a suitable nutrient medium with adequate

aeration and at a suitable temperature, preferably of from 20°C to 35°C, until an optical density (OD_{550}) of from 0.1 to 1.0 is achieved. The age of the Bacillus cultures provided for the electroporation has a distinct effect on the transformation frequency. An optically density of the Bacillus cultures of from 0.1 to 0.3, but especially of 0.2, is therefore especially preferred. Attention is, however, drawn to the fact that it is also possible to achieve good transformation frequencies with Bacillus cultures from other growth phases, especially with overnight cultures (see Figure 2).

Generally, fresh cells or spores are used as starting material, but it is also equally possible to use deep-frozen cell material. The cell material is preferably cell suspensions of B. thuringiensis and/or B. cereus cells in suitable liquid media to which, advantageously, a certain amount of an "antifreeze solution" has been added.

Suitable antifreeze solutions are especially mixtures of osmotically active components and DMSO in water or a suitable buffer solution. Other suitable components that can be used in antifreeze solutions include sugars, polyhydric alcohols, such as, for example, glycerol, sugar alcohols, amino acids and polymers, such as, for example, polyethylene glycol.

If B. thuringiensis spores are used as starting material, they are first of all inoculated in a suitable medium and incubated overnight at a suitable temperature, preferably of from 25°C to 28°C, and with adequate aeration. This batch is then diluted and further treated in the manner described above.

To induce sporulation in B. thuringiensis it is possible to use any medium that causes such a sporulation. Within the scope of this invention a GYS medium according to [23] Yousten A.A. and Rogoff M.H., (1969) is preferred.

Oxygen is usually introduced into the culture medium by moving the culture, for example using a shaker, speeds of rotation of from 50 revs/min to 300 revs/min being preferred.

B. thuringiensis spores and vegetative microorganism cells are cultured within the scope of the present invention according to known generally customary processes, liquid nutrient media preferably being used for reasons of practicability.

The composition of the nutrient media may vary slightly depending on the strain of B. thuringiensis or B. cereus used. Generally, complex media with loosely defined, readily assimilable carbon (C-) and nitrogen (N-) sources are preferred, like those customarily used for culturing aerobic Bacillus species.

In addition, vitamins and essential metal ions are necessary, but these are usually contained in an adequate concentration as constituents or impurities in the complex nutrient media used.

If desired, the said constituents, such as, for example, essential vitamins and also Na^+, K^+, CU^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, $NH_4^{\oplus}$, PO_4^{3-}, SO_4^{2-}, Cl^-, CO_3^{2-} ions and the trace elements cobalt and manganese, zinc, etc., can be added in the form of their salts.

In addition to yeast extracts, yeast hydrolysates, yeast autolysates and yeast cells, especially suitable nitrogen sources are in particular soya meal, maize meal, oatmeal, edamine (enzymatically digested lactalbumin), peptone, casein hydrolysate, corn steep liquors and meat extracts, without the subject of the invention being in any way limited by this list of examples.

The preferred concentration of the mentioned N-sources is from 1.0 g/l to 20 g/l.

Suitable C-sources are especially glucose, lactose, sucrose, dextrose, maltose, starch, cerelose, cellulose and malt extract. The preferred concentration range is from 1.0 g/l to 20 g/l.

Apart from complex nutrient media it is obviously also possible to use semi- or fully-synthetic media that contain the above-described nutrients in a suitable concentration.

Apart from the LB medium preferably used within the scope of the present invention it is also possible to use any other culture medium suitable for culturing B. thuringiensis and/or B. cereus, such as, for example, Antibiotic Medium 3, SCGY medium, etc.. Sporulated B. thuringiensis cultures are preferably stored on GYS media (inclined agar) at a temperature of 4°C.

After the cell culture has reached the desired cell density, the cells are harvested by means of centrifugation and suspended in a suitable buffer solution that has preferably been cooled beforehand with ice.

In the course of the investigations, the temperature proved not to be critical and is therefore freely selectable within a broad range. A temperature range of from 0°C to 35°C, preferably from 2°C to 15°C and more especially a temperature of 4°C, is preferred. The incubation period of the Bacillus cells before and after electroporation has only a slight effect on the transformation frequency attainable (see Table 1). Only an excessively long incubation results in a decrease in the transformation frequency. An incubation period of from 0.1 to 30 minutes, especially of 10 minutes, is preferred. In the course of the investigations, the temperature proved not to be critical and is therefore freely selectable within a broad range. A temperature range of from 0°C to 35°C, preferably from 2°C to 15°C and more especially a temperature of 4°C, is preferred. This operation can be repeated one or more times. Buffer solutions that are especially suitable within the scope of this invention are osmotically stabilised phosphate buffers that contain as stabilising agent sugars such as, for example, glucose or saccharose, or sugar alcohols, such as, for example, mannitol, and have pH values set to from 5.0 to 8.0. More especially preferred are phosphate buffers of the PBS type having a pH value of from 5.0 to 8.0, preferably of from 5.5 to 6.5, that contain saccharose as stabilising agent in a concentration of from 0.1M to 1.0M, but preferably of from 0.3M to 0.5M (see Figures 3 and 4).

Aliquots of the suspended Bacillus cells are then transferred into cuvettes or any other suitable vessels and incubated together with a DNA sample for a suitable period, preferably for a period of from 0.1 to 30 minutes, but especially of from 5 to 15 minutes, and at a suitable temperature, preferably at a temperature of from 0°C to 35°C, but especially at a temperature of from 2°C to 15°C and more especially at a temperature of 4°C.

When operating at low temperatures it is advantageous to use cuvettes that have already been precooled, or any other suitable precooled vessels.

Over a wide range there is a linear relationship between the number of transformed cells and the DNA concentration used for the electroporation, the number of transformed cells increasing as the DNA concentration increases (see Figure 5). The DNA concentration preferred within the scope of this invention is in a range of from 1 ng to 20 µg. A DNA concentration of from 10 ng to 2 µg is especially preferred.

Subsequently the entire batch containing B. thuringiensis and/or B. cereus cells and plasmid DNA or another suitable DNA sample is introduced into an electroporation apparatus and subjected to electroporation, that is to say is briefly exposed to an electric pulse.

Electroporation apparatus suitable for use in the process of the invention is already available from a variety of manufacturers, such as, for example, from Bio Rad (Richmond, CA, USA; "Gene Pulser Apparatus"), Biotechnologies and Experimental Research Inc. (San Diego, CA, USA; "BTX Transfector 100"), Promega (Madison, WI, USA; "X-Cell 2000 Electroporation System"), etc..

It is obviously also possible to use any other suitable apparatus in the process of the invention.

Various pulse forms can be used, for example rectangular pulses or alternatively exponentially decaying pulses.

The latter are preferred within the scope of this invention. They are produced by the discharging of a capacitor and are characterised by an initially very rapid increase in voltage and by a subsequent exponential decaying phase as a function of resistance and capacitance. The time constant RC provides a measure of the length of the exponential decay time. It corresponds to the time necessary for the voltage to decay to 37 % of the initial voltage (V_o).

One parameter decisive in influencing the bacterial cell concerns the strength of the electric field acting on the cells, which is calculated from the ratio of the voltage applied to the distance between the electrode plates.

Also of great importance in this connection is the exponential decay time, which depends on the configuration of the apparatus used (for example the capacitance of the capacitor) and on other parameters, such as, for example, the composition of the buffer solution or the volume of cell suspension provided for the electroporation.

In the course of the investigations it has been demonstrated, for example, that reducing by half the volume of the cell suspension provided for the electroporation results in an increase in the transformation frequency by a factor of 10.

A prolongation of the exponential decay time by way of an optimisation of the buffer solution used also results in a distinct increase in the transformation frequency.

All measures that result in a prolongation of the exponential decay time and consequently in an increase in the transformation frequency are therefore preferred within the scope of this invention.

The decay time preferred within the scope of the process of the invention is from approximately 2 ms to approximately 50 ms, but especially from approximately 8 ms to approximately 20 ms. Most especially preferred is an exponential decay time of from approximately 10 ms to approximately 12 ms.

Within the scope of the present invention, the bacterial cells are acted upon for short periods by very high electric field strengths by means of brief discharge(s) of a capacitor across the DNA-containing cell suspension; as a result of this, the permeability of the B. thuringiensis cells is briefly and reversibly increased. The electroporation parameters are so coordinated with each other in the course of the process of the invention that optimum absorption into the Bacillus cells of the DNA located in the electroporation buffer is ensured.

The capacitance setting of the capacitor within the scope of this invention is advantageously from 1 µF to 250 µF, but especially from 1 µF to 50 µF and more especially is 25 µF. The choice of the initial voltage is not critical, and is therefore freely selectable, within wide ranges. An initial voltage V_o of from 0.2 kV to 50 kV, but especially of from 0.2 kV to 2.5 kV and more especially of from 1.2 kV to 1.8 kV, is preferred. The distance between the electrode plates depends, inter alia, on the size of the electroporation apparatus. It is advantageously from 0.1 cm to 1.0 cm, preferably from 0.2 cm to 1.0 cm, and more especially is 0.4 cm. The field strength values that act on the cell suspension result from the distance between the electrode plates and the initial voltage set in the capacitor. These values are advantageously in a range of from 100 V/cm to 50,000 V/cm. Field strengths of from 100 V/cm to 10,000 V/cm, but particularly of from 3,000 V/cm to 4,500 V/cm, are especially preferred.

The fine coordination of the freely selectable parameters, such as, for example, capacitance, initial voltage, distance between plates etc., depends to a certain extent on the architecture of the apparatus used and can therefore vary from case to case within certain limits. In certain cases, therefore, it is possible to exceed or fall below the limiting values indicated, should this be necessary in order to achieve optimum field strengths.

The actual electroporation operation can be repeated one or more times until the optimum transformation frequency for the system in question has been achieved.

Following the electroporation, the treated Bacillus cells can advantageously be reincubated, preferably for a period of from 0.1 to 30 minutes, at a temperature of from 0°C to 35°C, preferably from 2°C to 15°C. The electroporated cells are then diluted with a suitable medium and incubated again for a suitable period, preferably from 2 to 3 hours, with adequate aeration and at a suitable temperature, preferably of from 20°C to 35°C.

The B. thuringiensis cells are then plated out onto solid media that contain as an additive an agent suitable for selecting the new DNA sequences introduced into the bacterial cell. Depending on the nature of the DNA used, the said agent may be, for example, an antibiotically active compound or a dye, inter alia. Antibiotics selected from the group consisting of tetracycline, kanamycin, chloramphenicol and erythromycin are especially preferred within the scope of this invention for the selection of Bacillus thuringiensis and/or B. cereus cells.

Also preferred are chromogenic substrates, such as, for example, X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside), which can be detected by way of a specific colour reaction.

Other phenotypic markers are known to the skilled person and can also be used within the scope of this invention.

It is possible to use any nutrient medium suitable for culturing B. thuringiensis cells, to which one of the conventionally employed solidifying media, such as, for example, agar, agarose, gelatin, etc., is added.

The process parameters described hereinbefore in detail for B. thuringiensis are applicable in the same manner to B. cereus cells.

Unlike the processes hitherto available in the prior art, the process of the invention for the transformation of B. thuringiensis and B. cereus described hereinbefore is not limited to the use of specific natural

plasmids occurring in B. thuringiensis and/or B. cereus but is applicable to all types of DNA.

It is accordingly now possible for the first time to transform B. thuringiensis and/or B. cereus in a controlled manner, it being possible to use apart from homologous plasmid DNA, that is to say plasmid DNA occurring naturally in B. thuringiensis or the closely related B. cereus, also plasmid DNA of heterologous origin.

This may be either plasmid DNA that occurs naturally in an organism other than B. thuringiensis or the closely related B. cereus, such as, for example, plasmids pUB110 and pC194 from Staphylococcus aureus ([24]Horinouchi S. and Weisblum B., 1982; [25]Polak J. and Novick R.P., 1982) and plasmid pIM13 from B. subtilis ([26]Mahler J. and Halvorson H.O., 1980), which are capable of replicating in B. thuringiensis and/or B. cereus, or hybrid plasmid DNA constructed by recombinant DNA technology from homologous plasmid DNA or from heterologous plasmid DNA or alternatively from a combination of homologous and heterologous plasmid DNA. The last-mentioned hybrid plasmid DNA is better suited for work with recombinant DNA than the natural isolates.

There may be mentioned by way of example here, without the subject of the present application in any way being limited, the plasmids pBD64 ([27]Gryczan T. et al., 1980), pBD347, pBD348 and pUB1664.

The cloning vectors already established for B. subtilis, such as, for example, pBD64, may be of particular importance for carrying out the cloning experiments in various B. thuringiensis and B. cereus strains.

Apart from plasmid DNA, it is now possible within the scope of the present invention to introduce any other DNA into B. thuringiensis and B. cereus by transformation. The transformed DNA can replicate either autonomously or integrated in the chromosome. It may be, for example, a vector DNA derived not from a plasmid but from a phage.

The present invention also relates to the construction of bifunctional vectors ("shuttle" vectors).

Especially preferred within the scope of this invention is the construction and use of bifunctional (hybrid) plasmid vectors, so-called "shuttle" vectors, that are capable of replicating in one or in several heterologous host organisms apart from in B. thuringiensis or the closely related B. cereus, and that are identifiable both in homologous and in heterologous host systems.

Heterologous host organisms are to be understood within the scope of this invention as all those organisms that do not belong to the B. thuringiensis/B. cereus group and that are capable of maintaining in a stable condition a self-replicating DNA.

According to the above definition it is therefore possible for both prokaryotic and eukaryotic organisms to function as heterologous host organisms. At this point there may be mentioned by way of example, as representatives from the prokaryotic host organism group, individual examples from the genera Bacillus, such as, for example, B. subtilis or B. megaterium, Staphylococcus, such as, for example, S. aureus, Streptococcus, such as, for example. Streptococcus faecalis, Streptomyces, such as, for example Streptomyces spp., Pseudomonas, such as, for example, Pseudomonas spp., Escherichia, such as, for example, E. coli, Agrobacterium, such as, for example, A. tumefaciens or A. rhizogenes, Salmonella, Erwinia, etc. From the eukaryotic host group there may be mentioned especially yeasts and animal and plant cells. This list of examples is not final and is not intended to limit the subject of the present invention in any way. Other suitable representatives from the prokaryotic and eukaryotic host organism groups are known to the skilled person.

Especially preferred within the scope of this invention are B. subtilis or B. megaterium, Pseudomonas spp., and especially E. coli from the group of prokaryotic hosts as well as yeasts and animal or plant cells from the group of eukaryotic hosts.

More especially preferred are bifunctional vectors that are capable of replicating in both B. thuringiensis and/or B. cereus cells as well as in E. coli.

The present invention also includes the use of the said bifunctional vectors for the transformation of B. thuringiensis and B. cereus.

"Shuttle" vectors are constructed using recombinant DNA technology, plasmid and/or vector DNA of homologous (B. thuringiensis, B. cereus) or heterologous origin initially being cleaved using suitable restriction enzymes and then those DNA fragments containing the functions essential for replication in the respective desired host system being joined to one another again in the presence of suitable enzymes.

The afore-mentioned heterologous host organisms can act as a source of plasmid- and/or vector DNA of heterologous origin.

The joining of the various DNA fragments must be effected in such a manner that the functions essential for replication in the different host systems are retained.

In addition, obviously also plasmid DNA and/or vector DNA of purely heterologous origin can be used for the construction of "shuttle" vectors, but at least one of the heterologous fusion partners must contain regions of DNA that render possible a replication in homologous B. thuringiensis/B. cereus host systems.

As a source of plasmid DNA and/or vector DNA of heterologous origin that is nevertheless capable of replicating in a B. thuringiensis/B. cereus host system there may be mentioned at this point, by way of example, a few representatives from the group of gram-positive bacteria, selected from the group consisting of the genera Staphylococcus, such as, for example, Staphylococcus aureus, Streptococcus, such as, for example, Streptococcus faecalis, Bacillus, such as, for example, Bacillus megaterium or B. subtilis, Streptomyces, such as, for example, Streptomyces spp., etc. In addition to the representatives from the group of gram-positive bacteria listed here by way of example, there is a whole

series of other organisms known to the skilled person that can be used in the process of the invention.

The present invention thus accordingly also relates to a process for the production of bifunctional vectors that are suitable for transforming B. thuringiensis and/or B. cereus which comprises
a) first of all breaking down plasmid DNA of homologous or heterologous origin into fragments using suitable restriction enzymes and
b) then joining to one another again, in the presence of suitable enzymes, those fragments containing the functions essential for replication and selection in the respective desired host system, this being effected in such a manner that the functions essential for replication and selection in the various host systems are retained.

In this manner bifunctional plasmids are obtained that contain, in addition to the functions necessary for replication in B. thuringiensis or B. cereus, further DNA sequences that ensure replication in at least one other heterologous host system.

To ensure rapid and efficient selection of the bifunctional vectors in both homologous and heterologous host system(s) it is advantageous to provide the said vectors with specific selectable markers that can be used in B. thuringiensis and/or B. cereus as well as in heterologous host system(s), that is to say that render possible a rapid and uncomplicated selection. Especially preferred within the scope of this invention is the use of DNA sequences coding for antibiotic resistances, especially DNA sequences that code for resistance to antibiotics selected from the group consisting of kanamycin, tetracycline, chloramphenicol, erythromycin etc..

Also preferred are genes that code for enzymes with a chromogenic substrate, such as for example, X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside). The transformed colonies can then be detected very easily by way of a specific colour reaction.

Other phenotypic marker genes are known to the skilled person and can also be used within the scope of this invention.

Especially preferred within the scope of this invention is the construction of "shuttle" vectors that, in addition to DNA sequences that permit replication in B. thuringiensis or B. cereus or in both host systems, also contain regions of DNA that are necessary for replication in other bacterial host systems, such as, for example, in B. subtilis, B. megaterium, Pseudomonas spp., E. coli, etc..

Also preferred are "shuttle" vectors that replicate on the one hand either in B. thuringiensis or B. cereus or in both, and on the other hand in eukaryotic host systems selected from the group consisting of yeast, animal and plant cells, etc..

More especially preferred is the construction of "shuttle" vectors that, in addition to DNA sequences that are necessary for replication of the said vectors in B. thuringiensis or B. cereus or in both systems, also contain DNA sequences that render possible replication of the said "shuttle" vectors in E. coli.

Examples of such starting plasmids for the construction of "shuttle" vectors for the B. thuringiensis-B. cereus/E. coli system, which must not, however, be regarded as in any way limiting, are the B. cereus plasmid pBC16, and the plasmid pUC8 derived from the E. coli plasmid pBR322 ([28] Vieira J. and Messing J., 1982).

The present invention also relates to bifunctional ("shuttle") vectors that, in addition to the functions essential for replication and selection in homologous and heterologous host systems, also contain one or more genes in expressible form or other useful DNA sequences. This invention also includes processes for the production of these vectors, which comprise inserting the said genes or other useful DNA sequences into these bifunctional vectors with the aid of suitable enzymes.

Using the "shuttle" vectors of the invention and the afore-described transformation process it is thus now possible for the first time to introduce into B. thuringiensis and/or B. cereus cells by transformation,

with a high degree of efficiency, DNA sequences that have been cloned outside B. thuringiensis cells in a foreign host system.

Accordingly it is now possible for the first time for genes or other useful DNA sequences, especially also those having a regulatory function, to be introduced in a stable manner into B. thuringiensis and B. cereus cells and, if desired, expressed therein, as a result of which B. thuringiensis and B. cereus cells with novel and desirable properties are obtained.

Both homologous and heterologous gene(s) or DNA and synthetic gene(s) or DNA according to the definition given within the scope of the present invention, as well as combinations of the said DNAs, can be used as genes in the process of the invention.

The coding DNA sequence can be constructed exclusively from genomic DNA, from cDNA or from synthetic DNA. Another possibility is the construction of a hybrid DNA sequence consisting of both cDNA and of genomic DNA and/or synthetic DNA, or alternatively a combination of those DNAs.

In that case, the cDNA may originate from the same gene as the genomic DNA, or alternatively both the cDNA and the genomic DNA may originate from different genes. In any case, however, both the genomic DNA and/or the cDNA may each be prepared individually from the same or from different genes.

If the DNA sequence contains parts of more than one gene, these genes may originate from one and the same organism, from several organisms that belong to different strains, or to varieties of the same kind or different species of the same genus, or from organisms that belong to more than one genus of the same or of another taxonomic unit.

In order to ensure the expression of the said structural genes in the bacterial cell, the coding gene sequences must first of all be operably joined to expression sequences capable of functioning in B. thuringiensis and/or B. cereus cells.

The hybrid gene constructs of the present invention thus contain, in addition to the structural gene(s), expression signals that include both promoter and terminator sequences as well as other regulatory sequences of 3' and 5' untranslated regions.

Especially preferred within the scope of this invention are the natural expression signals of B. thuringiensis and/or B. cereus themselves and mutants and variants thereof that are substantially homologous with the natural sequence. Within the scope of this invention, one DNA sequence is substantially homologous with a second DNA sequence when at least 70 %, preferably at least 80 %, but especially at least 90 %, of the active regions of the DNA sequence are homologous. According to the present definition of the expression "substantially homologous", two different nucleotides in a DNA sequence of a coding region are regarded as homologous if the exchange of the one nucleotide for the other is a silent mutation.

Most especially preferred is the use of sporulation-dependent promoters of B. thuringiensis that ensure expression as a function of the sporulation.

Especially preferred for the transformation of B. thuringiensis or B. cereus within the scope of this invention is the use of DNA sequences that code for a δ-endotoxin.

The coding region of the chimaeric gene of the invention preferably contains a nucleotide sequence coding for a polypeptide that occurs naturally in B. thuringiensis or, alternatively, for a polypeptide that is substantially homologous therewith, that is to say that at least has substantially the toxicity properties of a crystalline δ-endotoxin protein of B. thuringiensis. Within the scope of the present invention, by definition a polypeptide has substantially the toxicity properties of the crystalline δ-endotoxin protein of B. thuringiensis if it has an insecticidal activity against a similar spectrum of insect larvae to that of the crystalline protein of a sub-species of B. thuringiensis. Some suitable sub-species are, for example, those selected from the group

consisting of kurstaki, berliner, alesti, tolworthi, sotto, dendrolimus, tenebrionis and israelensis. The preferred subspecies for Lepidoptera larvae is kurstaki and, especially, kurstaki HD1.

The coding region may thus be a region that occurs naturally in B. thuringiensis. Altenatively, the coding region can if desired also contain a sequence that is different from the sequence in B. thuringiensis but that is equivalent to it on account of the degeneration in the genetic code.

The coding region of the chimaeric gene can also code for a polypeptide that is different from a naturally occurring crystalline δ-endotoxin protein but that still has substantially the insect-toxicity properties of the crystalline protein. Such a coding sequence will normally be a variant of a natural coding region. A "variant" of a natural DNA sequence within the scope of this invention should, by definition, be understood as a modified form of a natural sequence that, however, still fulfils the same function. The variant may be a mutant or a synthetic DNA sequence and is substantially homologous with the corresponding natural sequence. Within the scope of this invention a DNA sequence is substantially homologous with a second DNA sequence when at least 70 %, preferably at least 80 %, but especially at least 90 %, of the active regions of the DNA sequence are homologous. According to the present definition of the expression "substantially homologous", two different nucleotides in a DNA sequence of a coding region are regarded as homologous if the exchange of one nucleotide for the other is a silent mutation.

Within the scope of the present invention, it is accordingly possible to use any chimaeric gene coding for an amino acid sequence that has the insecticidal properties of a B. thuringiensis δ-endotoxin and that meets the disclosed and claimed requirements. Especially preferred is the use of a nucleotide sequence that is substantially homologous at least with the part or the parts of the natural sequence that is (are) responsible for the insecticidal activity and/or the host specificity of the B. thuringiensis toxin.

The polypeptide expressed by the chimaeric gene as a rule also has at least some immunological properties in common with a natural crystalline protein, because it has at least some of the same antigenic determinants.

Accordingly, the polypeptide that is encoded by the said chimaeric gene is preferably structurally related to the δ-endotoxin of the crystalline protein produced by B. thuringiensis. B. thuringiensis produces a crystalline protein with a subunit that corresponds to a protoxin having a molecular weight (MW) of approximately from 130,000 to 140,000. This subunit can be cleaved by proteases or by alkali into insecticidal fragments having a MW of 70,000 and possibly even less.

For the construction of chimaeric genes in which the coding region includes such fragments of the protoxin or even smaller parts, fragmenting the coding region can be continued for as long as the fragments or parts of those fragments still have the necessary insecticidal activity. The protoxin, insecticidal fragments of the protoxin and insecticidal parts of those fragments can be joined to other molecules, such as polypeptides and proteins.

Coding regions suitable for use within the scope of the process of the invention can be obtained from genes of B. thuringiensis that code for the crystalline toxin gene (Whiteley et al., PCT application WO86/01536 and US Patents 4 448 885 and 4 467 036). A preferred nucleotide sequence that codes for a crystalline protein is located between nucleotides 156 and 3623 in formula I or is a shorter sequence that codes for an insecticidal fragment of such a crystalline protein ([5] Geiser et al., 1986 and EP 238 441).

Formel I

```
        10          20          30          40          50          60
GTTAACACCC TGGGTCAAAA ATTGATATTT AGTAAAATTA GTTGCACTTT GTGCATTTTT

        70          80          90         100         110         120
TCATAAGAIG AGTCATATGT TTTAAATTGT AGTAATGAAA AACAGTATTA TATCATAATG
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130 140 150 160 170 180
AATTGGTATC TTAATAAAAG AGATGGAGGT AACTTATGGA TAACAATCCG AACATCAATG

190 200 210 220 230 240
AATGCATTCC TTATAATTGT TTAAGTAACC CTGAACTAGA AGTATTAGGT GGAGAAAGAA

250 260 270 280 290 300
TAGAAACTGG TTACACCCCA ATCGATATTT CCTTGTCGCT AACGCAATTT CTTTTGAGTG

310 320 330 340 350 360
AATTTGTTCC CGGTGCTGGA TTTGTGTTAG GACTAGTTGA TATAATATGG GGAATTTTTG

370 380 390 400 410 420
GTCCCTCTCA ATGGGACGCA TTTCTTGTAC AAATTGAACA GTTAATTAAC CAAAGAATAG

430 440 450 460 470 480
AAGAATTCGC TAGGAACCAA GCCATTTCTA GATTAGAAGG ACTAAGCAAT CTTTATCAAA

490 500 510 520 530 540
TTTACGCAGA ATGTTTTAGA GAGTGGGAAG CAGATCCTAC TAATCCAGCA TTAAGAGAAG

550 560 570 580 590 600
AGATGCGTAT TCAATTCAAT GACATGAACA GTGCCCTTAC AACCGCTATT CCTCTTTTTG

610 620 630 640 650 660
CAGTTCAAAA TTATCAAGTT CCTCTTTTAT CAGTATATGT TCAAGCTGCA AATTTACATT

670 680 690 700 710 720
TATCAGTTTT GAGAGATGTT TCAGTGTTTG GACAAAGGTG GGGATTTGAT GCCGCGACTA

730 740 750 760 770 780
TCAATAGTCG TTATAATGAT TTAACTAGGC TTATTGGCAA CTATACAGAT CATGCTGTAC

790 800 810 820 830 840
GCTGGTACAA TACGGGATTA GAGCGTGTAT GGGGACCGGA TTCTAGAGAT TGGATAAGAT

850 860 870 880 890 900
ATAATCAATT TAGAAGAGAA TTAACACTAA CTGTATTAGA TATCGTTTCT CTATTTCCGA

910 920 930 940 950 960
ACTATGATAG TAGAACGTAT CCAATTCGAA CAGTTTCCCA ATTAACAAGA GAAATTTATA

970 980 990 1000 1010 1020
CAAACCCAGT ATTAGAAAAT TTTGATGGTA GTTTTCGAGG CTCGGCTCAG GGCATAGAAG

1030 1040 1050 1060 1070 1080
GAAGTATTAG GAGTCCACAT TTGATGGATA TACTTAACAG TATAACCATC TATACGGATG

1090 1100 1110 1120 1130 1140
CTCATAGAGG AGAATATTAT TGGTCAGGGC ATCAAATAAT GGCTTCTCCT GTAGGGTTTT

1150 1160 1170 1180 1190 1200
CGGGGCCAGA ATTCACTTTT CCGCTATATG GAACTATGGG AAATGCAGCT CCACAACAAC

1210 1220 1230 1240 1250 1260
GTATTGTTGC TCAACTAGGT CAGGGCGTGT ATAGAACATT ATCGTCCACT TTATATAGAA

1270 1280 1290 1300 1310 1320
GACCTTTTAA TATAGGGATA AATAATCAAC AACTATCTGT TCTTGACGGG ACAGAATTTG

1330 1340 1350 1360 1370 1380
CTTATGGAAC CTCCTCAAAT TTGCCATCCG CTGTATACAG AAAAAGCGGA ACGGTAGATT

1390 1400 1410 1420 1430 1440
CGCTGGATGA AATACCGCCA CAGAATAACA ACGTGCCACC TAGGCAAGGA TTTAGTCATC

1450 1460 1470 1480 1490 1500
GATTAAGCCA TGTTTCAATG TTTCGTTCAG GCTTTAGTAA TAGTAGTGTA AGTATAATAA

1510 1520 1530 1540 1550 1560
GAGCTCCTAT GTTCTCTTGG ATACATCGTA GTGCTGAATT TAATAATATA ATTCCTTCAT

1570 1580 1590 1600 1610 1620
CACAAATTAC ACAAATACCT TTAACAAAAT CTACTAATCT TGGCTCTGGA ACTTCTGTCG

1630 1640 1650 1660 1670 1680
TTAAAGGACC AGGATTTACA GGAGGAGATA TTCTTCGAAG AACTTCACCT GGCCAGATTT

1690 1700 1710 1720 1730 1740
CAACCTTAAG AGTAAATATT ACTGCACCAT TATCACAAAG ATATCGGGTA AGAATTCGCT

1750 1760 1770 1780 1790 1800
ACGCTTCTAC CACAAATTTA CAATTCCATA CATCAATTGA CGGAAGACCT ATTAATCAGG

1810 1820 1830 1840 1850 1860
GGAATTTTTC AGCAACTATG AGTAGTGGGA GTAATTTACA GTCCGGAAGC TTTAGGACTG

1870 1880 1890 1900 1910 1920
TAGGTTTTAC TACTCCGTTT AACTTTTCAA ATGGATCAAG TGTATTTACG TTAAGTGCTC

1930 1940 1950 1960 1970 1980
ATGTCTTCAA TTCAGGCAAT GAAGTTTATA TAGATCGAAT TGAATTTGTT CCGGCAGAAG

1990 2000 2010 2020 2030 2040
TAACCTTTGA GGCAGAATAT GATTTAGAAA GAGCACAAAA GGCGGTGAAT GAGCTGTTTA

2050 2060 2070 2080 2090 2100
CTTCTTCCAA TCAAATCGGG TTAAAAACAG ATGTGACGGA TTATCATATT GATCAAGTAT

2110 2120 2130 2140 2150 2160
CCAATTTAGT TGAGTGTTTA TCTGATGAAT TTTGTCTGGA TGAAAAAAAA GAATTGTCCG

2170 2180 2190 2200 2210 2220
AGAAAGTCAA ACATGCGAAG CGACTTAGTG ATGAGCGGAA TTTACTTCAA GATCCAAACT

2230 2240 2250 2260 2270 2280
TTAGAGGGAT CAATAGACAA CTAGACCGTG GCTGGAGAGG AAGTACGGAT ATTACCATCC

2290 2300 2310 2320 2330 2340
AAGGAGGCGA TGACGTATTC AAAGAGAATT ACGTTACGCT ATTGGGTACC TTTGATGAGT

2350 2360 2370 2380 2390 2400
GCTATCCAAC GTATTTATAT CAAAAAATAG ATGAGTCGAA ATTAAAAGCC TATACCCGTT

2410 2420 2430 2440 2450 2460
ACCAATTAAG AGGGTATATC GAAGATAGTC AAGACTTAGA AATCTATTTA ATTCGCTACA

2470 2480 2490 2500 2510 2520
ATGCCAAACA CGAAACAGTA AATGTGCCAG GTACGGGTTC CTTATGGCCG CTTTCAGCCC

2530 2540 2550 2560 2570 2580
CAAGTCCAAT CGGAAAATGT GCCCATCATT CCCACATTT CTCCTTGGAC ATTGATGTTG

2590 2600 2610 2620 2630 2640
GATGTACAGA CTTAAATGAG GACTTAGGTG TATGGGTGAT ATTCAAGATT AAGACGCAAG

2650 2660 2670 2680 2690 2700
ATGGCCATGC AAGACTAGGA AATCTAGAAT TTCTCGAAGA GAAACCATTA GTAGGAGAAG

2710 2720 2730 2740 2750 2760
CACTAGCTCG TGTGAAAAGA GCGGAGAAAA AATGGAGAGA CAAACGTGAA AAATTGGAAT

2770 2780 2790 2800 2810 2820
GGGAAACAAA TATTGTTTAT AAAGAGGCAA AAGAATCTGT AGATGCTTTA TTTGTAAACT

2830 2840 2850 2860 2870 2880
CTCAATATGA TAGATTACAA GCGGATACCA ACATCGCGAT GATTCATGCG GCAGATAAAC

2890 2900 2910 2920 2930 2940
GCGTTCATAG CATTCGAGAA GCTTATCTGC CTGAGCTGTC TGTGATTCCG GGTGTCAATG

2950 2960 2970 2980 2990 3000
CGGCTATTTT TGAAGAATTA GAAGGGCGTA TTTTCACTGC ATTCTCCCTA TATGATGCGA

3010 3020 3030 3040 3050 3060
GAAATGTCAT TAAAAATGGT GATTTTAATA ATGGCTTATC CTGCTGGAAC GTGAAAGGGC

3070 3080 3090 3100 3110 3120
ATGTAGATGT AGAAGAACAA AACAACCACC GTTCGGTCCT TGTTGTTCCG GAATGGGAAG

3130 3140 3150 3160 3170 3180
CAGAAGTGTC ACAAGAAGTT CGTGTCTGTC CGGGTCGTGG CTATATCCTT CGTGTCACAG

3190 3200 3210 3220 3230 3240
CGTACAAGGA GGGATATGGA GAAGGTTGCG TAACCATTCA TGAGATCGAG AACAATACAG

3250 3260 3270 3280 3290 3300
ACGAACTGAA GTTTAGCAAC TGTGTAGAAG AGGAAGTATA TCCAAACAAC ACGGTAACGT

3310 3320 3330 3340 3350 3360
GTAATGATTA TACTGCGACT CAAGAAGAAT ATGAGGGTAC GTACACTTCT CGTAATCGAG

3370 3380 3390 3400 3410 3420
GATATGACGG AGCCTATGAA AGCAATTCTT CTGTACCAGC TGATTATGCA TCAGCCTATG

3430 3440 3450 3460 3470 3480
AAGAAAAAGC ATATACAGAT GGACGAAGAG ACAATCCTTG TGAATCTAAC AGAGGATATG

3490 3500 3510 3520 3530 3540
GGGATTACAC ACCACTACCA GCTGGCTATG TGACAAAAGA ATTAGAGTAC TTCCCAGAAA

3550 3560 3570 3580 3590 3600
CCGATAAGGT ATGGATTGAG ATCGGAGAAA CGGAAGGAAC ATTCATCGTG GACAGCGTGG

3610 3620 3630 3640 3650 3660
AATTACTTCT TATGGAGGAA TAATATATGC TTTATAATGT AAGGTGTGCA AATAAAGAAT

3670 3680 3690 3700 3710 3720
GATTACTGAC TTGTATTGAC AGATAAATAA GGAAATTTTT ATATGAATAA AAAACGGGCA

3730 3740 3750 3760 3770 3780
TCACTCTTAA AAGAATGATG TCCGTTTTTT GTATGATTTA ACGAGTGATA TTTAAATGTT

3790 3800 3810 3820 3830 3840
TTTTTTGCGA AGGCTTTACT TAACGGGGTA CCGCCACATG CCCATCAACT TAAGAATTTG

3850 3860 3870 3880 3890 3900
CACTACCCCC AAGTGTCAAA AAACGTTATT CTTTCTAAAA AGCTAGCTAG AAAGGATGAC

3910 3920 3930 3940 3950 3960
ATTTTTTATG AATCTTTCAA TTCAAGATGA ATTACAACTA TTTTCTGAAG AGCTGTATCG

3970 3980 3990 4000 4010 4020
TCATTTAACC CCTTCTCTTT TGGAAGAACT CGCTAAAGAA TTAGGTTTTG TAAAAAGAAA

4030 4040 4050 4060 4070 4080
ACGAAAGTTT TCAGGAAATG AATTAGCTAC CATATGTATC TGGGGCAGTC AACGTACAGC

4090 4100 4110 4120 4130 4140
GAGTGATTCT CTCGTTCGAC TATGCAGTCA ATTACACGCC GCCACAGCAC TCTTATGAGT

4150 4160 4170 4180 4190 4200
CCAGAAGGAC TCAATAAACG CTTTGATAAA AAAGCGGTTG AATTTTTGAA ATATATTTTT

4210 4220 4230 4240 4250 4260
TCTGCATTAT GGAAAAGTAA ACTTTGTAAA ACATCAGCCA TTTCAAGTGC AGCACTCACG

4270 4280 4290 4300 4310 4320
TATTTTCAAC GAATCCGTAT TTTAGATGCG ACGATTTTCC AAGTACCGAA ACATTTAGCA

4330 4340 4350 4360
CATGTATATC CTGGGTCAGG TGGTTGTGCA CAAACTGCAG

The coding region defined by nucleotides 156 to 3623 of formula I codes for a polypeptide of formula II.

Formel II

Met Asp Asn Asn Pro Asn Ile Asn Glu Cys	10
Ile Pro Tyr Asn Cys Leu Ser Asn Pro Glu	20
Val Glu Val Leu Gly Gly Glu Arg Ile Glu	30
Thr Gly Tyr Thr Pro Ile Asp Ile Ser Leu	40
Ser Leu Thr Gln Phe Leu Leu Ser Glu Phe	50
Val Pro Gly Ala Gly Phe Val Leu Gly Leu	60
Val Asp Ile Ile Trp Gly Ile Phe Gly Pro	70
Ser Gln Trp Asp Ala Phe Leu Val Gln Ile	80
Glu Gln Leu Ile Asn Gln Arg Ile Glu Glu	90
Phe Ala Arg Asn Gln Ala Ile Ser Arg Leu	100
Glu Gly Leu Ser Asn Leu Tyr Gln Ile Tyr	110
Ala Glu Ser Phe Arg Glu Trp Glu Ala Asp	120
Pro Thr Asn Pro Ala Leu Arg Glu Glu Met	130
Arg Ile Gln Phe Asn Asp Met Asn Ser Ala	140
Leu Thr Thr Ala Ile Pro Leu Phe Ala Val	150
Gln Asn Tyr Gln Val Pro Leu Leu Ser Val	160
Tyr Val Gln Ala Ala Asn Leu His Leu Ser	170
Val Leu Arg Asp Val Ser Val Phe Gly Gln	180
Arg Trp Gly Phe Asp Ala Ala Thr Ile Asn	190
Ser Arg Tyr Asn Asp Leu Thr Arg Leu Ile	200
Gly Asn Tyr Thr Asp His Ala Val Arg Trp	210
Tyr Asn Thr Gly Leu Glu Arg Val Trp Gly	220
Pro Asp Ser Arg Asp Trp Ile Arg Tyr Asn	230
Gln Phe Arg Arg Glu Leu Thr Leu Thr Val	240
Leu Asp Ile Val Ser Leu Phe Pro Asn Tyr	250
Asp Ser Arg Thr Tyr Pro Ile Arg Thr Val	260
Ser Gln Leu Thr Arg Glu Ile Tyr Thr Asn	270
Pro Val Leu Glu Asn Phe Asp Gly Ser Phe	280
Arg Gly Ser Ala Gln Gly Ile Glu Gly Ser	290
Ile Arg Ser Pro His Leu Met Asp Ile Leu	300
Asn Ser Ile Thr Ile Tyr Thr Asp Ala His	310
Arg Gly Glu Tyr Tyr Trp Ser Gly His Gln	320
Ile Met Ala Ser Pro Val Gly Phe Ser Gly	330
Pro Glu Phe Thr Phe Pro Leu Tyr Gly Thr	340
Met Gly Asn Ala Ala Pro Gln Gln Arg Ile	350

Val Ala Gln Leu Gly Gln Gly Val Tyr Arg	360
Thr Leu Ser Ser Thr Leu Tyr Arg Arg Pro	370
Phe Asn Ile Gly Ile Asn Asn Gln Gln Leu	380
Ser Val Leu Asp Gly Thr Glu Phe Ala Tyr	390
Gly Thr Ser Ser Asn Leu Pro Ser Ala Val	400
Tyr Arg Lys Ser Gly Thr Val Asp Ser Leu	410
Asp Glu Ile Pro Pro Gln Asn Asn Asn Val	420
Pro Pro Arg Gln Gly Phe Ser His Arg Leu	430
Ser His Val Ser Met Phe Arg Ser Gly Phe	440
Ser Asn Ser Ser Val Ser Ile Ile Arg Ala	450
Pro Met Phe Ser Trp Ile His Arg Ser Ala	460
Glu Phe Asn Asn Ile Ile Pro Ser Ser Gln	470
Ile Thr Gln Ile Pro Leu Thr Lys Ser Thr	480
Asn Leu Gly Ser Gly Thr Ser Val Val Lys	490
Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu	500
Arg Arg Thr Ser Pro Gly Gln Ile Ser Thr	510
Leu Arg Val Asn Ile Thr Ala Pro Leu Ser	520
Gln Arg Tyr Arg Val Arg Ile Arg Tyr Ala	530
Ser Thr Thr Asn Leu Gln Phe His Thr Ser	540
Ile Asp Gly Arg Pro Ile Asn Gln Gly Asn	550
Phe Ser Ala Thr Met Ser Ser Gly Ser Asn	560
Leu Gln Ser Gly Ser Phe Arg Thr Val Gly	570
Phe Thr Thr Pro Phe Asn Phe Ser Asn Gly	580
Ser Ser Val Phe Thr Leu Ser Ala His Val	590
Phe Asn Ser Gly Asn Glu Val Tyr Ile Asp	600
Arg Ile Glu Phe Val Pro Ala Glu Val Thr	610
Phe Glu Ala Glu Tyr Asp Leu Glu Arg Ala	620
Gln Lys Ala Val Asn Glu Leu Phe Thr Ser	630
Ser Asn Gln Ile Gly Leu Lys Thr Asp Val	640
Thr Asp Tyr His Ile Asp Gln Val Ser Asn	650
Leu Val Glu Cys Leu Ser Asp Glu Phe Cys	660
Leu Asp Glu Lys Lys Glu Leu Ser Glu Lys	670
Val Lys His Ala Lys Arg Leu Ser Asp Glu	680
Arg Asn Leu Leu Gln Asp Pro Asn Phe Arg	690
Gly Ile Asn Arg Gln Leu Asp Arg Gly Trp	700
Arg Gly Ser Thr Asp Ile Thr Ile Gln Gly	710
Gly Asp Asp Val Phe Lys Glu Asn Tyr Val	720

Thr Leu Leu Gly Thr Phe Asp Glu Cys Tyr	730
Pro Thr Tyr Leu Tyr Gln Lys Ile Asp Glu	740
Ser Lys Leu Lys Ala Tyr Thr Arg Tyr Gln	750
Leu Arg Gly Tyr Ile Glu Asp Ser Gln Asp	760
Leu Glu Ile Tyr Leu Ile Arg Tyr Asn Ala	770
Lys His Glu Thr Val Asn Val Pro Gly Thr	780
Gly Ser Leu Trp Pro Leu Ser Ala Pro Ser	790
Pro Ile Gly Lys Cys Ala His His Ser His	800
His Phe Ser Leu Asp Ile Asp Val Gly Cys	810
Thr Asp Leu Asn Glu Asp Leu Gly Val Trp	820
Val Ile Phe Lys Ile Lys Thr Gln Asp Gly	830
His Ala Arg Leu Gly Asn Leu Glu Phe Leu	840
Glu Glu Lys Pro Leu Val Gly Glu Ala Leu	850
Ala Arg Val Lys Arg Ala Glu Lys Lys Trp	860
Arg Asp Lys Arg Glu Lys Leu Glu Trp Glu	870
Thr Asn Ile Val Tyr Lys Glu Ala Lys Glu	880
Ser Val Asp Ala Leu Phe Val Asn Ser Gln	890
Tyr Asp Arg Leu Gln Ala Asp Thr Asn Ile	900
Ala Met Ile His Ala Ala Asp Lys Arg Val	910
His Ser Ile Arg Glu Ala Tyr Leu Pro Glu	920
Leu Ser Val Ile Pro Gly Val Asn Ala Ala	930
Ile Phe Glu Glu Leu Glu Gly Arg Ile Phe	940
Thr Ala Phe Ser Leu Tyr Asp Ala Arg Asn	950
Val Ile Lys Asn Gly Asp Phe Asn Asn Gly	960
Leu Ser Cys Trp Asn Val Lys Gly His Val	970
Asp Val Glu Glu Gln Asn Asn His Arg Ser	980
Val Leu Val Val Pro Glu Trp Glu Ala Glu	990
Val Ser Gln Glu Val Arg Val Cys Pro Gly	1000
Arg Gly Tyr Ile Leu Arg Val Thr Ala Tyr	1010
Lys Glu Gly Tyr Gly Glu Gly Cys Val Thr	1020
Ile His Glu Ile Glu Asn Asn Thr Asp Glu	1030
Leu Lys Phe Ser Asn Cys Val Glu Glu Glu	1040
Val Tyr Pro Asn Asn Thr Val Thr Cys Asn	1050
Asp Tyr Thr Ala Thr Gln Glu Glu Tyr Glu	1060
Gly Thr Tyr Thr Ser Arg Asn Arg Gly Tyr	1070
Asp Gly Ala Tyr Glu Ser Asn Ser Ser Val	1080
Pro Ala Asp Tyr Ala Ser Ala Tyr Glu Glu	1090

Lys Ala Tyr Thr Asp Gly Arg Arg Asp Asn	1100
Pro Cys Glu Ser Asn Arg Gly Tyr Gly Asp	1110
Tyr Thr Pro Leu Pro Ala Gly Tyr Val Thr	1120
Lys Glu Leu Glu Tyr Phe Pro Glu Thr Asp	1130
Lys Val Trp Ile Glu Ile Gly Glu Thr Glu	1140
Gly Thr Phe Ile Val Asp Ser Val Glu Leu	1150
Leu Leu Met Glu Glu End	1156

In order to introduce a chimaeric gene into B. thuringiensis or B. cereus cells by transformation using the process of the invention, the gene is preferably first of all inserted into a vector. The insertion is especially preferably into a bifunctional vector of the invention.

If the corresponding gene is not available in an amount sufficient for the insertion into the Bacillus cells, the vector can first of all be amplified by replication in a heterologous host cell. Bacterial cells or yeast cells are best suited for the amplification of genes. When a sufficient amount of the gene is available it is inserted into the Bacillus cells. The insertion of the gene into B. thuringiensis or B. cereus cells can be carried out with the same vector as was used for the replication, or with a different vector. The bifunctional vectors of the invention are especially suitable.

A few examples of bacterial host cells that are suitable for replication of the chimaeric gene include bacteria selected from the genera Escherichia, such as E. coli, Agrobacterium, such as A. tumefaciens or A. rhizogenes, Pseudomonas, such as Pseudomonas spp., Bacillus, such as B. megaterium or B. subtilis, etc.. As a result of the transformation process of the invention it is now possible for the first time, within the scope of this invention, also to use B. thuringiensis and B. cereus themselves as host cells. Processes for cloning heterologous genes in bacteria are described in US Patents 4 237 224 and 4 468 464.

The replication of genes in E. coli that code for the crystalline protein of B. thuringiensis is described by [29] Wong et al. (1983).

Some examples of yeast host cells that are suitable for the replication of the genes of the invention include those selected from the genus Saccharomyces (European Patent Application EP 0 238 441).

Any vector into which the chimaeric gene can be inserted and which is replicated in a suitable host cell, such as in bacteria or yeast, can be used for the amplification of the genes of the invention. The vector may be derived, for example, from a phage or from a plasmid. Examples of vectors that are derived from phages and that can be used within the scope of this invention are vectors derived from M13- and from λ-phages. Some suitable vectors derived from M13 phages include M13mp18 and m13mp19. Some suitable vectors derived from λ-phages include λgt11, λgt7 and λCharon4.

Of the vectors that are derived from plasmids and are especially suitable for replication in bacteria, there may be mentioned here by way of example pBR322 ([30]Bolivar et al., 1977), pUC18 and pUC19 ([31]Norrander et al., 1983) and Ti-plasmids ([32]Bevan et al., 1983), without the subject of the invention being in any way limited thereby. Preferred vectors for the amplification of genes in bacteria are pBR322, pUC18 and pUC19.

Without any limitation being implied, especially direct cloning vectors, such as, for example, pBD347, pBD348, pBD64 and pUB1664, and especially "shuttle" vectors, which have already been described in detail hereinbefore, may be mentioned for cloning directly in B. thuringiensis and/or B. cereus.

Especially preferred within the scope of this invention are the bifunctional ("shuttle") vectors pXI61 (=pK61) and pXI93 (=pK93) which, introduced by transformation into B. thuringiensis var. kurstaki HD1cryB and B. cereus 569K, have been deposited at the "Deutsche Sammlung von Mikroorganismen" (Braunschweig, Federal Republic of Germany), recognised as an International Depository, in accordance with the requirements of the Budapest Treaty under the number DSM 4573 (pXI61, introduced by transformation into B. thuringiensis var. kurstaki HD1cryB) and DSM 4571

(pXI93, introduced by transformation into B. thuringiensis var. kurstaki HD1cryB) and DSM 4573 (pXI93, introduced by transformation into B. cereus 569K).

In order to construct a chimaeric gene suitable for replication in bacteria, a promoter sequence, a 5' untranslated sequence, a coding sequence and a 3' untranslated sequence are inserted into a vector or are assembled in the correct sequence in one of the afore-described vectors. Suitable vectors according to the invention are those that are capable of being replicated in the host cell.

The promoter, the 5' untranslated region, the coding region and the 3' untranslated region can, if desired, first of all be combined in one unit outside the vector and then inserted into the vector. Alternatively, parts of the chimaeric gene can also be inserted into the vector individually.

In the case of B. thuringiensis and B. cereus cloning vectors this process step can be omitted since the entire unit isolated from B. thuringiensis, consisting of a 5' untranslated region, the coding region and a 3' untranslated region, can be inserted into the vector.

The vector furthermore preferably also contains a marker gene which confers on the host cell a property by which it is possible to recognise the cells transformed with the vector. Marker genes that code for an antibiotic resistance are preferred. Some examples of suitable antibiotics are ampicillin, chloramphenicol, erythromycin, tetracycline, hygromycin, G 418 and kanamycin.

Also preferred are marker genes that code for enzymes having a chromogenic substrate, such as, for example, X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside). The transformed colonies can then be detected very easily by way of a specific colour reaction.

The insertion of the gene into, or the assembly of the gene in, the vector is carried out by way of standard processes, for example using recombinant DNA ([33] Maniatis et al., 1982) and using homologous recombination ([34] Hinnen et al., 1978).

The recombinant DNA technology processes are based on the vector first of all being cleaved and the desired DNA sequence being inserted between the cleaved portions of the vector; the ends of the desired DNA sequence are then joined to the corresponding ends of the vector.

The vector is preferably cleaved with suitable restriction endonucleases. Suitable restriction endonucleases are, for example, those that form blunt ends, such as Sma I, Hpa I and Eco RV, as well as those that form cohesive ends, such as Eco RI, Sac I and Bam HI.

The desired DNA sequence normally exists as a region of a larger DNA molecule, such as a chromosome, a plasmid, a transposon or a phage. The desired DNA sequence is in these cases excised from its original source and, if desired, so modified that its ends can be joined to those of the cleaved vector. If the ends of the desired DNA sequence and of the cleaved vector are blunt ends, then they can, for example, be joined to one another with ligases specific for blunt ends, such as T4 DNA ligase.

The ends of the desired DNA sequence can also be joined in the form of cohesive ends to the ends of the cleaved vector, in which case a ligase specific for cohesive ends, which may also be T4 DNA ligase, is used. Another suitable ligase specific for cohesive ends is, for example, the E. coli DNA ligase.

Cohesive ends are advantageously formed by cleaving the desired DNA sequence and the vector with the same restriction endonuclease, in which case the desired DNA sequence and the cleaved vector have cohesive ends that are complementary to each other.

The cohesive ends can also be constructed by adding complementary homopolymer tails to the ends of the desired DNA sequence and of the cleaved vector with the aid of terminal deoxynucleotidyl transferase.

Alternatively, cohesive ends can be produced by adding a synthetic oligonucleotide sequence that is recognised by a particular restriction endonuclease and is known as a linker, and cleaving the sequence with the endonuclease (see, for example, [33] Maniatis et al., 1982).

It is thus now possible for the first time, within the scope of this invention, genetically to modify B. thuringiensis genes, and especially δ-endotoxin-encoding DNA sequences, outside B. thuringiensis, to clone those genes and then to return them into B. thuringiensis and/or B. cereus cells, where the said δ-endotoxin genes can be expressed (in a homologous bacterial host system).

This means that it is now possible also for the genome of B. thuringiensis to be manipulated genetically in a specifically controlled manner by first of all generating large amounts of plasmid material in a foreign cloning system and then introducing this into B. thuringiensis by transformation.

The possibility of modifying the δ-endotoxin genes and the control sequences regulating the expression of those genes is of particular interest here.

Apart from chimaeric genes, it is obviously also possible for any other chimaeric genetic construct to be inserted into Bacillus thuringiensis and/or Bacillus cereus cells using the process of the invention.

It is thus, for example, conceivable, using the process of the invention, to insert non-coding "anti-sense" DNA into the genome of a Bacillus thuringiensis and/or Bacillus cereus cell, so that in the course of the expression of the said "anti-sense" DNA a mRNA is transcribed that inhibits the expression of the corresponding "sense" DNA. In this manner it is possible to inhibit in a specifically controlled manner the expression in Bacillus thuringiensis and/or Bacillus cereus of certain undesired genes.

Furthermore, apart from the preparation of improved, well-defined B. thuringiensis strains for the preparation of improved bioinsecticides, it is now also possible to use B. thuringiensis as a general host for cloning and, if desired, expressing heterologous and/or homologous genes.

In a specific and preferred embodiment of the process of the invention it is furthermore now possible for the first time to clone new genes, and especially new protoxin genes, directly in the natural host, that is to say in B. thuringiensis or B. cereus.

In the search for new protoxin genes, first of all a gene library of B. thuringiensis is created.

In a first process step, the total DNA of a protoxin-producing B. thuringiensis strain is isolated by processes that are known per se and then broken down into individual fragments. The B. thuringiensis DNA can be fragmented either mechanically, for example by the action of shearing forces, or, preferably, by digestion with suitable restriction enzymes. Digestion of the DNA sample is partial or complete, depending on the choice of enzymes. Within the scope of this invention, the use of restriction enzymes that contain quaternary recognition sites and/or result in a partial digestion of the B. thuringiensis DNA are especially preferred, such as, for example, the restriction enzyme Sau IIIA, but this preference does not imply any limitation. Obviously, it is also possible to use any other suitable restriction enzyme in the process of the invention.

The restriction fragments obtained in the afore-described manner are then separated according to size by processes known per se. Size-dependent separation of DNA fragments is usually effected by centrifuging processes, such as, for example, saccharose gradient centrifugation, or by electrophoretic processes, such as agarose gel electrophoresis, or by a combination of those processes.

Those fractions containing fragments of the correct size, that is to say fragments that on account of their size are capable of coding for a protoxin, are pooled and used for the next process steps.

The previously isolated fragments are first of all inserted into suitable cloning vectors using standard processes, and then inserted directly into Bacillus thuringiensis or B. cereus, but preferably into protoxin-free strains of Bacillus thuringiensis, using the transformation process of the invention.

The vectors used may be either gram-positive plasmids, such as, for example, pBC16, pUB110, pC194, or the "shuttle" vectors described in detail hereinbefore. The shuttle vector pXI200, which is described in detail hereinafter (see Example 9.1), is especially preferred within the scope of this invention. Suitable vectors preferably contain DNA sequences that ensure easy identification of the transformed vector-containing clones from among the immense number of untransformed clones. Especially preferred are DNA sequences coding for a specific marker that on expression results in an easily selectable feature, such as, for example an antibiotic resistance. There may be mentioned by way of example here a resistance to ampicillin, chloramphenicol, erythromycin, tetracycline, hygromycin, G418 or kanamycin.

Also preferred are marker genes that code for enzymes having a chromogenic substrate, such as, for example, X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside). The transformed colonies can then be detected very easily by way of a specific colour reaction.

After electroporation the treated Bacillus thuringiensis or B. cereus cells are transferred to a selective sporulation medium and are incubated until sporulation is complete at a temperature of from 10°C to 40°C, preferably from 20°C to 35°C, and more especially at a temperature of from 29°C to 31°C. The sporulation medium contains as selective substance preferably one of the above-mentioned antibiotics, depending on the vector used, and a suitable solidifying agent, such as, for example, agar, agarose, gelatin etc..

In the course of sporulation, autolysis of the sporulating cells occurs, which is advantageous in industrial scale processing for the subsequent screening since breaking open the cells artificially is dispensed with.

In clones that contain the desired protoxin gene and are expressed under the control of their natural promoter, the crystalline proteins formed are freely accessible in the medium. These crystalline proteins which exist freely in the medium can then be immobilised, for example with the aid of membrane filters or by other suitable measures. Suitable membrane filters are, for example, nylon or nitrocellulose membranes. Membranes of this kind are freely available on the market.

The crystalline proteins immobilised in this manner can then be located and identified very simply in a suitable screening process.

Immunological screening using protoxin-specific antibodies is preferred within the scope of this invention. Immunological screening processes are known and are described in detail, for example, in [35] Young et al., 1983. The use of monoclonal antibodies that recognise quite specifically a particular region of the protein molecule is especially preferred within the scope of the process of the invention. These antibodies can be used either on their own or in the form of a mixture. It is, of course, also possible, however, to use polyclonal antisera for the immunological screening. Mixtures based on monoclonal and polyclonal antibodies are also possible.

Processes for the production of monoclonal antibodies to Bacillus thuringiensis protoxin proteins are known and are described in detail, for example, in [36] Huber-Lukaç (1984) and in [37] Huber-Lukač et al, (1986). These processes can also be used in the present case.

The immunological screening process based on antibodies is part of the present invention.

It is obviously also possible within the scope of this invention to use other suitable screening processes for locating novel DNA sequences in B. thuringiensis and/or B. cereus.

Bacillus thuringiensis and B. cereus cells that have been transformed using the afore-described process, and the toxins produced by these transformed Bacillus cells, are excellently suitable for controlling insects, but especially for controlling insects of the orders Lepidoptera, Diptera and Coleoptera.

The present invention accordingly also relates to a method of controlling insects which comprises treating insects or the locus thereof
a) with B. thuringiensis or B. cereus cells, or with a mixture of the two, that have been transformed with a recombinant DNA molecule containing a structural gene that codes for a δ-endotoxin polypeptide occurring naturally in B. thuringiensis or for a polypeptide essentially homologous therewith; or alternatively
b) with a cell-free crystalline body preparation containing a protoxin that is produced by the said transformed Bacillus cells.

The present invention also includes insecticidal compositions that, in addition to the conventionally employed carriers, dispersants or carriers and dispersants, contain
a) B. thuringiensis or B. cereus cells, or a mixture of the two, that have been transformed with a recombinant DNA molecule containing a structural gene that codes for a δ-endotoxin polypeptide occurring naturally in B. thuringiensis or for a polypeptide essentially homologous therewith; or alternatively
b) a cell-free crystalline body preparation containing a protoxin that is produced by the said transformed Bacillus cells.

For use as insecticides, the transformed microorganisms containing the recombinant B. thuringiensis toxin gene, preferably transformed living or dead B. thuringiensis or B. cereus cells, including mixtures of living and dead B. thuringiensis and B. cereus cells, as well as the toxin proteins produced by the said transformed cells, are used in unmodified form or, preferably, together with adjuvants customarily employed in the art of formulation, and are formulated in a manner known per se, for example into suspension concentrates, coatable pastes, directly sprayable or dilutable solutions, wettable powders, soluble powders, dusts, granulates, and also encapsulations in, for example, polymer substances.

As with the nature of the compositions, the methods of application, such as spraying, atomising, dusting, scattering, coating or pouring, are chosen in accordance with the intended objectives and the prevailing circumstances.

Furthermore it is obviously also possible to use insecticidal mixtures consisting of transformed living or dead B. thuringiensis and/or B. cereus cells and cell-free crystalline body preparations containing a protoxin produced by the said transformed Bacillus cells.

The formulations, that is to say the compositions or preparations containing the transformed living or dead Bacillus cells or mixtures thereof and also the toxin proteins produced by the said transformed Bacillus cells and, where appropriate, solid or liquid adjuvants, are prepared in known manner, for example by intimately mixing the transformed cells and/or toxin proteins with solid carriers and, where appropriate, surface-active compounds (surfactants).

The solid carriers used e.g. for dusts and dispersible powders, are normally natural mineral fillers such as calcite, talcum, kaolin, montmorillonite or attapulgite. In order to improve the physical properties it is also possible to add highly dispersed silicic acid or highly dispersed absorbent polymers. Suitable granulated adsorptive carriers are porous types, for example pumice, broken brick, sepiolite or bentonite; and suitable nonsorbent carriers are, for example, calcite or sand. In addition, a great number of pregranulated materials of inorganic or organic nature can be used, e.g. especially dolomite or pulverised plant residues.

Suitable surface-active compounds are non-ionic, cationic and/or anionic surfactants having good dispersing and wetting properties. The term "surfactants" will also be understood as comprising mixtures of surfactants.

Both so-called water-soluble soaps and also water-soluble synthetic surface-active compounds are suitable anionic surfactants.

Suitable soaps are the alkali metal salts, alkaline earth metal salts or unsubstituted or substituted ammonium salts of higher fatty acids (C_{10}-C_{22}), e.g. the sodium or potassium salts of oleic or stearic acid or of natural fatty acid mixtures which can be obtained e.g. from coconut oil or tallow oil. Mention may also be made of fatty acid methyltaurin salts, such as, for example, the sodium salt of cis-2-(methyl-9-octadecenylamino)-ethanesulfonic acid (content in formulations preferably approximately 3 %).

More frequently, however, so-called synthetic surfactants are used, especially fatty sulfonates, fatty sulfates, sulfonated benzimidazole derivatives or alkylarylsulfonates or fatty alcohols, such as, for example, 2,4,7,9-tetramethyl-5-decyne-4,7-diol (content in formulations preferably approximately 2 %).

The fatty sulfonates or sulfates are usually in the form of alkali metal salts, alkaline earth metal salts or unsubstituted or substituted ammonium salts and contain a C_8-C_{22}alkyl radical which also includes the alkyl moiety of acyl radicals, e.g. the sodium or calcium salt of lignosulfonic acid, of dodecylsulfate or of a mixture of fatty alcohol sulfates obtained from natural fatty acids. These compounds also comprise the salts of sulfated and sulfonated fatty alcohol/ethylene oxide adducts. The sulfonated benzimidazole derivatives preferably contain 2 sulfonic acid groups and one fatty acid radical containing 8 to 22 carbon atoms. Examples of alkylarylsulfonates are the sodium, calcium or triethanolamine salts of dodecylbenzenesulfonic acid, dibutylnaphthalenesulfonic acid, or of a condensate of naphthalenesulfonic acid and formaldehyde.

Also suitable are corresponding phosphates, e.g. salts of the phosphoric acid ester of an adduct of p-nonylphenol with 4 to 14 moles of ethylene oxide.

Non-ionic surfactants are preferably polyglycol ether derivatives of aliphatic or cycloaliphatic alcohols, saturated or unsaturated fatty acids and alkylphenols, said derivatives containing 3 to 30 glycol ether groups and 8 to 20 carbon atoms in the (aliphatic) hydrocarbon moiety and 6 to 18 carbon atoms in the alkyl moiety of the alkylphenols.

Further suitable non-ionic surfactants are the water-soluble adducts of polyethylene oxide with polypropylene glycol, ethylenediaminopolypropylene glycol and alkylpolypropylene glycol containing 1 to 10 carbon atoms in the alkyl chain, which adducts contain 20 to 250 ethylene glycol ether groups and 10 to 100 propylene glycol ether groups. These compounds usually contain 1 to 5 ethylene glycol units per propylene glycol unit.

Examples of non-ionic surfactants are nonylphenolpolyethoxyethanols, castor oil polyglycol ethers, polypropylene/polyethylene oxide adducts, tributylphenoxypolyethoxyethanol, polyethylene glycol and octylphenoxypolyethoxyethanol. Fatty acid esters of polyoxyethylene sorbitan, e.g. polyoxyethylene sorbitan trioleate, are also suitable non-ionic surfactants.

Cationic surfactants are preferably quaternary ammonium salts which contain, as N-substituent, at least one C_8-C_{22}alkyl radical and, as further substituents, unsubstituted or halogenated lower alkyl, benzyl or hydroxy-lower alkyl radicals. The salts are preferably in the form of halides, methylsulfates or ethylsulfates, e.g. stearyltrimethylammonium chloride or benzyldi(2-chloroethyl)ethylammonium bromide.

The surfactants customarily employed in the art of formulation are described, inter alia, in the following publications:

[38] 1986 International McCutcheon's Emulsifiers & Detergents, The Manufacturing Confectioner Publishing Co., Glen Rock, NJ, USA; Helmut Stache "Tensid-Taschenbuch" Carl Hanser-Verlag Munich/Vienna 1981.

The agrochemical compositions usually contain 0.1 to 99 %, preferably 0.1 to 95 %, of the transformed living or dead Bacillus cells or mixtures thereof or of the toxin proteins produced by the said transformed Bacillus cells, 99.9 to 1 %, preferably 99.8 to 5 %, of a solid or liquid adjuvant, and 0 to 25 %, preferably 0.1 to 25 %, of a surfactant.

Whereas commercial products will preferably be formulated as concentrates, the end user will normally employ dilute formulations.

The compositions may also contain further auxiliaries such as stabilisers, antifoams, viscosity regulators, binders, tackifiers as well as fertilisers or other active ingredients for obtaining special effects.

The transformed living or dead Bacillus cells or mixtures thereof containing the recombinant B. thuringiensis toxin genes, as well as the toxin proteins produced by the said transformed Bacillus cells, are excellently suitable for controlling insect pests. Plant-destructive insects of the order Lepidoptera should preferably be mentioned here, especially those of the genera Pieris, Heliothis, Spodoptera and Plutella, such as, for example, Pieris brassicae, Heliothis virescens, Heliothis zea, Spodoptera littoralis and Plutella xylostella.

Other insect pests that can be controlled by the afore-described insecticidal preparations are, for example, beetles of the order Coleoptera, especially those of the Chrysomelidae family, such as, for example, Diabrotica undecimpunctata, D. longicornis, D. virgifera, D. undecimpunctata howardi, Agelastica alni, Leptinotarsa decemlineata etc., as well as insects of the order Diptera, such as, for example, Anopheles sergentii, Uranatenia unguiculata, Culex univittatus, Aedes aegypti, Culex pipiens, etc..

The amounts in which the Bacillus cells or the toxin proteins produced by them are used depends on the respective conditions, such as, for example, the weather conditions, the soil conditions, the plant growth and the time of application.

Formulation Examples for material containing B. thuringiensis toxin

In the following Formulation Examples the term "Bacillus cells" is used to mean those B. thuringiensis and/or B. cereus cells containing a recombinant B. thuringiensis gene of the invention. (The figures given are percentages by weight throughout).

F1. Granulates	a)	b)
Bacillus cells and/or toxin protein produced by these cells	5 %	10 %
kaolin	94 %	-
highly dispersed silicic acid	1 %	-
attapulgite	-	90 %

The Bacillus cells and/or toxin protein produced by these cells are first of all suspended in methylene chloride, then the suspension is sprayed onto the carrier, and the suspending agent is subsequently evaporated off in vacuo.

F2. Dusts	a)	b)
Bacillus cells and/or toxin protein produced by these cells	2 %	5 %
highly dispersed silicic acid	1 %	5 %
talcum	97 %	-
kaolin	-	90 %

Ready-for-use dusts are obtained by intimately mixing the carriers with the Bacillus cells and/or with toxin protein produced by these cells.

F3. Wettable powders	a)	b)	c)
Bacillus cells and/or toxin protein produced by these cells	25 %	50 %	75 %
sodium lignosulfonate	5 %	5 %	-
sodium laurylsulfate	3 %	-	5 %
sodium diisopropylnaphthalene-sulfonate	-	6 %	10 %

octylphenol polyethylene glycol ether (7-8 moles of ethylene oxide)	-	2 %	-
highly dispersed silicic acid	5 %	10 %	10 %
kaolin	62 %	27 %	-

The Bacillus cells and/or toxin protein produced by these cells are carefully mixed with the adjuvants and the resulting mixture is then thoroughly ground in a suitable mill, affording wettable powders, which can be diluted with water to give suspensions of the desired concentration.

F4. Extruder granulates

Bacillus cells and/or toxin protein produced by these cells	10 %
sodium lignosulfonate	2 %
carboxymethylcellulose	1 %
kaolin	87 %

The Bacillus cells and/or toxin protein produced by these cells are mixed with the adjuvants, carefully ground, and the mixture is subsequently moistened with water. The mixture is extruded and then dried in a stream of air.

F5. Coated granulate

Bacillus cells and/or toxin protein produced by these cells	3 %
polyethylene glycol 200	3 %
kaolin	94 %

The homogeneously mixed Bacillus cells and/or toxin protein produced by these cells are uniformly applied, in a mixer, to the kaolin moistened with the polyethylene glycol. Non-dusty coated granulates are obtained in this manner.

F6. Suspension concentrate

Bacillus cells and/or toxin protein produced by these cells	40 %
ethylene glycol	10 %
nonylphenol polyethylene glycol (15 moles of ethylene oxide)	6 %
alkylbenzenesulfonic acid triethanolamine salt*	3 %
carboxymethylcellulose	1 %
silicone oil in the form of a 75 % aqueous emulsion	0.1 %
water	39 %

*Alkyl is preferably linear alkyl having from 10 to 14, especially from 12 to 14, carbon atoms, such as, for example, n-dodecylbenzenesulfonic acid triethanolamine salt.

The homogeneously mixed Bacillus cells and/or toxin protein produced by these cells are intimately mixed with the adjuvants, giving a suspension concentrate from which suspensions of any desired concentration can be obtained by dilution with water.

Examples

General recombinant DNA techniques

Since many of the recombinant DNA techniques used in this invention are routine for the skilled person, a brief description of the techniques generally used is given in the following so that these general details need not be given in the Embodiment Examples themselves. Unless expressly indicated otherwise, all of these methods are described in the reference work by [33] Maniatis et al., 1982.

A. Cleaving with restriction endonucleases

The reaction mixture will typically contain about 50 µg/ml to 500 µg/ml DNA in the buffer solution recommended by the manufacturer, New England Biolabs, Beverly, MA.. From 2 to 5 units of restriction endonuclease are added for every µg of DNA and the reaction mixture is incubated at the temperature recommended by the manufacturer for from one to three hours. The reaction is stopped by heating at 65°C for 10 minutes or by extraction with phenol, followed by precipitation of the DNA with ethanol. This technique is also described on pages 104 to 106 of the [33] Maniatis et al. reference work.

B. Treatment of the DNA with polymerase to produce blunt ends

50 µg/ml to 500 µg/ml DNA fragments are added to a reaction mixture in the buffer recommended by the manufacturer, New England Biolabs. The reaction mixture contains all four deoxynucleotide triphosphates in concentrations of 0.2 mM. An appropriate DNA polymerase is added and the reaction is carried out for 30 minutes at 15°C and is then stopped by heating for 10 minutes at 65°C. For fragments obtained by cleaving with restriction endonucleases that produce 5' cohesive ends, such as Eco RI and Bam HI, the large fragment, or Klenow fragment, of DNA polymerase is used. For fragments obtained using endonucleases that produce 3' cohesive ends, such as Pst I and Sac I, T4 DNA polymerase is used. The use of these two enzymes is described on pages 113 to 121 of the [33] Maniatis et al. reference work.

C. Agarose gel electrophoresis and cleaning DNA fragments to remove gel contaminants

Agarose gel electrophoresis is carried out in a horizontal apparatus as described on pages 150 to 163 of the [33] Maniatis et al. reference work. The buffer used corresponds to the Tris-borate buffer or Tris-acetate described therein. The DNA fragments are stained with 0.5 µg/ml ethidium bromide which either is present in the gel or tank buffer during electrophoresis or is not added until after electrophoresis, as desired. The DNA is made visible by illumination with long-wave ultra-violet light. If the

fragments are to be separated from the gel, an agarose that gels at low temperature, obtainable from Sigma Chemical, St. Louis, Missouri, is used. After electrophoresis, the desired fragment is excised, placed in a small plastics tube, heated at 65°C for about 15 minutes, extracted three times with phenol and precipitated twice with ethanol. This method has been changed slightly compared with the method described by [33] Maniatis et al. on page 170.

Alternatively, the DNA can be isolated from the agarose gel with the aid of the 'Geneclean Kit' (Bio 101 Inc., La Jolla, CA, USA).

D. Removal of 5' terminal phosphates from DNA fragments

During the plasmid cloning steps, treatment of the plasmid vector with phosphatase reduces the recircularisation of the vector (discussed on page 13 of the [33] Maniatis et al. reference work). After cleaving the DNA with the appropriate restriction endonuclease, one unit of calf intestinal alkaline phosphatase, which can be obtained from Boehringer-Mannheim, Mannheim, is added. The DNA is incubated for one hour at 37°C and then extracted twice with phenol and precipitated with ethanol.

E. Joining of DNA fragments

If fragments having complementary cohesive ends are to be joined to one another, about 100 ng of each fragment are incubated in a reaction mixture of from 20 µl to 40 µl with about 0.2 unit of T4 DNA ligase from New England Biolabs in the buffer recommended by the manufacturer. The incubation is carried out for from 1 to 20 hours at 15°C. If DNA fragments having blunt ends are to be joined, they are incubated as described above except that the amount of T4 DNA ligase is increased to from 2 to 4 units.

F. Transformation of DNA in E. coli

E. coli strain HB101 is used for most experiments. DNA is introduced into E. coli using the calcium chloride process described by [33] Maniatis et al., pages 250 to 251.

G. Screening of E. coli for plasmids

After transformation, the resulting colonies of E. coli are examined for the presence of the desired plasmid by a rapid plasmid isolation process. Two commonly used processes are described on pages 366 to 369 of the [33] Maniatis et al. reference work.

H. Large-scale isolation of plasmid DNA

Processes for the large-scale isolation of plasmids from E. coli are described on pages 88 to 94 of the [33] Maniatis et al. reference work.

Media and Buffer Solutions

LB medium	[g/l]
tryptone	10
yeast extract	5
NaCl	5

Antibiotic medium No. 3 (Difco Laboratories)

	[g/l]
bovine meat extract	1.5
yeast extract	1.5
peptone	5
glucose	1
NaCl	3.5
K_2HPO_4	3.68
KH_2PO_4	1.32

SCGY medium [g/l]

casamino acids	1
yeast extract	0.1
glucose	5
K_2HPO_4	14
KH_2PO_4	6
Na_3-citrate	1
$(NH_4)_2SO_4$	2
$MgSO_4 \cdot 7\ H_2O$	0.2

GYS medium ([22] Yousten & Rogoff, 1969)

[g/l]

glucose	1
yeast extract	2
$(NH_4)_2SO_4$	2
K_2HPO_4	0.5
$MgSO_4 \cdot 7\ H_2O$	0.2
$CaCl_2 \cdot 2\ H_2O$	0.08
$MnSO_4 \cdot H_2O$	0.05

pH adjusted to 7.3 before autoclaving.

PBS buffer [mM]

saccharose	400
$MgCl_2$	1
phosphate buffer, pH 6.0	7

TBST buffer	[mM]
Tween 20*	0.05 % (w/v)
Tris/HCl* (pH 8.0)	10
NaCl	150

*Tween 20: polyethoxysorbitan laurate
*Tris/HCl: α,α,α-Tris(hydroxymethyl)methylaminohydrochloride

The internal reference pK chosen for designating the plasmids in the Priority Document has been replaced in the Auslandsfassung (foreign filing text) by the officially recognised reference pXI.

Also, the designation for the asporogenic B. thuringiensis HD1 mutants used in the Embodiment Examples has been changed from cryß to cryB.

Example 1: Transformation of B. thuringiensis using electroporation

Example 1.1: 10 ml of an LB medium (tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l) are inoculated with spores of B. thuringiensis var. kurstaki HD1cryB ([39] Stahly D.P. et al., 1978), a plasmid-free variant of B. thuringiensis var. kurstaki HD1.

This batch is incubated overnight at a temperature of 27°C using a rotary shaker at 50 revs/min. Subsequently the B. thuringiensis culture is diluted 100-fold in from 100 ml to 400 ml of LB medium, and further cultured at a temperature of 30°C using a rotary shaker at 250 revs/min until an optical density (OD_{550}) of 0.2 is reached.

The cells are harvested by centrifugation and suspended in 1/40 volume of an ice-cooled PBS buffer (400 mM saccharose, 1 mM $MgCl_2$, 7 mM phosphate buffer pH 6.0). Centrifugation and subsequent suspension of the harvested B. thuringiensis cells in PBS buffer is repeated once more.

The cells pretreated in this manner can be electroporated either directly, or alternatively after the addition of glycerin to the buffer

solution [20 % (w/v)], and are stored at from -20°C to -70°C, and used at a later point in time.

800 µl aliquots of the ice-cooled cells are then transferred into precooled cuvettes, 0.2 µg pBC16 plasmid DNA ([40] Bernhard K. et al., 1978) (20 µg/ml) is subsequently added, and the entire batch is incubated at 4°C for 10 minutes.

If deep-frozen cell material is used, a suitable aliquot of frozen cells is first thawed in ice or at room temperature. The further treatment is analogous to the procedure used for fresh cell material.

The cuvette is then introduced into an electroporation apparatus and the B. thuringiensis cells present in the suspension are electroporated by the action of voltages of from 0.1 kV to 2.5 kV from a single discharge of a capacitor.

The capacitor used has a capacitance of 25 µF and the distance between the electrodes in the cuvette is 0.4 cm, which, when discharge occurs results, depending on the setting, in an exponentially decreasing field strength with initial peak values of from 0.25 kV/cm to 6.25 kV/cm. The exponential decay time lies in the range of from 10 ms to 12 ms.

An electroporation apparatus from the firm Bio Rad ("Gene Pulser Apparatus", #165-2075, Bio Rad, 1414 Harbour Way South, Richmond, CA 94804, USA), for example, can be used for the described electroporation experiments.

It is obviously also possible to use any other suitable apparatus in the process of the invention.

After a further 10 minutes' incubation at 4°C, the cell suspension is diluted with 1.2 ml of LB medium, and incubated for 2 hours at a temperature of 30°C using a rotary shaker at 250 revs/min.

Suitable dilutions are then plated out onto LB agar (LB medium solidified with agar, 15 g/l), which contains as an additive an antibiotic suitable

for the selection of the newly obtained plasmid. In the case of pBC16 this is tetracycline, which is added to the medium in a concentration of 20 mg/l.

The transformation frequencies achieved for B. thuringiensis HD1cryB and pBC16 as a function of the initial voltage applied for a given distance between plates are reproduced in Figure 1.

The expression of the inserted DNA can be detected by way of the tetracycline resistance that occurs. As soon as 2 hours after the introduction by transformation of pBC16 into B. thuringiensis a complete phenotypic expression of the newly introduced tetracycline resistance occurs (see Table 2).

Example 1.2: The transformation of B. thuringiensis cells is carried out in exactly the same manner as that described in Example 1.1, except that the volume of the cell suspension provided for the electroporation is in this case 400 µl.

The transformation frequency can be increased by a factor of 10 by this measure.

Example 2: Transformation of B. thuringiensis HD1cryB with a number of different plasmids

Most of the tests are carried out with plasmid pBC16, a naturally occurring plasmid of B. cereus. In addition, however, other naturally occurring plasmids can also be successfully inserted into B. thuringiensis cells, such as, for example, pUB110 ([25]Polack J. and Novik R.P., 1982), pC194 ([24]Horinouchi S. and Weisblum B., 1982) and pIM13 ([26]Mahler I. and Halvorson H.O. 1980) (see Table 3).

Also, variants of these plasmids that are better suited than the natural isolates for work with recombinant DNA can be introduced by transformation into the B. thuringiensis strain HD1cryB using the process of the invention, such as, for example, the B. subtilis cloning vector pBD64 ([27]Gryczan T. et al., 1980) and plasmids pBD347, pBD348 and pUB1664 (see

Table 3; plasmids pBD347, pBD348 and pUB1664 can be obtained from Dr. W. Schurter, CIBA-GEIGY AG, Basle).

The transformation results in Table 3 show clearly that using the transformation process of the invention, transformation frequencies are achieved that, with one exception, are all in the range of from 10^5 to 10^7, irrespective of the plasmid DNA used.

Example 3: Construction of a "shuttle" vector for Bacillus thuringiensis

Existing bifunctional vectors for E. coli and B. subtilis, such as, for example, pHV33 ([41] Primrose S.B. and Ehrlich S.D., Plasmid, 6: 193-201, 1981) are not suitable for B. thuringiensis HD1cryB (see Table 3).

For the construction of a potent bifunctional vector, first of all the large Eco RI fragment of pBC16 is inserted with the aid of T4 DNA ligase into the Eco RI site of plasmid pUC8 ([28] Vieira J. and Messing J. 1982). E. coli cells are then transformed with this construct. A construct recognised as correct by restriction analysis is designated pXI62.

The removal of the Eco RI cleavage site situated distally from the pUC8 polylinker region then follows. pXI62 is linearised by a partial Eco RI digestion. The cohesive Eco RI ends are made up with Klenow polymerase and joined together again with T4 DNA ligase. After introduction into E. coli by transformation, a construct recognised as correct by restriction analysis is selected and designated pXI61. A map of pXI61 with the cleavage sites of restriction enzymes that cleave pXI61 only once, is shown in Figure 6.

This construct can be introduced directly into B. thuringiensis HD1cryB using the transformation process described in Example 1.

On account of the strong restriction barriers in B. thuringiensis strains, the transformation rates are lower when using pXI61 DNA originating from E. coli than when using plasmid DNA originating from B. thuringiensis HD1cryB (see Table 3). Nevertheless pXI61 proves to be very suitable for carrying out cloning experiments in B. thuringiensis.

Example 4: Insertion of the Kurhdl delta-endotoxin gene into strains of B. thuringiensis and B. cereus

The DNA sequence coding for a Kurhdl delta-endotoxin protein used within the scope of this invention for insertion and expression in B. thuringiensis and B. cereus originates from plasmid pK36, which was deposited on 4th March 1986 under the Deposit Number DSM 3668 in accordance with the requirements of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patenting, at the Deutsche Sammlung von Mikroorganismen, Federal Republic of Germany, which is recognised as an International Depository.

A detailed description of the process for identifying and isolating the δ-endotoxin genes and for the construction of plasmid pK36 is contained in European Patent Application EP 0 238 441 and is a part of the present invention in the form of a reference.

pK36 plasmid DNA is completely digested with the restriction enzymes Pst I and Bam HI and the 4.3 Kb fragment, which contains the Kurhdl delta-endotoxin gene (cf. formula I), is isolated from an agarose gel. This fragment is then inserted into pXI61, which has previously been digested with Pst I and Bam HI and treated with alkaline phosphatase from calf's stomach. After the transformation of E. coli HB101, a construct recognised as correct by restriction analysis is isolated and designated pXI93. A restriction map of pXI93 is reproduced in Figure 7.

pXI93 can be introduced into B. thuringiensis HDlcryB in 2 different ways.

a) B. thuringiensis cells are transformed directly with a pXI93 isolate of E. coli using the transformation process of the invention described in Example 1.

b) pXI93 is first of all introduced into B. subtilis cells by transformation, as described by Chang and Cohen, 1979. The complete and intact pXI93 plasmid DNA contained in a transformant is isolated and then introduced into B. thuringiensis HDlcryB by transformation using the electroporation process described in Example 1.

Both methods result in transformants that contain the intact pXI93 plasmid, which can be demonstrated by restriction analysis.

Example 5: Evidence of the expression of the delta-endotoxin gene in B. thuringiensis

Sporulating cultures of B. thuringiensis HD1cryB, HD1cryB (pXI61), HD1cryB (pXI93) and HD1 are compared under a phase contrast microscope at a magnification of 400. The typical bipyrimidal protein crystals can be detected only in the strain containing pXI93 and in HD1. Extracts from the same cultures are separated electrophoretically on an SDS polyacrylamide gel. A protein band of 130,000 Dalton, which corresponds to the Kurhd1 gene product, could be detected on the gel only for the strain containing plasmid pXI93 and in HD1 (Figure 8a).

In a Western blot analysis (Figure 8b), this 130,000 Dalton protein and its degradation products react specifically with polyclonal antibodies that have been prepared previously against crystalline protein of B. thuringiensis var. kurstaki HD1 in accordance with the process described by [42] Huber-LukačH., 1982. A detailed description of this process can be found in European Patent Application EP 238 441, which is a part of this invention in the form of a reference. Located on plasmid pXI93, upstream of the toxin-encoding region, is a 156 Bp DNA region, which contains the afore-described sporulation-dependent tandem promoter ([29] Wong H.C. et al., 1983). This sequence is adequate for a high expression of the delta-endotoxin gene in B. thuringiensis HD1cryB and B. cereus 569K.

Example 6: Evidence of the toxicity of recombinant B. thuringiensis HD1cryB (pXI93)

B. thuringiensis HD1cryB and HD1cryB (pXI93) are cultured at 25°C in sporulation medium (GYS medium). When sporulation is complete, which is checked using a phase contrast microscope, spores and (if present) protoxin crystals are harvested by centrifugation and spray-dried. The resulting powder is admixed in various concentrations with the food of L-1 larvae of Heliothis virescens (tobacco budworm). The mortality of the larvae is ascertained after six days.

As expected, the protoxin gene-free strain HDlcryB is non-toxic to Heliothis virescens, whilst the strain transformed with plasmid pXI93 causes a dosage-dependent mortality of H. virescens (Table 4). This demonstrates that recombinant strains produced by the electroporation process can actually be used as bioinsecticides.

Example 7: Electroporation of various B. thuringiensis and B. spec. strains

The transformation protocol for B. thuringiensis HDlcryB described under Example 1 can also be applied to other strains.

All tested strains of B. thuringiensis var. kurstaki can be very simply and efficiently transformed by this process (Table 5).

Excellent transformation frequencies can also be achieved with a laboratory strain of B. cereus. The same applies also to other tested B. thuringiensis varieties (var. israelensis, var. kurstaki). By contrast, transformation of B. subtilis by the electroporation process is very poor.

Using the protoplast-dependent PEG method for B. subtilis, on the other hand, transformation rates of 4 x 10^6/µg plasmid DNA were achieved.

The low transformation rates of B. subtilis obtained using the electroporation technique are not associated with incorrectly selected parameters, such as, for example, an unsuitable voltage, or with a high mortality rate caused by electric pulses, as can be seen from Figure 9.

Example 8: Transformation of B. thuringiensis HDlcryB with the ß-galactosidase gene

8.1. Insertion of a Bam HI restriction cleavage site directly before the first AUG codon of the B. thuringiensis protoxin gene

Before the ß-galactosidase gene from the plasmid piWiTh5 (obtainable from Dr. M. Geiser, CIBA-GEIGY AG, Basle, Switzerland) can be joined to the

promoter of the Kurhdl δ-endotoxin gene of B. thuringiensis, the DNA sequence of the protoxin gene located in the region of the AUG start codon must first be modified.

This modification is carried out by oligonucleotide-directed mutagenesis, using the single-stranded phage M13mp8, which contains the 1.8 . . Hinc II-Hind III fragment, of the δ-endotoxin gene containing the 5' region of that gene.

First of all 3 µg of plasmid pK36 (cf. Example 4) are digested with the restriction enzymes Hind III and Hinc II. The resulting 1.8 kb fragment is purified by agarose gel electrophoresis and then isolated from the gel.

In parallel with this, 100 ng of M13mp8 RF phage DNA (Biolab, Tozer Road, Beverly MA, 01915, USA or any other manufacturer) are digested with the restriction enzymes Sma I and Hind III, treated with phenol, and precipitated by the addition of ethanol. The phage DNA treated in this manner is then mixed with 200 ng of the previously isolated protoxin fragment and joined thereto by the addition of T4 DNA ligase.

After the transfection of E. coli JM103, 6 white plaques are selected and analysed by restriction mapping.

An isolate in which the join between the ß-galactosidase gene and the promoter of the Kurhdl δ-endotoxin gene of B. thuringiensis has been carried out correctly is selected and designated M13mp8/Hinc-Hind.

An oligonucleotide with the following sequence is synthesized using a DNA synthesizing apparatus ("APPLIED BIOSYSTEM DNA SYNTHESIZER"):

(5') GTTCGGATTGGGATCCATAAG (3')

This synthetic oligonucleotide is complementary to the M13mp8/Hinc-Hind DNA in a region that extends from position 153 to position 173 of the Kurdhl δ-endotoxin gene (cf. formula I). The oligonucleotide sequence reproduced above has a "mismatch" in positions 162 and 163, however,

compared with the sequence reproduced in formula I, so that the formation of a Bam HI restriction cleavage site is necessary. The general procedure for the mutagenesis is described by J. M. Zoller and M. Smith ([43] J.M. Zoller and M. Smith; 19). Approximately 5 µg of single-stranded M13mp18/Hinc-Hind phage DNA is mixed with 0.3 µg of phosphorylated oligonucleotides in a total volume of 40 µl. This mixture is heated for 5 minutes at 65°C, cooled first to 50°C and then, gradually, to 4°C. Buffer, nucleotide triphosphates, ATP, T4 DNA ligase and the large fragment of DNA polymerase are then added and the batch is incubated overnight at 15°C in the manner described ([43] J.M. Zoller and M. Smith). After agarose gel electrophoresis, circular double-stranded DNA is purified and inserted into E. coli strain JM103 by transfection. As an alternative, the E. coli strain JM107 can be used.

The resulting plaques are examined for sequences that hybridize with ^{32}P-labelled oligonucleotide; the phages are examined by DNA restriction endonuclease analysis.

A phage that contains a correct construct in which a Bam HI cleavage site is located directly before the first AUG codon of the protoxin gene is designated M13mp8/Hinc-Hind/Bam.

8.2. Joining the ß-galactosidase gene to the δ-endotoxin promoter

8.2.1: The δ-endotoxin promoter is on a 162 Bp Eco RI/Bam HI fragment of the M13mp8/Hinc-Hind/Bam phage DNA. RF phage DNA is digested with restriction enzyme Bam HI. The projections resulting at the 5' ends are removed by treatment with "Mung Bean" nuclease (Biolabs) in accordance with the manufacturer's instructions. Subsequently, the DNA is digested with the restriction endonuclease Eco RI and, after carrying out agarose gel electrophoresis, the 162 Bp fragment is isolated from the agarose gel.

The ß-galactosidase gene is isolated from plasmid piWiTh5. piWiTh5 DNA is first of all cleaved at the single Hind III cleavage site. The 3' recessed ends are made up using the Klenow fragment of DNA polymerase (cf. [33] Maniatis et al., 1983, page 113-114) and the modified DNA is then

digested with the restriction enzyme Sal I. The DNA fragment containing the ß-galactosidase gene is isolated by agarose gel electrophoresis.

The vector pXI61 (cf. Example 3) is digested with the restriction enzymes Eco RI and Sal I and the two previously isolated fragments are inserted into the vector pXI61.

After transformation of this ligation mixture in the E. coli strain HB101 or JM107, the correctly joined clones are selected by restriction analysis and by their ß-galactosidase activity with respect to the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside). A clone containing a correct genetic construct is designated pXI80.

8.2.2: In an alternative embodiment, the 162 Bp Eco RI/Bam HI fragment containing the δ-endotoxin promoter is isolated by cleavage of M13mp8/Hinc-Hind/Bam with Eco RI and Bam HI, followed by separation by gel electrophoresis.

The ß-galactosidase gene is isolated from plasmid piWiTh5 in this instance too (cf. Example 8.1.). In this case, the plasmid DNA is digested with the restriction enzymes Bam HI and Bgl II and the large fragment is eluted from the agarose gel after gel electrophoresis.

The vector pHY300 PLK (#PHY-001; Toyobo Co., Ltd., 2-8 Dojima Hama 2-Chome, Kita-ku, Osaka, 530 Japan), which can be obtained commercially (cf. Example 9.1), is digested with the restriction enzymes Eco RI and Bgl II. The two previously isolated fragments are then inserted into the vector pHY300 PLK.

The entire ligation mixture is then introduced by transformation into the E. coli strain JM107 (Bethesda Research Laboratories (BRL), 411 N, Stonestreet Avenue, Rockville, MD 20850, USA). A clone having a ß-galactosidase activity is further analysed by restriction digestions. A clone containing a correct genetic construct is designated pXI101.

8.3. Introduction by transformation into B. subtilis and B. thuringiensis of plasmid pXI80 or pXI101

pXI80 or pXI101 plasmid DNA is first of all introduced into B. subtilis protoplasts by transformation according to a known test protocol described by Chang and Cohen ([13] Chang and Cohen, 1979).

A correct clone is selected, the DNA to be transformed is isolated by standard processes and introduced by transformation into B. thuringiensis HDlcryB cells by way of electroporation (cf. Example 1).

The transformed B. thuringiensis cells are plated out onto GYS agar (sporulation medium), which contains X-gal as an additive.

Correctly transformed clones turn blue when sporulation commences.

A B. thuringiensis HDlcryB strain transformed by the pXI61 vector, on the other hand, remains white under the same conditions.

Restriction analysis shows that with correctly transformed clones, an intact pXI80 or pXI101 plasmid is present in the B. thuringiensis cells.

8.4. ß-galactosidase gene under the control of a sporulation-dependent promoter

B. thuringiensis HDlcryB cells containing plasmid pXI80 or pXI101 are cultured on GYS medium in the manner described hereinbefore. At intervals during the growth phase (both during the vegetative growth phase and during the sporulation phase) a ß-galactosidase assay is carried out in accordance with the test protocol described by [44] J.H. Miller ("Experiments in Molecular Genetics", Cold Spring Harbor Laboratory, 1972, Experiment 48 and 49).

The individual differences from the above-mentioned test protocol concern the use of X-gal as chromogenic substrate and the measurement of the coloured hydrolysis product, which is formed by the cells after approximately 1 hour.

The cells are then removed by centrifugation, and the optical density of the supernatant is ascertained at a wavelength of 650 nm (OD_{650}).

An increase in the optical density as a function of sporulation is observed. The non-transformed B. thuringiensis cells, on the other hand, cannot hydrolyse the chromogenic substrate X-gal.

Example 9: Creation of gene banks in Bacillus thuringiensis

9.1. Construction of pXI200

Plasmid pXI200 is a derivative of plasmid pHY300 PLK, which can be obtained commercially from Toyobo Co., Ltd. (#PHY-001; Toyobo Co., Ltd., 2-8 Dojima Hama 2-Chome, Kita-ku, Osaka, 530 Japan). Plasmid pHY300, the construction of which is described in European Patent Application EP 162 725, contains both an ampicillin (amp^R) and a tetracycline ($tetr^R$) resistance gene.

Plasmid pHY300 PLK is completely digested with Bgl I and Pvu I. The resulting restriction fragments are then separated by agarose gel electrophoresis. The 4.4 Kb fragment is isolated from the agarose gel, purified and then religated with T4 DNA ligase.

The whole ligation batch is introduced by transformation into E. coli HB101. After incubation of the transformed E. coli HB101 cells at 37°C on a selective L-agar containing 20 µg/ml tetracycline, the tetracycline-resistant (Tcr) transformants are selected. It is then possible to isolate from an ampicillin-sensitive (Aps) clone (100 µg/ml ampicillin) a plasmid that has lost the Pst I cleavage site in the Apr gene together with the 0.3 Kb Pvu I/Bgl I fragment. This plasmid is designated pXI200.

9.2 Cloning protoxin genes of Bacillus thuringiensis var. kurstaki HD1 in Bacillus thuringiensis HD1cryB

The total DNA (50 µg) of Bacillus thuringiensis var kurstaki HD1 is completely digested by incubation with the restriction enzymes Pst 1 and Hpa 1. The restriction fragments so obtained are transferred to a continuous saccharose gradient [5 % (w/v) - 23 % (w/v)] where they are separated according to size by density gradient centrifugation and

collected in 500 µl fractions. The centrifugation is carried out in a TST 41-rotor (Kontron Ausschwingrotor) at a temperature of 15°C at max 2.4 x 10^5 g for a period of 16 hours. Subsequently, in order to determine the fragment size aliquots, each of 50 µl, are transferred to an agarose gel [0.8 % (w/v) agarose in Tris acetate EDTA or Tris borate EDTA; see [33] Maniatis et al., 1982]. Those fractions containing fragments between 3 Kb and 6 Kb are pooled and concentrated to a volume of 10 µl by ethanol precipitation.

5 µg of the "shuttle" vector pXI200 described in Example 9.1 are digested with the restriction enzymes Pst 1 and Sma 1. The 5' phosphate groups of the resulting restriction fragments are then removed by treatment with calf intestinal alkaline phosphatase. 0.2 µg to 0.3 µg of the previously isolated HD1 DNA is then mixed with 0.5 µg of the pXI200 vector DNA and incubated overnight at 14°C with the addition of 0.1 U of T4 DNA ligase (so-called "Weiss Units"; one unit of T4 DNA ligase corresponds to an enzymatic activity sufficient to convert 1 nM [32P] from pyrophosphate at a temperature of 37°C and within a period of 20 minutes into a Norit-absorbable material). The entire ligation batch is then introduced by transformation directly into Bacillus thuringiensis HD1cryB cells by means of electroporation (cf. Example 1). The electroporated B. thuringiensis cells are then plated out onto a selective sporulation agar containing 20 µg/ml of tetracycline as selecting agent, and incubated at a temperature of 25°C until sporulation is complete.

9.3. Manufacture of monoclonal antibodies to B. thuringiensis protoxin protein

The manufacture of monoclonal antibodies to δ-endotoxin of Bacillus thuringiensis var. kurstaki HD1 is carried out analogously to the description in [36] Huber-Lukač (1984) and in [37] Huber-Lukačet al., (1986).

The hybridoma cells used for the antibody manufacture are fusion products of Sp2/0-Ag myeloma cells (described in [45] Shulman et al., 1978; can be obtained at the "American Type Culture Collection" in Rockville, Maryland, USA) and splenocytes of Balb/c mice that have previously been immunised with δ-endotoxin of B. thuringiensis var. kurstaki HD1.

In this manner it is possible to obtain monoclonal antibodies that are directed specifically against the δ-endotoxin of B. thuringiensis. Especially preferred are monoclonal antibodies that either bind specifically to an epitope in the N-terminal half of the protoxin protein (for example antibody 54.1 of the Huber-Lukač et al., 1986 reference), or recognise an epitope in the part of the protein constant in Lepidoptera-active protoxins, the C-terminal half (for example antibody 83.16 of the Huber-Lukač et al., 1986 reference).

It is, however, also entirely possible for other monoclonal or also polyclonal antibodies to be used for the subsequent immunological screening (cf. Example 9.4).

9.4. Immunological Screening

The monoclonal antibodies produced in accordance with Example 9.3, or other suitable monoclonal antibodies, are used for the immunological screening.

First of all, the crystalline proteins present in free form after the sporulation of the B. thuringiensis cells are bound by means of transfer membranes (for example Pall Biodyne transfer membrane; Pall Ultrafine Filtration Corporation, Glen Cove, N.Y.) by applying the filter membranes to the plates for a period of approximately 5 minutes. The filters are subsequently washed for 5 minutes with TBST buffer [0.05 % (w/v) Tween 20, 10 mM Tris/HCl (pH 8.0), 150 mM NaCl in bidist. H_2O] and then, in order to block non-specific binding, incubated in a mixture of TBST buffer and 1 % (w/v) skimmed milk for from 15 to 30 minutes.

The filters prepared in this manner are then incubated overnight with the protoxin-specific antibodies [antibody mixture of 54.1 and 83.16 ([37] Huber-Lukač et al., (1986)]. The unbound antibodies are removed by washing the filter three times with TBST buffer for from 5 to 10 minutes each time. To detect the antibody-bound protoxin the filters are incubated with a further antibody. The secondary antibody used is an anti-mouse antibody labelled with alkaline phosphatase, which can be obtained commercially, for example, from Bio-Rad [Katalog #170-6520,

goat's anti-mouse IgG(H+L)-alkaline phosphatase conjugate]. After an incubation period of 30 minutes the unbound secondary antibodies are removed in the manner described above by washing the filters with TBST buffer three times (for from 5 to 10 minutes each time). The filters are then incubated with a substrate mixture consisting of NBT ['p-nitro blue tetrazolium chloride; nitro-blue tetrazolium chloride] and BCIP [5-bromo-4-chloro-3-indolylphosphate-p-toluidine salt]. The enzymatic reaction is carried out in accordance with the manufacturer's instructions [Bio-Rad; 1414 Harbour Way South, Richmond CA, 94804, USA].

Positive, that is to say protoxin-containing clones, can be recognised very easily by their violet colouring. This occurs as a result of the enzymatic reaction of the alkaline phosphatase with the afore-mentioned substrate mixture. Between 800 and 1000 transformants result from the transformation described in Example 9.2 with the ligation batch indicated in that Example. Of these transformants 2 colonies exhibit clearly positive signals in the above-described enzyme reaction.

Plasmid DNA is isolated from positive clones in which expression of the protoxin gene could be detected by way of the described enzyme reaction. The cloned protoxin genes can be further characterised and ultimately identified by restriction analysis and comparison with known restriction maps.

Both clones contain a recombinant plasmid with an insert of 4.3 Kb. The subsequent restriction digestions with Hind III, Pvu II, Eco RI and Xba I permit identification of the gene on the insert by comparison with the known restriction maps of the endotoxin genes of B. thuringiensis var. kurstaki HD1. In both cases the gene is the Kurhd1 gene, which is also known as the 5.3 Kb protoxin gene and is described in [5] Geiser et al., 1986.

This gene, cloned directly in B. thuringiensis and identified by immunological screening, furthermore hybridises with a 1847 Bp Bam HI/Hind III fragment of the 5.3 Kb gene in plasmid pK36 ([5] Geiser et al., 1986). In

the SDS/PAGE, both clones exhibit a band of 130,000 Dalton typical of the protoxin, which in a Western blot ([46] Towbin et al., 1979) react specifically with the afore-described (see Example 9.4) monoclonal antibodies.

Tables

Table 1: Influence of the incubation time at 4°C, before and after electroporation, on the transformation frequency. B. thuringiensis HD1cryB was transformed using the electroporation process with 0.2 µg pBC16 per batch.

Example	1	2	3	4	5	6	7	8
preincubation * (minutes)	0	5	10	20	20	20	20	20
subsequent incubation ** (minutes)	20	20	20	20	0	5	10	20
Transformation frequency (Transformants /µg Plasmid DNA)	2.6×10^6	2.1×10^6	2.2×10^6	2.3×10^6	2.5×10^6	1.9×10^6	3.3×10^6	1.7×10^6

* Incubation at 4°C between the addition of DNA and electroporation

** Incubation at 4°C between electroporation and the beginning of the expression period

Table 2: Expression of the tetracycline resistance of pBC16 after introduction into B. thuringiensis HD1cryB by transformation

B. thuringiensis HD1cryB was transformed with pBC16 plasmid DNA using the electroporation protocol according to the invention. After various incubation periods in LB medium at 30°C, the transformed cells are selected by plating out onto LB agar containing 20 µg/ml tetracycline.

Time taken to express tetracycline resistance (hours)	Transformation frequency (Transformants/µgDNA)	Number of living cells
0.5	0	4×10^8
1	1.6×10^6	10^9
2	8.8×10^6	1.4×10^9
3	8×10^6	1.6×10^9

Table 3: Transformation of the B. thuringiensis strain HD1cryB with various plasmids

Plasmid	Origin	resistance marker[1] gram negative	gram positive	Transformation[2] frequency
		naturally occuring plasmids		
pBC16	B. cereus	-	Tc	1.9×10^6
pUB110	Staphylococcus aureus	-	Km, Ble	3.3×10^6 *
pC194	S. aureus	-	Cm	6×10^6 *
pIM13	B. subtilis	-	Em	1.8×10^5
		modified plasmids/cloning vectors		
pBD64	pUB110 replicon	-	Km, Cm	5×10^6
pBD347	pIM13 replicon,	-	Cm	2.9×10^5
pBD348	pIM13 replicon,	-	Em, Cm	1.1×10^5
pUB1664	pUB110 replicon,	-	Cm, Em	3.5×10^4
		"shuttle" vectors		
pHV33	pBR322/pC194,	Amp, Tc	Cm	< 50*
pK61	pUC8/ pBC16,	Amp	Tc	2.8×10^4

1: Tc: tetracycline; Km: kanamycin; Ble: bleomycin; Cm: chloramphenicol; Em: erythromycin

2: All plasmid DNA originates from B. thuringiensis HD1cryB with the exception of * isolated from B. subtilis LBG4468.

Table 4: Biotest of B. thuringiensis HD1cryB and HD1cryB (pXI93) against Heliothis virescens.

Spray-dried sporulated cultures (spores and (if present) protoxin crystals) are admixed, in the amounts indicated, with the food of L-1 larvae of Heliothis virescens.

Concentration of spores and protoxin crystals (µg/g food)	Mortality (%) of H. virescens caused by:	
	HD1 cryB	HD1 cryB (pXI93)
200	0	57
100	0	43
50	3	27
25	0	10
12.5	0	0

Table 5: Transformability of strains of B. thuringiensis, B. cereus and B. subtilis. All strains were transformed with plasmid pBC16 in accordance with the electroporation process described under Example 1

Strain	Transformation[1] frequency
B. thuringiensis var. kurstaki	
HD1cryB	1
HD1 dipel	0.25
HD1-9	0.9
HD 73	0.1
HD 191	0.5
B. thuringiensis var. thuringiensis	
HD 2-D6-4	13.8
B. thuringiensis var. israelensis	
LBG B-4444	2.6
B. cereus	
569 K	7.5
B. subtilis	
LBG B-4468	0.0002

[1] relative values based on the transformation frequency, defined as 1, achieved with B. thuringiensis var. kurstaki HD1cryB.

Deposit of Microorganisms

A culture of each of the microorganisms listed in the following that are used within the scope of the present invention has been deposited at the "Deutsche Sammlung von Mikroorganismen", recognised as an International Depository, in Braunschweig, Federal Republic of Germany, in accordance with the requirements of the Budapest Treaty for the International

Recognition of the Deposit of Microorganisms for the Purposes of Patenting. A declaration concerning the viability of the deposited samples has been issued by the said International Depository.

Deposit of Micoorganisms

Microorganisms	Deposit Date	Deposit Number	Date of the viability certificate
HB 101 (pK36) (E. coli HB101 transformed with pK36 plasmid DNA)	4. March 1986	DSM 3668	7. March 1986
*HD1 cryß (Bacillus thuringiensis var. kurstaki HD1 cryß	4. May 1988	DSM 4574	4. May 1988
*HD1 cryß (*pK 61) (B. thuringiensis HD1 cryß transformed with *pK61 plasmid DNA)	4. May 1988	DSM 4572	4. May 1988
*HD1 cryß (*pK 93) (B. thuringiensis HD1 cryß transformed with *pK93 plasmid DNA)	4. May 1988	DSM 4571	4. May 1988
569 K (Bacillus cereus 569 K)	4. May 1988	DSM 4575	4. May 1988
569 K (*pK 93) (B. cereus 569 K transformed with *pK93 plasmid DNA)	4. May 1988	DSM 4573	4. May 1988

The internal reference pK selected for the designation of the plasmids in the Priority Document has been replaced for the Auslandsfassung (foreign filing text) by the officially recognised designation pXI.

Also, the designation for the asporogenic B. thuringiensis HD1 mutants used in the Embodiment Examples has been changed from cryß to cryB.

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The claims defining the invention are as follows:

1. A process for inserting and cloning DNA sequences in gram positive bacteria selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus*, comprising:

(a) isolating the DNA to be introduced;

(b) cloning the thus isolated DNA in a cloning vector that is capable of replicating in a bacterial host cell selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus* cells in a heterologous cloning system;

(c) directly introducing the thus cloned vector DNA into the said bacterial cell via electroporation at a transformation rate sufficient to overcome the restriction barrier present in the said bacterial cells; and

(d) cultivating the thus transformed bacterial cells and isolating the thus cloned vector DNA.

2. A process for inserting, cloning and expressing DNA sequences in gram positive bacteria selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus*, comprising:

(a) isolating the DNA to be introduced and optionally ligating the thus isolated DNA with expression sequences that are capable of functioning in bacterial cells selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus* cells;

(b) cloning the thus isolated DNA in a cloning vector that is capable of replicating in a bacterial host cell selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus* cells in a heterologous cloning system;

(c) directly introducing the thus cloned vector DNA into the said bacterial cell via electroporation at a transformation rate sufficient to overcome the restriction barrier present in the said bacterial cells; and

(d) cultivating the thus transformed bacterial cells and isolating the thus cloned vector DNA and the expressed gene product.

3. A process according to claim 2, wherein said transforming comprises:

(a) preparing a suspension of host cells in an aerated medium sufficient to allow for growth of the cells;

(b) separating the grown cells from the cell suspension and resuspending the grown cells in an inoculation buffer;

(c) adding a DNA sample comprising the cloned DNA in a concentration suitable for the electroporation to the buffer;

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(d) introducing the batch of step (c) into an electroporation apparatus;

(e) subjecting the thus introduced batch to at least one capacitor discharge to produce a high electric field strength that is sufficient to render the bacterial cell wall permeable to the DNA to be introduced, for a period of time sufficient to transform the bacterial host cells with the recombinant DNA;

(h) selecting the thus transformed bacterial host cells.

4. A process according to claim 3, which comprises using *B. thuringiensis* spores as starting material for the preparation of the cell suspension of step (a).

5. A process according to claim 3, which comprises using thawed bacterial cells, which cells have previously been deep-frozen, as starting material for the preparation of the cell suspension of step (a).

6. A process according to claim 3, wherein the culture medium of step (a) comprises

(a) complex nutrient media with readily assimilable carbon and nitrogen sources that are conventionally employed for culturing aerobic Bacillus species; or

(b) fully synthetic or semi-synthetic nutrient media that contain

(b_1) a complex or alternatively a defined readily assimilable carbon and nitrogen source or a combination of the two and also

(b_2) essential vitamins and metal ions.

7. A process according to claim 3, wherein in step (a) the said *Bacillus* cells are grown until an optical density [OD_{550}] of from 0.1 to 1.0 is achieved.

8. A process according to claim 3, wherein the inoculation buffer of step (b) is a phosphate buffer that has been osmotically stabilized by addition of at least one osmotic stabilizing agent.

9. A process according to claim 8, wherein the said phosphate buffer contains sugars or sugar alcohols as an osmotic stabilizing agent.

10. A process according to claim 9, wherein the said stabilizing agent is saccharose, which is present in a concentration of from 0.1 M to 1.0 M.

11. A process according to claim 8, wherein the said phosphate buffer has a pH value of from pH 5.0 to pH 8.0.

12. A process according to claim 3, wherein the incubation of the bacterial cells is carried out at a temperature of from 0°C to 35°C before, during and after electroporation.

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13. A process according to claim 12, wherein the incubation of the bacterial cells is carried out at a temperature of from 2°C to 15°C before, during and after electroporation.

14. A process according to claim 3, wherein the concentration of the added DNA sample is from 1 ng to 20µg.

15. A process according to claim 3, wherein the field strength are from 3000 V/cm to 4500 V/cm.

16. A process according to claim 3, wherein the exponential decay time of the pulse acting on the bacterial cell suspension lies within a range of from 2 ms to 50 ms.

17. A process according to claim 3, wherein selection of the transformed bacterial host cells comprises plating out the electroporated cells, after a suitable subsequent incubation phase, onto solid media containing an additive suitable for the selection of the transformed bacterial cells.

18. A process according to claim 17, wherein the said additive is an antibiotic suitable for the selection *B. thuringiensis* or *B. cereus* or both, selected from the group consisting of tetracycline, kanamycin, chloramphenicol, erythromycin.

19. A process according to claim 18, wherein the said additive is a chromogenic substrate suitable for the selection of *B. thuringiensis* or *B. cereus* or both.

20. A process according to any one of claims 1 or 2, wherein the DNA to be introduced into the said bacterial host cell is a recombinant DNA which is of homologous or heterologous origin or is a combination of homologous and heterologous DNA.

21. A process according to claim 20, wherein the said recombinant DNA contains one or more structural genes and 3' and 5' flanking regulatory sequences that are capable of functioning in the said bacterial host cells, which sequences are operably linked to the structural gene(s) and thus ensure the expression of the said structural gene(s) in said bacterial host cells.

22. A process according to claim 21, wherein the said structural gene codes for a δ-endotoxin polypeptide occurring naturally in *B. thuringiensis*, or for a polypeptide that has substantial structural homologies therewith and has still substantially the toxicity properties of the said crystalline δ-endotoxin polypeptide.

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23. A process according to claim 22, wherein the said δ-endotoxin-encoding DNA sequence is substantially homologous with at least the part or parts of the natural δ-endotoxin-encoding sequence that is (are) responsible for the insecticidal activity.

24. A process according to claim 22, wherein the said polypeptide is substantially homologous with a δ-endotoxin polypeptide of a suitable sub-species of *B. thuringiensis*, selected from the group consisting of *kurstaki, berliner, alesti, sotto, tolworthi, dendrolimus, tenebrionis* and *israelensis*.

25. A process according to claim 22, wherein the said δ-endotoxin-encoding DNA sequence is a DNA fragment of *B. thuringiensis* var. *kurstaki* HD1 located between nucleotides 156 and 3623 in formula I, or is any shorter DNA fragment that still codes for a polypeptide having insect-toxic properties:

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Formula I

```
        10         20         30         40         50
GTTAACACCC TGGGTCAAAA ATTGATATTT AGTAAAATTA GTTGCACTTT

        60         70         80         90        100
GTGCATTTTT TCATAAGATG AGTCATATGT TTTAAATTGT AGTAATGAAA

       110        120        130        140        150
AACAGTATTA TATCATAATG AATTGGTATC TTAATAAAAG AGATGGAGGT

       160        170        180        190        200
AACTTATGGA TAACAATCCG AACATCAATG AATGCATTCC TTATAATTGT

       210        220        230        240        250
TTAAGTAACC CTGAAGTAGA AGTATTAGGT GGAGAAAGAA TAGAAACTGG

       260        270        280        290        300
TTACACCCCA ATCGATATTT CCTTGTCGCT AACGCAATTT CTTTTGAGTG

       310        320        330        340        350
AATTTGTTCC CGGTGCTGGA TTTGTGTTAG GACTAGTTGA TATAATATGG

       360        370        380        390        400
GGAATTTTTG GTCCCTCTCA ATGGGACGCA TTTCTTGTAC AAATTGAACA

       410        420        430        440        450
GTTAATTAAC CAAAGAATAG AAGAATTCGC TAGGAACCAA GCCATTTCTA

       460        470        480        490        500
GATTAGAAGG ACTAAGCAAT CTTTATCAAA TTTACGCAGA ATCTTTTAGA

       510        520        530        540        550
GAGTGGGAAG CAGATCCTAC TAATCCAGCA TTAAGAGAAG AGATGCGTAT
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```
       560         570         580         590         600
TCAATTCAAT GACATGAACA GTGCCCTTAC AACCGCTATT CCTCTTTTTG

       610         620         630         640         650
CAGTTCAAAA TTATCAAGTT CCTCTTTTAT CAGTATATGT TCAAGCTGCA

       660         670         680         690         700
AATTTACATT TATCAGTTTT GAGAGATGTT TCAGTGTTTG GACAAAGGTG

       710         720         730         740         750
GGGATTTGAT GCCGCGACTA TCAATAGTCG TTATAATGAT TTAACTAGGC

       760         770         780         790         800
TTATTGGCAA CTATACAGAT CATGCTGTAC GCTGGTACAA TACGGGATTA

       810         820         830         840         850
GAGCGTGTAT GGGCACCGGA TTCTAGAGAT TGGATAAGAT ATAATCAATT

       860         870         880         890         900
TAGAAGAGAA TTAACACTAA CTGTATTAGA TATCGTTTCT CTATTTCCGA

       910         920         930         940         950
ACTATGATAG TAGAACGTAT CCAATTCGAA CAGTTTCCCA ATTAACAAGA

       960         970         980         990        1000
GAAATTTATA CAAACCCAGT ATTAGAAAAT TTTGATGGTA GTTTTCGAGG

      1010        1020        1030        1040        1050
CTCGGCTCAG GGCATAGAAG GAAGTATTAG GAGTCCACAT TTGATGGATA

      1060        1070        1080        1090        1100
TACTTAACAG TATAACCATC TATACGGATG CTCATAGAGG AGAATATTAT

      1110        1120        1130        1140        1150
TGGTCAGGGC ATCAAATAAT GGCTTCTCCT GTAGGGTTTT CGGGGCCAGA
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      1160        1170        1180        1190        1200
ATTCACTTTT CCGCTATATG GAACTATGGG AAATGCAGCT CCACAACAAC

      1210        1220        1230        1240        1250
GTATTGTTGC TCAACTAGGT CAGGGCGTGT ATAGAACATT ATCGTCCACT

      1260        1270        1280        1290        1300
TTATATAGAA GACCTTTTAA TATAGGGATA AATAATCAAC AACTATCTGT

      1310        1320        1330        1340        1350
TCTTGACGGG ACAGAATTTG CTTATGGAAC CTCCTCAAAT TTGCCATCCG

      1360        1370        1380        1390        1400
CTGTATACAG AAAAAGCGGA ACGGTAGATT CGCTGGATGA AATACCGCCA

      1410        1420        1430        1440        1450
CAGAATAACA ACGTGCCACC TAGGCAAGGA TTTAGTCATC GATTAAGCCA

      1460        1470        1480        1490        1500
TGTTTCAATG TTTCGTTCAG GCTTTAGTAA TAGTAGTGTA AGTATAATAA

      1510        1520        1530        1540        1550
GAGCTCCTAT GTTCTCTTGG ATACATCGTA GTGCTGAATT TAATAATATA

      1560        1570        1580        1590        1600
ATTCCTTCAT CACAAATTAC ACAAATACCT TTAACAAAAT CTACTAATCT

      1610        1620        1630        1640        1650
TGGCTCTGGA ACTTCTGTCG TTAAAGGACC AGGATTTACA GGAGGAGATA

      1660        1670        1680        1690        1700
TTCTTCGAAG AACTTCACCT GGCCAGATTT CAACCTTAAG AGTAAATATT

      1710        1720        1730        1740        1750
ACTGCACCAT TATCACAAAG ATATCGGGTA AGAATTCGCT ACGCTTCTAC
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      1760        1770        1780        1790        1800
CACAAATTTA CAATTCCATA CATCAATTGA CGGAAGACCT ATTAATCAGG

      1810        1820        1830        1840        1850
GGAATTTTTC AGCAACTATG AGTAGTGGGA GTAATTTACA GTCCGGAAGC

      1860        1870        1880        1890        1900
TTTAGGACTG TAGGTTTTAC TACTCCGTTT AACTTTTCAA ATGGATCAAG

      1910        1920        1930        1940        1950
TGTATTTACG TTAAGTGCTC ATGTCTTCAA TTCAGGCAAT GAAGTTTATA

      1960        1970        1980        1990        2000
TAGATCGAAT TGAATTTGTT CCGGCAGAAG TAACCTTTGA GGCAGAATAT

      2010        2020        2030        2040        2050
GATTTAGAAA GAGCACAAAA GGCGGTGAAT GAGCTGTTTA CTTCTTCCAA

      2060        2070        2080        2090        2100
TCAAATCGGG TTAAAAACAG ATGTGACGGA TTATCATATT GATCAAGTAT

      2110        2120        2130        2140        2150
CCAATTTAGT TGAGTGTTTA TCTGATGAAT TTTGTCTGGA TGAAAAAAAA

      2160        2170        2180        2190        2200
GAATTGTCCG AGAAAGTCAA ACATGCGAAG CGACTTAGTG ATGAGCGGAA

      2210        2220        2230        2240        2250
TTTACTTCAA GATCCAAACT TTAGAGGGAT CAATAGACAA CTAGACCGTG

      2260        2270        2280        2290        2300
GCTGGAGAGG AAGTACGGAT ATTACCATCC AAGGAGGCGA TGACGTATTC

      2310        2320        2330        2340        2350
AAAGAGAATT ACGTTACGCT ATTGGGTACC TTTGATGAGT GCTATCCAAC
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      2360        2370        2380        2390        2400
GTATTTATAT CAAAAAATAG ATGAGTCGAA ATTAAAAGCC TATACCCGTT

      2410        2420        2430        2440        2450
ACCAATTAAG AGGGTATATC GAAGATAGTC AAGACTTAGA AATCTATTTA

      2460        2470        2480        2490        2500
ATTCGCTACA ATGCCAAACA CGAAACAGTA AATGTGCCAG GTACGGGTTC

      2510        2520        2530        2540        2550
CTTATGGCCG CTTTCAGCCC CAAGTCCAAT CGGAAAATGT GCCCATCATT

      2560        2570        2580        2590        2600
CCCATCATTT CTCCTTGGAC ATTGATGTTG GATGTACAGA CTTAAATGAG

      2610        2620        2630        2640        2650
GACTTAGGTG TATGGGTGAT ATTCAAGATT AAGACGCAAG ATGGCCATGC

      2660        2670        2680        2690        2700
AAGACTAGGA AATCTAGAAT TTCTCGAAGA GAAACCATTA GTAGGAGAAG

      2710        2720        2730        2740        2750
CACTAGCTCG TGTGAAAAGA GCGGAGAAAA AATGGAGAGA CAAACGTGAA

      2760        2770        2780        2790        2800
AAATTGGAAT GGGAAACAAA TATTGTTTAT AAAGAGGCAA AAGAATCTGT

      2810        2820        2830        2840        2850
AGATGCTTTA TTTGTAAACT CTCAATATGA TAGATTACAA GCGGATACCA

      2860        2870        2880        2890        2900
ACATCGCGAT GATTCATGCG GCAGATAAAC GCGTTCATAG CATTCGAGAA

      2910        2920        2930        2940        2950
GCTTATCTGC CTGAGCTGTC TGTGATTCCG GGTGTCAATG CGGCTATTTT
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      2960        2970        2980        2990        3000
TGAAGAATTA GAAGGGCGTA TTTTCACTGC ATTCTCCCTA TATGATGCGA

      3010        3020        3030        3040        3050
GAAATGTCAT TAAAAATGGT GATTTTAATA ATGGCTTATC CTGCTGGAAC

      3060        3070        3080        3090        3100
GTGAAAGGGC ATGTAGATGT AGAAGAACAA AACAACCACC GTTCGGTCCT

      3110        3120        3130        3140        3150
TGTTGTTCCG GAATGGGAAG CAGAAGTGTC ACAAGAAGTT CGTGTCTGTC

      3160        3170        3180        3190        3200
CGGGTCGTGG CTATATCCTT CGTGTCACAG CGTACAAGGA GGGATATGGA

      3210        3220        3230        3240        3250
GAAGGTTGCG TAACCATTCA TGAGATCGAG AACAATACAG ACGAACTGAA

      3260        3270        3280        3290        3300
GTTTAGCAAC TGTGTAGAAG AGGAAGTATA TCCAAACAAC ACGGTAACGT

      3310        3320        3330        3340        3350
GTAATGATTA TACTGCGACT CAAGAAGAAT ATGAGGGTAC GTACACTTCT

      3360        3370        3380        3390        3400
CGTAATCGAG GATATGACGG AGCCTATGAA AGCAATTCTT CTGTACCAGC

      3410        3420        3430        3440        3450
TGATTATGCA TCAGCCTATG AAGAAAAAGC ATATACAGAT GGACGAAGAG

      3460        3470        3480        3490        3500
ACAATCCTTG TGAATCTAAC AGAGGATATG GGGATTACAC ACCACTACCA

      3510        3520        3530        3540        3550
GCTGGCTATG TGACAAAAGA ATTAGAGTAC TTCCCAGAAA CCGATAAGGT
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      3560        3570        3580        3590        3600
ATGGATTGAG ATCGGAGAAA CGGAAGGAAC ATTCATCGTG GACAGCGTGG

      3610        3620        3630        3640        3650
AATTACTTCT TATGGAGGAA TAATATATGC TTTATAATGT AAGGTGTGCA

      3660        3670        3680        3690        3700
AATAAAGAAT GATTACTGAC TTGTATTGAC AGATAAATAA GGAAATTTTT

      3710        3720        3730        3740        3750
ATATGAATAA AAAACGGGCA TCACTCTTAA AAGAATGATG TCCGTTTTTT

      3760        3770        3780        3790        3800
GTATGATTTA ACGAGTGATA TTTAAATGTT TTTTTTGCGA AGGCTTTACT

      3810        3820        3830        3840        3850
TAACGGGGTA CCGCCACATG CCCATCAACT TAAGAATTTG CACTACCCCC

      3860        3870        3880        3890        3900
AAGTGTCAAA AAACGTTATT CTTTCTAAAA AGCTAGCTAG AAAGGATGAC

      3910        3920        3930        3940        3950
ATTTTTTATG AATCTTTCAA TTCAAGATGA ATTACAACTA TTTTCTGAAG

      3960        3970        3980        3990        4000
AGCTGTATCG TCATTTAACC CCTTCTCTTT TGGAAGAACT CGCTAAAGAA

      4010        4020        4030        4040        4050
TTAGGTTTTG TAAAAAGAAA ACGAAAGTTT TCAGGAAATG AATTAGCTAC

      4060        4070        4080        4090        4100
CATATGTATC TGGGGCAGTC AACGTACAGC GAGTGATTCT CTCGTTCGAC

      4110        4120        4130        4140        4150
TATGCAGTCA ATTACACGCC GCCACAGCAC TCTTATGAGT CCAGAAGGAC
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      4160        4170        4180        4190        4200
TCAATAAACG CTTTGATAAA AAAGCGGTTG AATTTTTGAA ATATATTTTT

      4210        4220        4230        4240        4250
TCTGCATTAT GGAAAAGTAA ACTTTGTAAA ACATCAGCCA TTTCAAGTGC

      4260        4270        4280        4290        4300
NGCACTCACG TATTTTCAAC GAATCCGTAT TTTAGATGCG ACGATTTTCC

      4310        4320        4330        4340        4350
AAGTACCGAA ACATTTAGCA CATGTATATC CTGGGTCAGG TGGTTGTGCA

      4360
CAAACTGCAG
```

26. A process according to any one of claims 1 or 2, wherein the cloning vector used in step (b) is a bifunctional vector that apart from being capable of replicating in bacterial cells selected from the group consisting of *B. thuringiensis* and *B. cereus* cells is capable of replicating at least in one other heterologous host organism, and that is identifiable in both the homologous and the heterologous host system.

27. A process according to claim 26, wherein the said heterologous host organisms are

(a) prokaryotic organisms selected from the group consisting of the genera *Bacillus, Staphylococcus, Streptococcus, Streptomyces, Pseudomonas, Escherichia, Agrobacterium, Salmonella,* and *Erwinia* or

(b) eukaryotic organisms selected from the group consisting of yeast, animal and plant cells.

28. A process according to claim 27, wherein the said heterologous host organism is *E. coli*.

29. A bifunctional vector when used in a process according to any one of claims 1 or 2 that, apart from being capable of replicating in bacterial cells selected from the group consisting of *B. thuringiensis* and *B. cereus* cells, are capable of replicating in at least one other heterologous host organisms and that is identifiable in both the homologous and the heterologous host system and that comprises under the control of expression sequences that are capable of functioning in bacterial cells selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus* cells a structural gene encoding a δ-endotoxin

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polypeptide that occurs naturally in *B. thuringiensis*, or for a polypeptide that has substantial structural homologies therewith and has still substantially the toxicity properties of the said crystalline δ-endotoxin polypeptide.

30. A bifunctional vector according to claim 29, wherein the said expression sequences include a sporulation-dependent promoter of *B. thuringiensis*.

31. A bifunctional vector according to claim 29, wherein the said δ-endotoxin-encoding DNA sequence is substantially homologous with at least the part or parts of the natural δ-endotoxin-encoding sequence that is (are) responsible for the insecticidal activity.

32. A bifunctional vector according to claim 29, wherein the said polypeptide is substantially homologous with a δ-endotoxin polypeptide of a suitable sub-species of *B. thuringiensis*, selected from the group consisting of *kurstaki, berliner, alesti, sotto, tolworthi, dendrolimus, tenebrionis* and *israelensis*.

33. A bifunctional vector according to claim 29, wherein the said δ-endotoxin-encoding DNA sequence is a DNA fragment of *B. thuringiensis* var. *kurstaki* HD1 located between nucleotides 156 and 3623 in formula I, or is any shorter DNA fragment that still codes for a polypeptide having insect-toxic properties:

Formula I

```
          10          20          30          40          50
GTTAACACCC TGGGTCAAAA ATTGATATTT AGTAAAATTA GTTGCACTTT

          60          70          80          90         100
GTGCATTTTT TCATAAGATG AGTCATATGT TTTAAATTGT AGTAATGAAA

         110         120         130         140         150
AACAGTATTA TATCATAATG AATTGGTATC TTAATAAAAG AGATGGAGGT

         160         170         180         190         200
AACTTATGGA TAACAATCCG AACATCAATG AATGCATTCC TTATAATTGT
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       210         220         230         240         250
TTAAGTAACC CTGAAGTAGA AGTATTAGGT GGAGAAAGAA TAGAAACTGG

       260         270         280         290         300
TTACACCCCA ATCGATATTT CCTTGTCGCT AACGCAATTT CTTTTGAGTG

       310         320         330         340         350
AATTTGTTCC CGGTGCTGGA TTTGTGTTAG GACTAGTTGA TATAATATGG

       360         370         380         390         400
CGAATTTTTG GTCCCTCTCA ATGGGACGCA TTTCTTGTAC AAATTGAACA

       410         420         430         440         450
GTTAATTAAC CAAAGAATAG AAGAATTCGC TAGGAACCAA GCCATTTCTA

       460         470         480         490         500
GATTAGAAGG ACTAAGCAAT CTTTATCAAA TTTACGCAGA ATCTTTTAGA

       510         520         530         540         550
GAGTGGGAAG CAGATCCTAC TAATCCAGCA TTAAGAGAAG AGATGCGTAT

       560         570         580         590         600
TCAATTCAAT GACATGAACA GTGCCCTTAC AACCGCTATT CCTCTTTTTG

       610         620         630         640         650
CAGTTCAAAA TTATCAAGTT CCTCTTTTAT CAGTATATGT TCAAGCTGCA

       660         670         680         690         700
AATTTACATT TATCACTTTT GAGAGATGTT TCAGTGTTTG GACAAAGGTG

       710         720         730         740         750
GGGATTTGAT GCCGCGACTA TCAATAGTCG TTATAATGAT TTAACTAGGC

       760         770         780         790         800
TTATTGGCAA CTATACAGAT CATGCTGTAC GCTGGTACAA TACGGGATTA
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      810         820         830         840         850
GAGCGTGTAT GGGGACCGGA TTCTAGAGAT TGGATAAGAT ATAATCAATT

      860         870         880         890         900
TAGAAGAGAA TTAACACTAA CTGTATTAGA TATCGTTTCT CATTTCCGA

      910         920         930         940         950
ACTATGATAG TAGAACGTAT CCAATTCGAA CAGTTTCCCA ATTAACAAGA

      960         970         980         990        1000
GAAATTTATA CAAACCCAGT ATTAGAAAAT TTTGATGGTA GTTTTCGAGG

     1010        1020        1030        1040        1050
CTCGGCTCAG GGCATAGAAG GAAGTATTAG GAGTCCACAT TTGATGGATA

     1060        1070        1080        1090        1100
TACTTAACAG TATAACCATC TATACGGATG CTCATAGAGG AGAATATTAT

     1110        1120        1130        1140        1150
TGGTCAGGGC ATCAAATAAT GGCTTCTCCT GTAGGGTTTT CGGGGCCAGA

     1160        1170        1180        1190        1200
ATTCACTTTT CCGCTATATG GAACTATGGG AAATGCAGCT CCACAACAAC

     1210        1220        1230        1240        1250
GTATTGTTGC TCAACTAGGT CAGGGCGTGT ATAGAACATT ATCGTCCACT

     1260        1270        1280        1290        1300
TTATATAGAA GACCTTTTAA TATAGGGATA AATAATCAAC AACTATCTGT

     1310        1320        1330        1340        1350
TCTTGACGGG ACAGAATTTG CTTATGGAAC CTCCTCAAAT TTGCCATCCG

     1360        1370        1380        1390        1400
CTGTATACAG AAAAAGCGGA ACGGTAGATT CGCTGGATGA AATACCGCCA
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      1410        1420        1430        1440        1450
CAGAATAACA ACGTGCCACC TAGGCAAGGA TTTAGTCATC GATTAAGCCA

      1460        1470        1480        1490        1500
TGTTTCAATG TTTCGTTCAG GCTTTAGTAA TAGTAGTGTA AGTATAATAA

      1510        1520        1530        1540        1550
GAGCTCCTAT GTTCTCTTGG ATACATCGTA GTGCTGAATT TAATAATATA

      1560        1570        1580        1590        1600
ATTCCTTCAT CACAAATTAC ACAAATACCT TTAACAAAAT CTACTAATCT

      1610        1620        1630        1640        1650
TGGCTCTGGA ACTTCTGTCG TTAAAGGACC AGGATTTACA GGAGGAGATA

      1660        1670        1680        1690        1700
TTCTTCGAAG AACTTCACCT GGCCAGATTT CAACCTTAAG AGTAAATATT

      1710        1720        1730        1740        1750
ACTGCACCAT TATCACAAAG ATATCGGGTA AGAATTCGCT ACGCTTCTAC

      1760        1770        1780        1790        1800
CACAAATTTA CAATTCCATA CATCAATTGA CGGAAGACCT ATTAATCAGG

      1810        1820        1830        1840        1850
GGAATTTTTC AGCAACTATG AGTAGTGGGA GTAATTTACA GTCCGGAAGC

      1860        1870        1880        1890        1900
TTTAGGACTG TAGGTTTTAC TACTCCGTTT AACTTTTCAA ATGGATCAAG

      1910        1920        1930        1940        1950
TGTATTTACG TTAAGTGCTC ATGTCTTCAA TTCAGGCAAT GAAGTTTATA

      1960        1970        1980        1990        2000
TAGATCGAAT TGAATTTGTT CCGGCAGAAG TAACCTTTGA GGCAGAATAT
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2010 2020 2030 2040 2050
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2160 2170 2180 2190 2200
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      4260        4270        4280        4290        4300
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      4310        4320        4330        4340        4350
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      4360
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34. The bifunctional vector pX193 (pK93) introduced into *B. thuringiensis* var. *kurstaki* HD1cryB (DSM 4571) and *B. cereus* 569K (DSM 4573).

35. A bacterial host cell selected from the group consisting of *B. thuringiensis* and *B. cereus* cells prepared by a method as described in any one of claims 1 or 2 comprising a bifunctional vector according to any one of claims 29 to 33.

36. *B. thuringiensis* var. *kurstaki* HD1cryB according to claim 35, transformed with the bifunctional vector pX193 (pK93) and deposited under the number DSM 4571.

37. *B. cereus* 569K according to claim 35, transformed with the bifunctional vector pX193 (pK93) and deposited under the number DSM 4573.

38. A method of controlling insects which comprises treating insects or their habitat

(a) with a bacterial host cell according to claim 35, or with a mixture thereof, or alternatively

(b) with a cell-free crystall-body preparation containing a protoxin that is produced by a bacterial host cell according to claim 35.

39. A method according to claim 38, wherein the insects are insects of the orders *Lepidoptera, Diptera* or *Coleoptera*.

40. A method according to claim 39, wherein the insects are insects of the order *Lepidoptera*.

41. A composition for controlling insects, comprising

(a) a bacterial host cell according to claim 35, or a mixture thereof, or alternatively

(b) a cell-free crystall-body preparation containing a protoxin that is produced by a bacterial host cell according to claim 35, together with carriers, dispersing agents or carriers and dispersing agents conventionally employed.

42. A process according to claim 1, wherein the DNA of step (a) is obtainable by digesting total DNA of a bacterial donor selected from the group consisting of *Bacillus thuringiensis* and *B. cereus*.

43. A process for the identification of new δ-endotoxin encoding genes in *Bacillus thuringiensis*, which process comprises

(a) digesting the total DNA of *Bacillus thuringiensis* using suitable restriction enzymes;

(b) isolating from the resulting restriction fragments those of suitable size;

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(c) inserting the said fragments into a suitable vector;

(d) constructing a genomic DNA library by transforming *Bacillus thuringiensis* host cells with the said vector using a process according to claim 1;

(e) screening the thus obtainable DNA library for new δ-endotoxin encoding genes.

44. A process according to claim 43, wherein a bifunctional vector is used.

45. A process according to claim 43, wherein an immunological screening process is used to locate new δ-endotoxin encoding genes.

46. A process for inserting and cloning DNA sequences in gram positive bacteria selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus*, which method is substantially as hereinbefore described with reference to any one of Examples 1 to 4 or 7 to 9.

47. A process for inserting, cloning and expressing DNA sequences in gram positive bacteria selected from the group consisting of *Bacillus thuringiensis* and *Bacillus cereus*, which method is substantially as hereinbefore described with reference to any one of Examples 6, 8 or 9.

48. A bifunctional vector for inserting and cloning, or inserting, cloning and expressing, DNA sequences in gram positive bacteria selected from *Bacillus thuringiensis* or *Bacillus cereus*, which vector is substantially as hereinbefore described with reference to any one of Examples 3, 4 or 8.

49. A bifunctional vector for inserting and cloning, or inserting, cloning and expressing, DNA sequences in gram positive bacteria selected from *Bacillus thuringiensis* or *Bacillus cereus*, which vector is substantially as hereinbefore described with reference to Figure 6 or Figure 7.

50. A process for preparing a bifunctional vector for inserting and cloning, or inserting, cloning and expressing, DNA sequences in gram positive bacteria selected from *Bacillus thuringiensis* or *Bacillus cereus*, which process is substantially as hereinbefore described with reference to any one of Examples 3, 4 or 8.

51. A bacterial host cell selected from *B. thuringiensis* or *B. cereus* cells prepared by a process as described in claim 46 or claim 48 comprising a bifunctional vector according to claim 48.

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52. A composition for controlling insects, comprising

a) a bacterial host cell according to claim 51, or a mixture thereof; or alternatively

b) a cell-free crystal-body preparation containing a protoxin that is produced by a bacterial host cell according to claim 51,

together with carriers, dispersing agents or carriers and dispersing agents conventionally employed.

DATED this SECOND day of APRIL 1993
Ciba-Geigy AG

Patent Attorneys for the Applicant
SPRUSON & FERGUSON

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LMM/926Z

Fig. 1

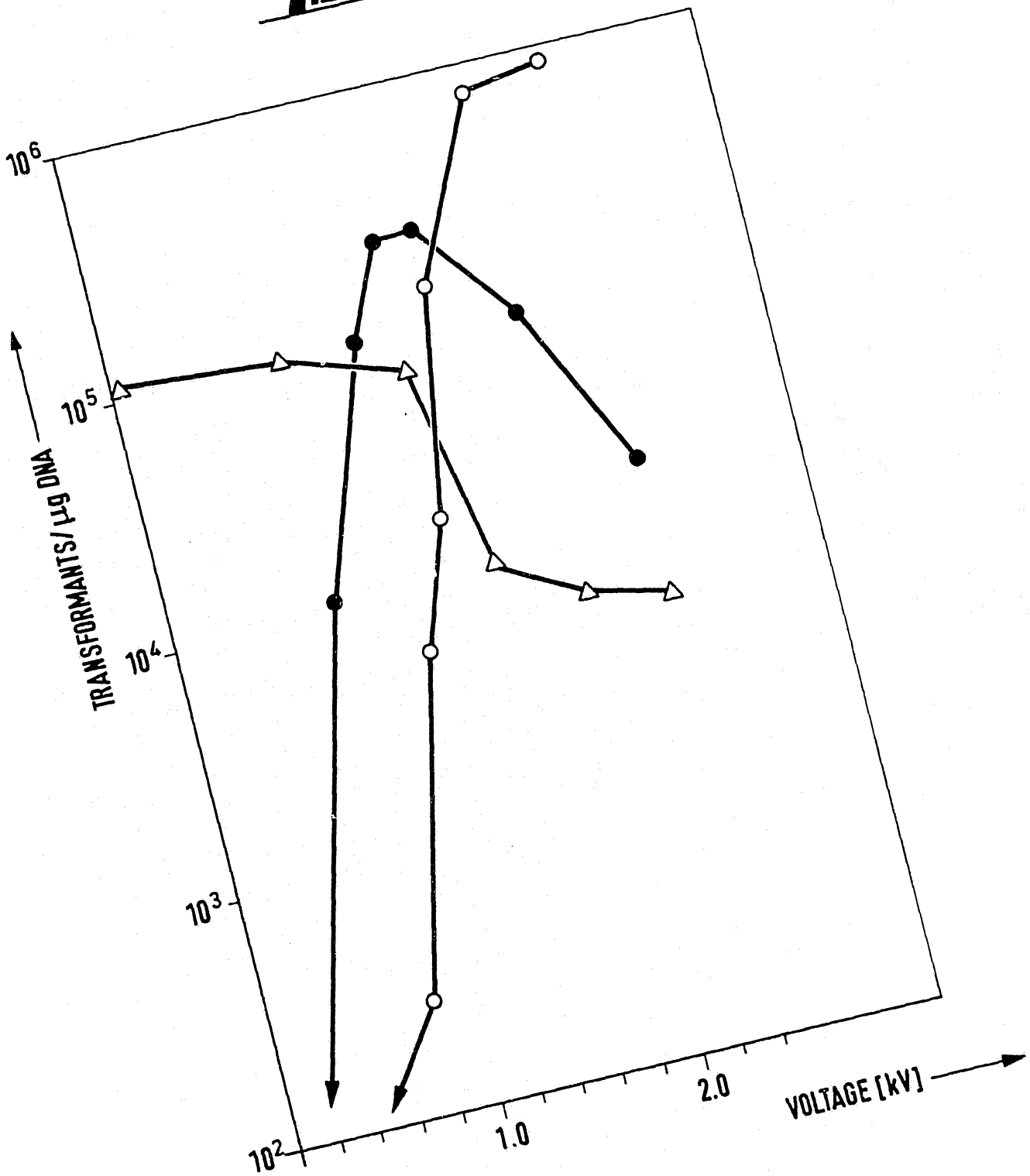


Fig. 2

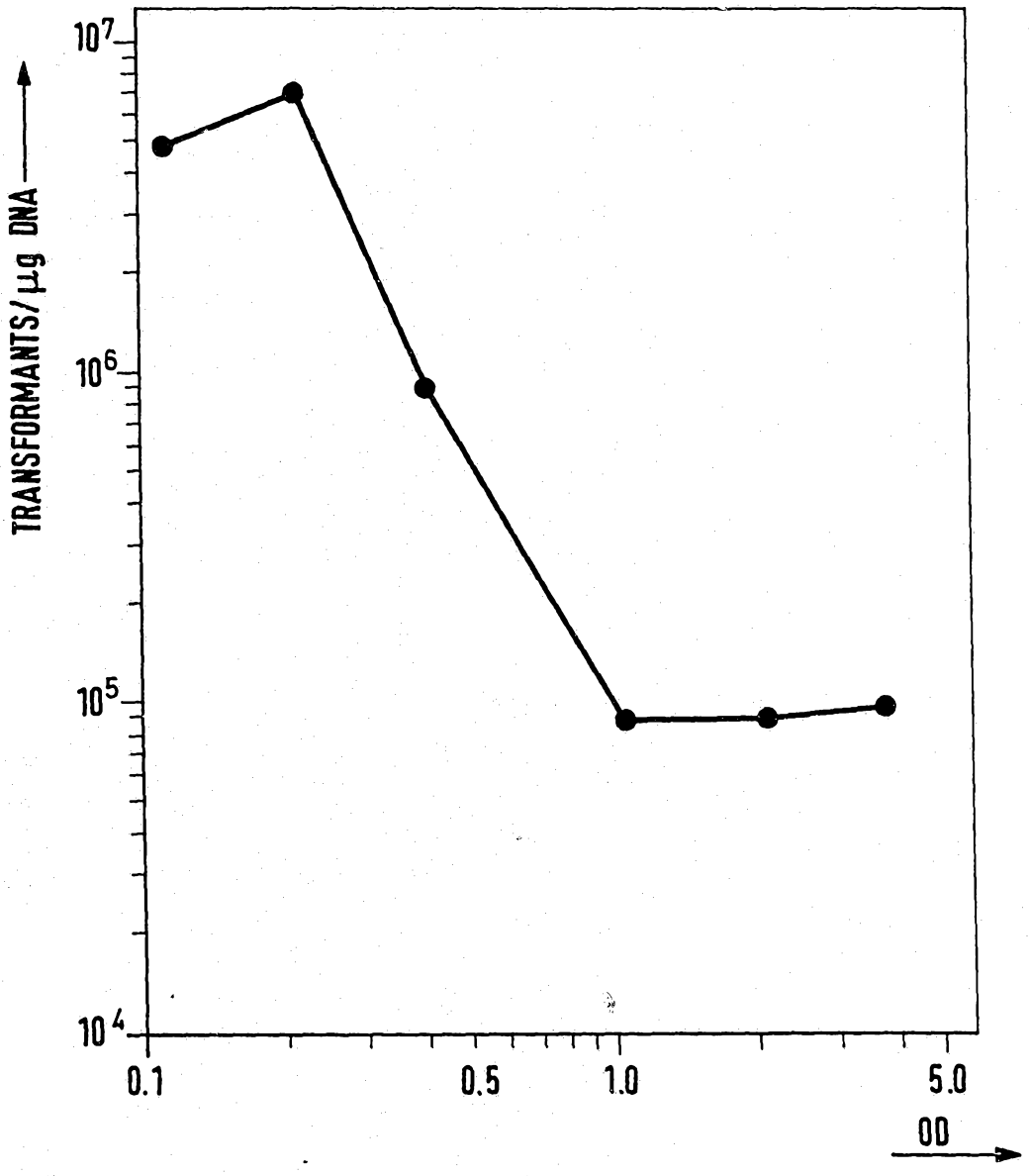


Fig. 3

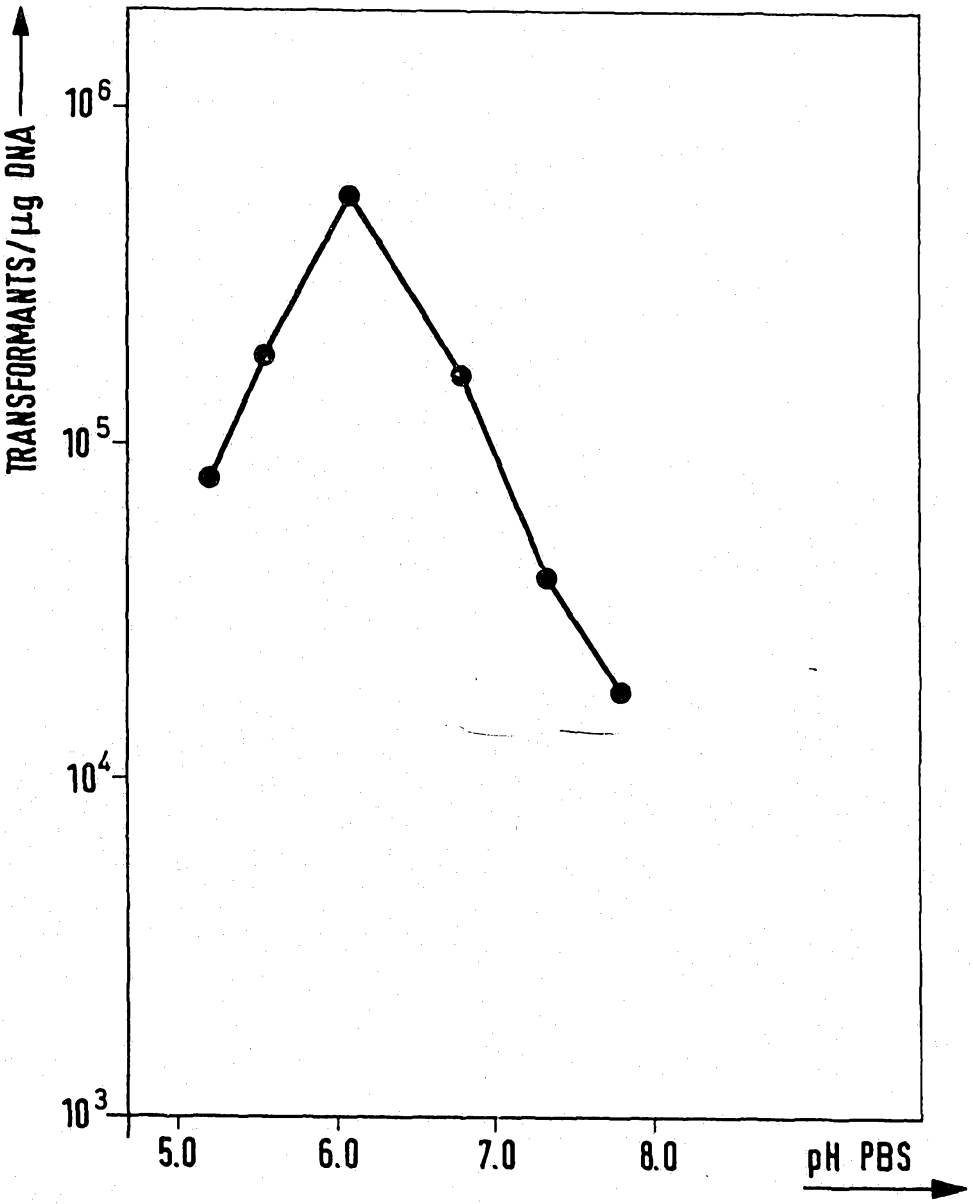
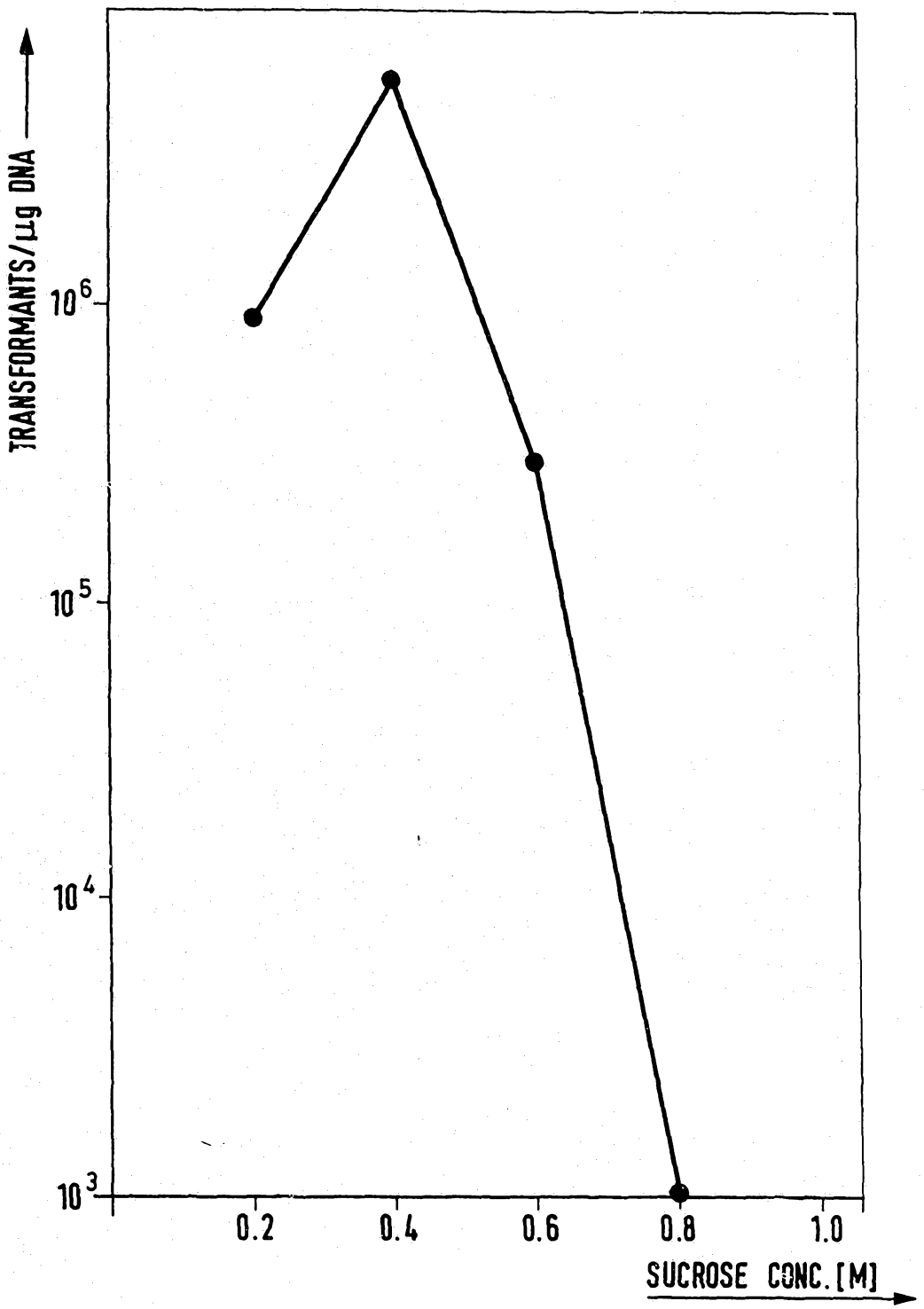


Fig. 4


TRANSFORMANTS/µg DNA
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10^5
10^4
10^3
0.2
0.4
0.6
0.8
1.0
SUCROSE CONC. [M]

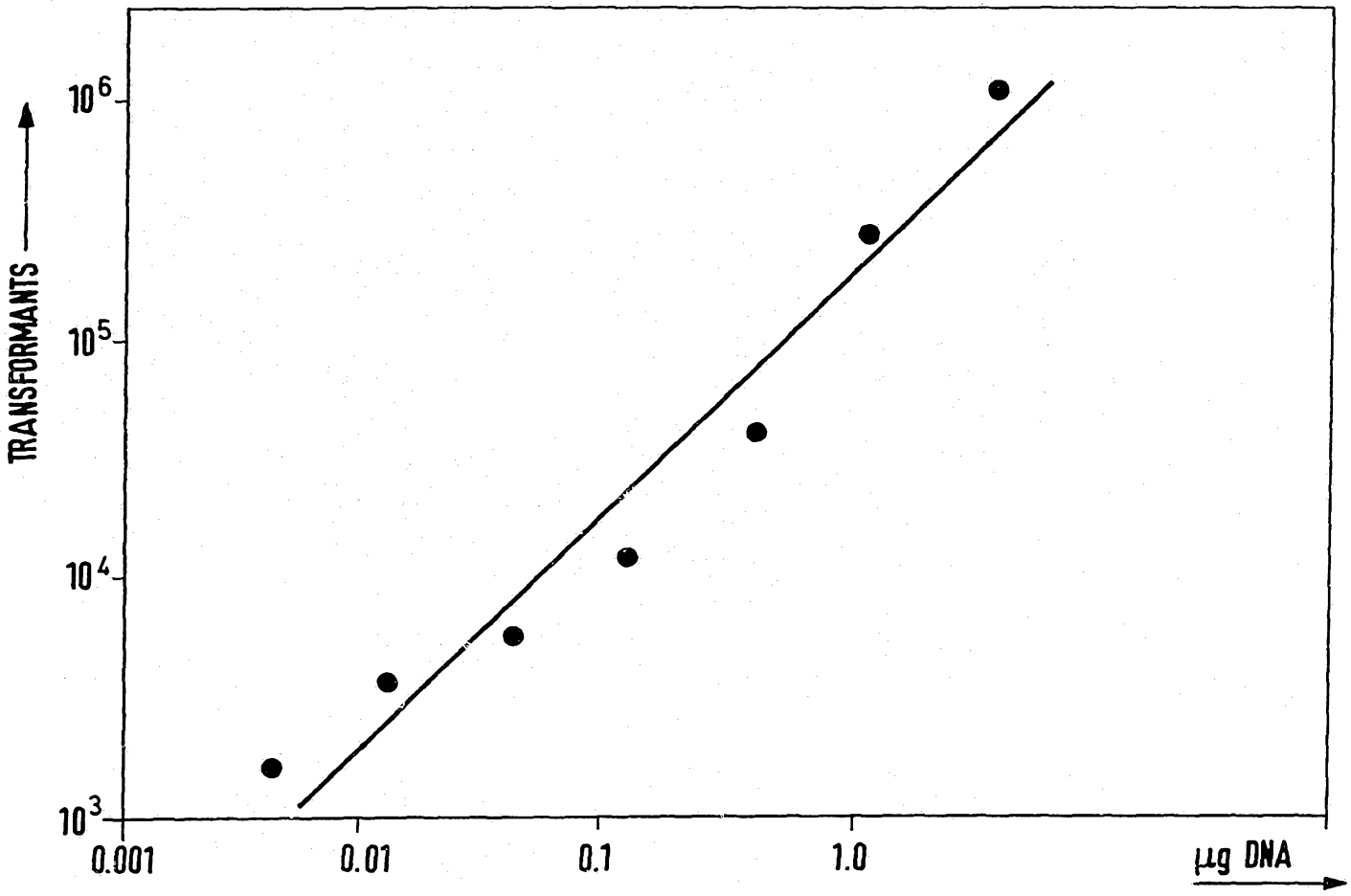
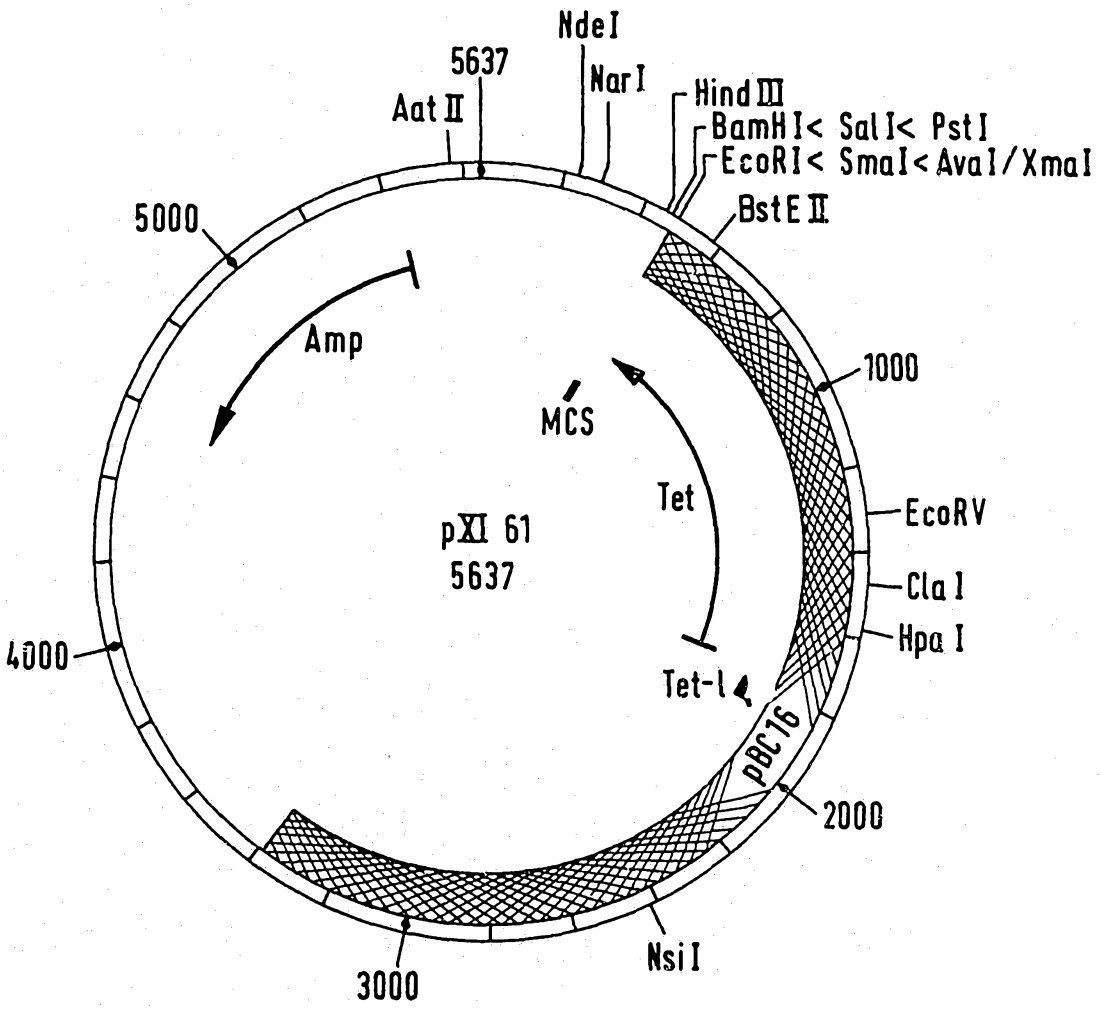


Fig. 5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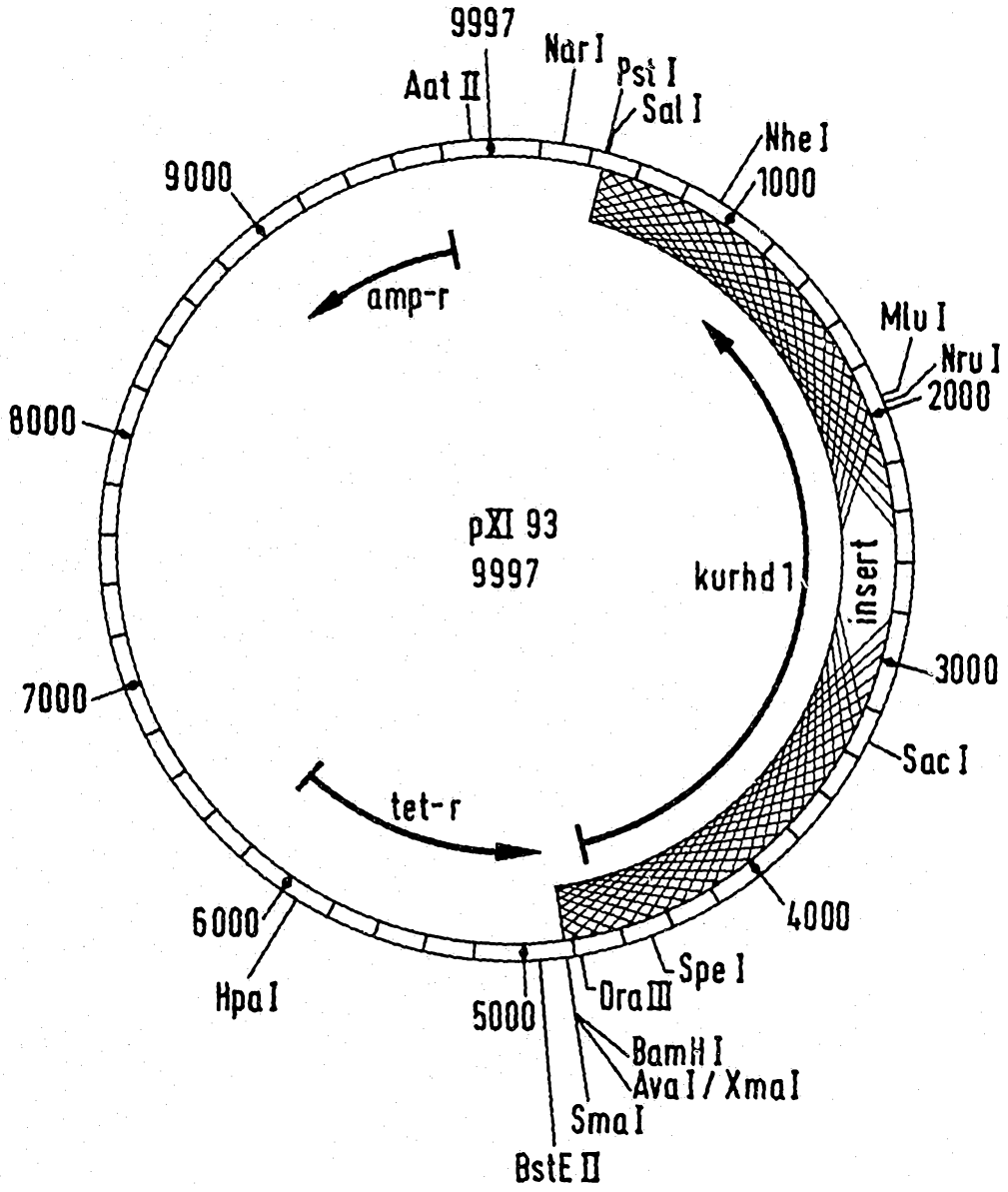
NarI
Aat II
Hind III
BamH I< Sal I< Pst I
EcoR I< Sma I< Ava I/Xma I
BstE II
5000
Amp
1000
MCS
Tet
pXI 61
5637
EcoRV
Cla I
Hpa I
4000
Tet-I
pBC16
2000
NsiI
3000

Fig. 6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Pst I
Sal I
Nhe I
1000
9000
amp-r
Mlu I
Nru I
2000
8000
pXI 93
9997
kurhd 1
insert
3000
7000
Sac I
tet-r
4000
6000
Spe I
Dra III
Hpa I
5000
BamH I
Ava I / Xma I
Sma I
BstE II

Fig. 1

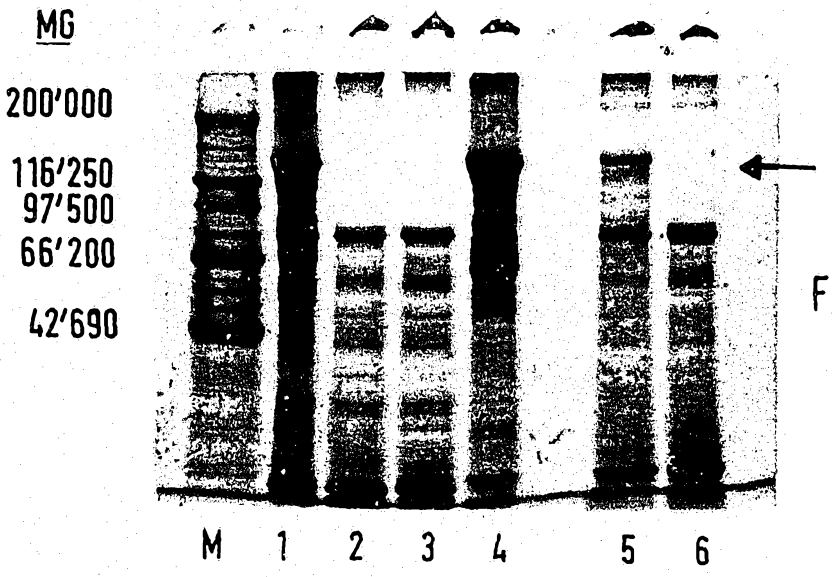


Fig. 8a

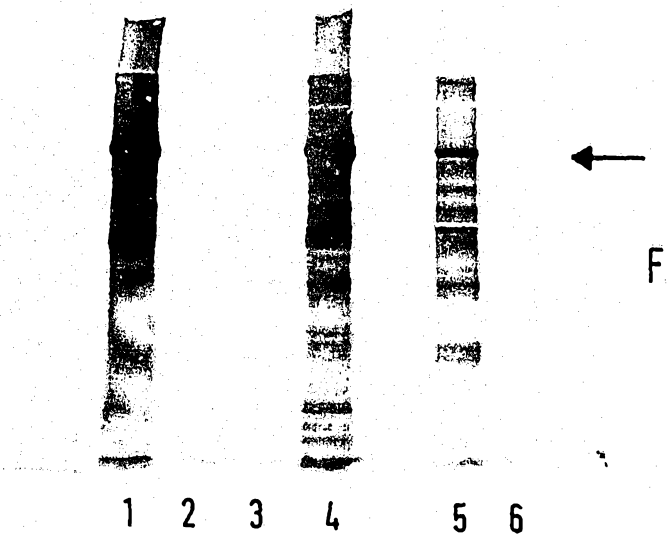
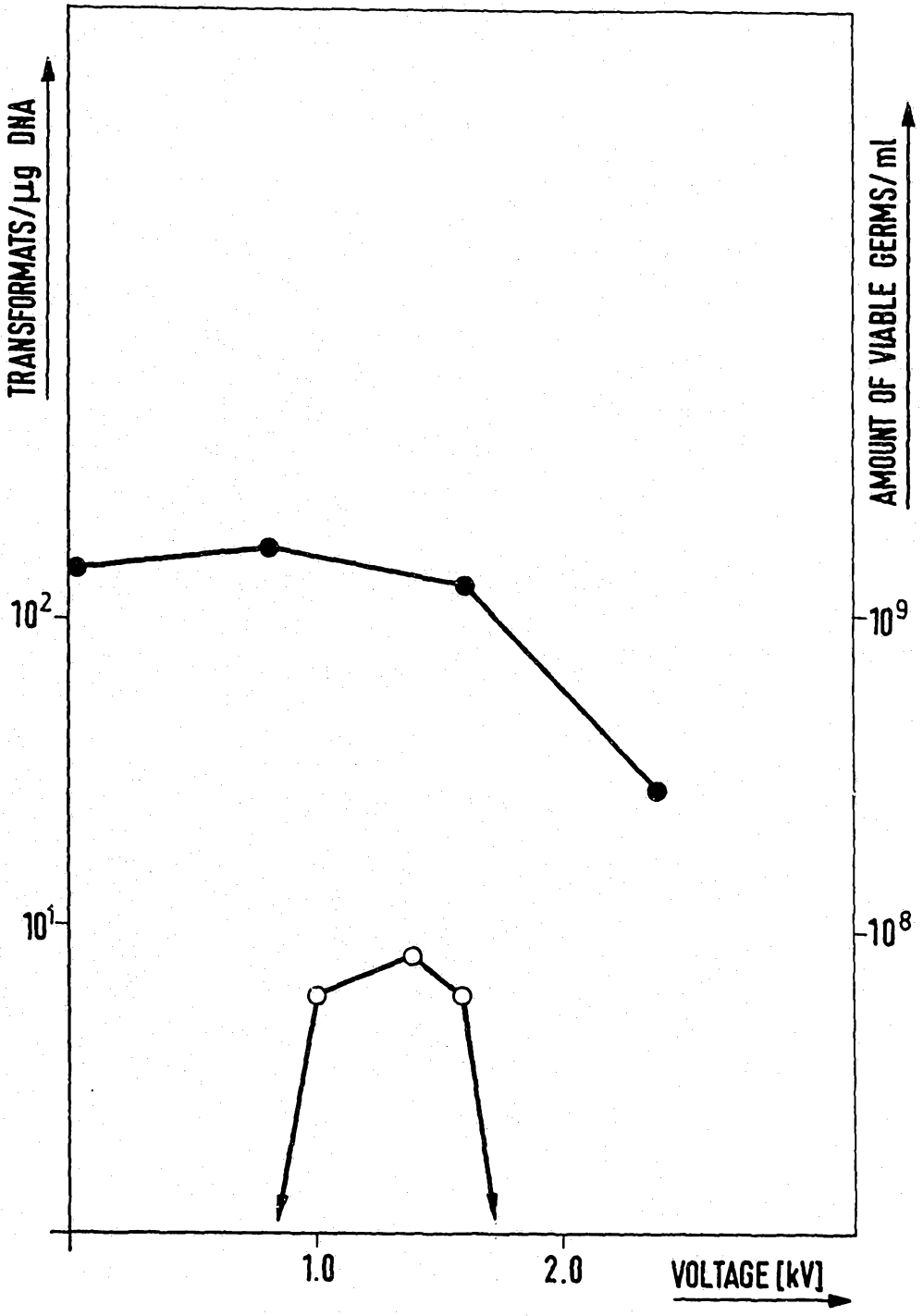


Fig. 8b

Fig. 9


TRANSFORMATS/µg DNA
AMOUNT OF VIABLE GERMS/ml
10^2
10^1
10^9
10^8
1.0
2.0
VOLTAGE [kV]