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(54) Title: ENTEROTOXIN-DEFICIENT BACILLUS (57) Abstract A enterotoxin-deficient mutant of a member strain of the <i>Bacillus cereus</i> group does not produce HBL enterotoxin, which has been regarded as a human pathogen found in member strains. An enterotoxin-deficient mutant is suitable for use as a biocontrol agent. Methods for making the mutant and for using the mutant are described.		

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ENTEROTOXIN-DEFICIENT BACILLUS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S.
Provisional Application No. 60/080,943 filed April 7, 1998.

5 STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR
 DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

Significant research has been conducted in recent
10 years on the use of biological agents to increase
agricultural productivity and efficiency. Biological
control based on the use of microorganisms to suppress
plant pests offers an attractive alternative to chemical
pesticides which are less favored than they have previously
15 been because of concerns about human health and
environmental quality. Several screening programs have
been used before to isolate biological agents which are
effective in the laboratory or in the field to combat pests
or facilitate plant growth.

20 "Biological control" or "biocontrol" is defined as
pathogen suppression by the use of a second organism.
Mechanisms of biological control are diverse. For example,
certain bacteria have been examined for their usefulness in
biological control of root rot in alfalfa. It is believed
25 that control is obtained by competition or antagonism
between the bacteria and the fungi on the surface of the
alfalfa roots. In contrast, a toxin produced by one
species of bacteria may be used to control another species
of bacteria or other microorganism that appears as a
30 pathogen. Bacterially produced antibiotics are examples of
such toxins. The toxin can be isolated from the species

producing it and administered directly, as is the common procedure with penicillin, or the species itself may be administered under appropriate circumstances to produce the toxin in situ. Once identified, such toxins produced by
5 soil-dwelling bacteria may have utility in diverse other areas as antifungal or antibiotic agents.

An example of a biological control agent into which significant scientific and economic development has occurred is *Bacillus thuringiensis*. *B. thuringiensis*
10 strains produce toxic proteins which have the ability to specifically kill certain insects. Significant further research has identified a large number of *B. thuringiensis* strains having variations in target range and efficacy. In addition, research has been conducted on methods for
15 stabilizing and applying such toxins, or strains harboring them, to a wide variety of field crop situations. It was also discovered that knowledge of *B. thuringiensis* strains was largely transferable to new strains since the toxins required for biological control and methods for preparing
20 inocula for use in the field were generally similar among strains.

B. cereus (Bc) is closely related to *B. thuringiensis* (Bt), which has been used for insect biocontrol for about 30 years. Bc and Bt are generally considered to be members
25 of a single species and the names are artifacts of history (Ash et al., 1991a,b; Carlson et al., 1994; Gordon, 1975; Logan and Berkeley, 1984; Lysenko, 1983; Priest, 1993; and Zahner et al., 1989). The major difference between Bc and Bt appears to be the presence of plasmids in the latter
30 that encode an insecticidal crystal toxin responsible for insect biocontrol by Bt. Bc is a known food contaminant that can cause diarrhea in humans. Outbreaks of Bc food poisoning are associated with improper food storage and handling and require ingestion of large numbers of Bc
35 spores (Gilbert, 1979). There have been no reported outbreaks of food poisoning associated with Bt use, presumably because it does not reach the human food supply

in sufficient populations to cause a problem.

Previously it has been found that a specific strain of *Bacillus cereus*, which has been referred to both as UW85 and by its ATCC designation 53522, has biocontrol efficacy in many applications. See, e.g., U.S. Patent No. 4,877,738. UW85 has proven to be an effective biocontrol agent of damping-off and root-rot diseases of soybeans and alfalfa under diverse field conditions in the upper Midwest U.S. (Handelsman et al., 1990; Osburn et al., 1995). The UW85 *B. cereus* strain protects alfalfa seedlings from damping off caused by *Phytophthora medicaginis* (Pmm), tobacco seedlings from *Phytophthora nicotianae*, cucumber fruits from rot caused by *Pythium aphanidermatum*, and peanuts from *Sclerotinia minor*. In a five-year field study on soybeans, UW85 applied either on the seed or in-furrow increased soybean yields significantly in all five years at a site with high disease pressure; yield enhancements were typically in the range of 35% to 139% depending on the severity of disease. In each year, the magnitude of the yield effect of UW85 was similar to that of Ridomil, the in-furrow formulation of metalaxyl, which controls Oomycete pathogens (Osburn et al., 1995). UW85 has been extensively field-tested and is currently in review by EPA for registration as a seed treatment for various crops.

UW85 produces two antifungal compounds that independently contribute to suppression of damping-off fungi. The more potent of these compounds, is zwittermicin A, a novel aminopolyol (Silo-Suh et al., 1994; He et al., 1994). Other, diverse strains of *B. cereus* found in all tested soils also suppress plant disease and produce zwittermicin A (Stabb et al., 1994; Raffel et al., 1996). Thus, this species has broad potential for biocontrol applications. The second compound is kanosamine, a well known aminoglycoside antibiotic.

Inoculation with *B. cereus* appears to have a negligible long-term impact on the environment. This

species is common and present in all soils we have tested at approximately 10^4 cfu/gm soil (Stabb et al., 1994; Raffel et al., 1996). Data suggest that roots have a carrying capacity for Bc since by the end of the growing
5 season, soybean roots derived from seeds treated with Bc UW85 had the same populations of Bc as untreated roots. However, the treated roots carried UW85 whereas untreated roots carried indigenous Bc strains (Halverson et al., 1993). Therefore, it appears that when UW85 is applied to
10 seeds, it colonizes the roots of the plant at low populations and then dies off to the background level by the end of the season, suggesting that it will have minimal environmental impact. We have shown that early in the season, treatment of seeds with UW85 can result in a change
15 in the microbial community on roots (Gilbert et al., 1993), but this effect does not seem to be sustained beyond the first 10 days (Raffel and Handelsman, unpublished). Overall, the existing data suggest that seed treatment with UW85 is a benign environmental event since Bc is a common
20 soil bacterium and UW85 does not maintain high populations for long, which is in concert with other studies of *Bacillus* (Kim et al., 1997).

There is significant interest in developing numerous strains of Bc as inoculants for disease suppression on
25 field crops, fruit and vegetable crops, greenhouse and ornamental production, and turf management, and in choosing strains adapted to particular host species or geographic locations. Other such strains have been identified. Thus, the invention disclosed herein will have application to
30 many strains of Bc, not just to UW85 and the other strains noted herein.

Biocontrol has long been thought to be safer for the environment and human health than synthetic pesticides (Cook et al. 1996; Benbrook et al., 1996). As more
35 bacterial biocontrol agents have reached the federal regulatory agencies for review, the relatedness of some of the bacteria and human pathogens has raised concerns by the

agencies themselves and the public. For example, a recent registration application for a soil isolate of *Burkholderia cepacia* (Parke et al., 1991) raised concern from the cystic fibrosis community because members of this bacterial
5 species are opportunistic pathogens of cystic fibrosis patients (Govan and Deretic, 1996).

There is some evidence to suggest that the *B. cepacia* biocontrol strains are genetically and physiologically different from the human isolates, but the risks associated
10 with use of these bacteria remain in question. This type of issue is likely to emerge more frequently as biocontrol research yields a greater diversity of strains. Many of the strains being developed for biocontrol are common soil bacteria, and many soil bacteria are, or have close
15 relatives that are, human pathogens. It is therefore essential to assess the environmental exposure associated with use of these organisms and determine whether that exposure is greater with biocontrol practices than with standard agricultural practices. Furthermore, there is an
20 excellent opportunity to use the powerful techniques of genetic engineering to make biocontrol organisms safer by deleting genes associated with unwanted activities. This will have two impacts: providing biocontrol agents that pass regulatory review, and providing the public with
25 examples of genetic engineering used to make the food supply safer.

Despite the good safety record of Bt, lingering concerns remain about registration of products containing Bt or Bc. Many strains of Bc and Bt produce an enterotoxin,
30 denoted HBL, that is associated with mammalian toxicity (Beecher and Macmillan, 1991; Beecher et al., 1995). All of the major Bt strains on the commercial market produce HBL (Damgaard, 1995) and, as a result, the EPA has expressed hesitation about future registration of Bt
35 strains. Bc is a recognized food toxicant and therefore must be demonstrated to be safe before it is used as an inoculant. There is strong evidence that HBL is

responsible for the toxicity of most pathogenic Bc strains because purified HBL mimics Bc toxicity and there is a high correlation between toxicity and HBL production among Bc strains. The two key issues with Bc inoculant strains are
5 the presence of the gene for enterotoxin production and any associated potential for human exposure to Bc from food grown from Bc-treated seed. It is not known whether other members of the so-called *Bacillus cereus* group, namely the *B. anthracis* strains, produce the enterotoxin, although
10 both *B. cereus* and *B. thuringiensis* do produce the enterotoxin.

HBL is a tripartite enterotoxin, that includes a binding component, B, and two lytic components, L₁ and L₂ (Beecher and Macmillan, 1991). All three components are
15 absolutely necessary for enterotoxigenic activity, as assessed by fluid accumulation in the ligated rabbit ileal loop assay and vascular permeability, which are currently the best animal models for Bc toxicity (Beecher et al., 1995; Glatz et al., 1974).

20 The genes encoding each of the components have been cloned and sequenced, and appear to be cotranscribed in an operon in the order L₂, L₁, B (Heinrichs et al., 1993; Ryan et al., 1997). Studies indicate that HBL production is a common trait among strains of *B. cereus* isolated from a
25 variety of sources, including flour, rice, dairy products, and soil (Carlson et al., 1994; Damgaard et al., 1996; Granum et al., 1993; Lee et al., 1995; Mosso et al., 1989; Te Giffel et al., 1997; Yusuf et al., 1992).

A PCR screen of nearly 175 soil and root isolates for
30 *hblA* using primers derived from the sequence of the *hblA* gene published by Heinrichs et al., 1993 showed that approximately 70% contain *hblA*, the gene that encodes the B component (Raffel and Handelsman, unpublished). UW85 contains this gene and produces the toxin when grown in
35 tryptic soy broth as shown by ELISA with antiserum against HBL.

In addition to being the most important and widespread

toxin in Bc, HBL is the only known mammalian toxin in strain UW85. In addition to being important biologically, HBL is important from a regulatory perspective. EPA requests data about the production of HBL (and not about
5 other toxins) prior to registration of Bc and Bt strains.

What is lacking in the art is a strain in the *Bacillus cereus* group having utility as a biocontrol agent that is also deficient in the HBL enterotoxin. The present invention responds to that regulatory concern as well as
10 the scientific evidence that HBL is important in mammalian toxicity by Bc.

BRIEF SUMMARY OF THE INVENTION

The present invention is summarized in that a mutant *Bacillus* strain is deficient in the enterotoxin HBL. The
15 invention is further summarized in that the mutant strain is obtained by altering at least one of three polynucleotide sequences that otherwise would encode a component of the HBL enterotoxin, so that the polynucleotide sequence can no longer encode a functional
20 component and, therefore, an active HBL enterotoxin cannot be produced.

The present invention is further summarized in that a genetic construct for use in accordance with the present invention contains an allele of a polynucleotide sequence
25 that encodes at least one inactive component of HBL enterotoxin.

It is an advantage of the present invention that the enterotoxin can be eliminated from the *Bacillus* without adding any new DNA in the organism. Therefore, under the
30 current EPA definition of genetic engineering (Federal Register 1997, 17910-17958), the resulting mutants are not considered genetically engineered and will not be subject to any regulations that do not apply to the wild type strain.

35 Other objects, advantages and features of the invention will become apparent upon consideration of the

following detailed description taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1 depicts the construction of vector
5 (pUC18::*hblA1*) that contains an *hblA* deletion allele, denoted *hblA1*, in accordance with the present invention.

Fig. 2 depicts the coding sequence of *hblA*. The underlining represents the portion of *hblA* that is deleted in the preferred embodiment of the present invention.

10 Fig. 3 depicts the construction of a vector (pKM10::*hblA1*) containing an *hblA* deletion allele in accordance with the present invention.

Fig. 4 depicts the wild type *hbl* operon of a *Bacillus* chromosome (top), a vector comprising the *hblA1* mutation
15 integrated into the *Bacillus* chromosome after a single recombination event (center), and the *hblA1* mutation integrated into the *Bacillus* chromosome after a second recombination event that excises the vector sequences from the chromosome (bottom).

20 DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the present invention is a mutant member of the *Bacillus cereus* group that is deficient in HBL enterotoxin. An enterotoxin-deficient mutant of the present invention does not produce an active HBL
25 enterotoxin, although it may produce fewer than all three components of wild-type HBL enterotoxin. However, since all three distinct components of the enterotoxin are required to produce an active toxin, a mutant of the present invention can encompass a mutation in any one or
30 more of the polynucleotides that encode the components.

The mutation in any of the polynucleotides can be a point mutation, a deletion, an insertion or a rearrangement that is sufficient to render the component ineffective in combination with the remaining components to form active
35 HBL enterotoxin. Although the applicants have exemplified

below a defined deletion mutant of the *hblA* gene, the invention can readily be practiced using a different deletion, or another type of mutation in *hblA*, or with a comparable mutation in *hblC* or *hblD*. The mutant strain can
5 alternatively include a change in a sequence that encodes a protein or peptide that acts in trans to modify one or more of the HBL components. In addition to any change that inactivates a component, the polynucleotide encoding the component can also include additional changes that may not
10 otherwise alter the function of the component. Such mutants would fall within the scope of the invention as long as they are unable to produce HBL enterotoxin by virtue of a change in at least one polynucleotide that encodes a component of the enterotoxin. Isolated
15 preparations of naturally occurring mutants can also fall within the scope of the present invention.

The enterotoxin-deficient mutants of the present invention can be mutants of any member of the *Bacillus cereus* group of bacteria, including *B. cereus*, *B.*
20 *thuringiensis* and *B. anthracis*. Preferably, the mutant is also characterized by having a biological control activity when used as an active agent in an inoculum, as described *infra*.

In a second aspect, the invention is a method for
25 producing a enterotoxin-deficient mutant of the present invention, wherein the method comprises the step of modifying in a *Bacillus* strain at least one polynucleotide that encodes a component of the HBL enterotoxin. The component can be modified by altering the polynucleotide
30 that encodes the component, for example, by gene replacement. A suitable method for gene replacement, described in the accompanying Examples, employs a vector carrying a desired mutation that alters the HBL enterotoxin component. The vector can be cured from cells at a non-
35 permissive temperature, and further permits selection of mutants on the basis of resistance or sensitivity to an antibiotic.

The invention has particular utility when applied in strains of *Bacillus cereus* that produce biocontrol antibiotics such as zwittermicin A. Such strains include, but are not limited to, *Bacillus cereus* strains UW85, AS4-
5 12, M1-9, and DGA34. These strains are disclosed in U.S. Patent No. 4,877,738; 5,552,138; 5,700,462; and 5,736,382 (which issues on April 7, 1998), respectively, all of which are incorporated herein by reference in their entirety. One or more mutations that inactivate at least one component of
10 the three components that comprise the HBL enterotoxin can be introduced into a zwittermicin-producing strain, thereby eliminating the enterotoxin from the strain and obviating safety concerns about the human pathogenicity of the modified strain. Since *B. thuringiensis* is very closely
15 related to *B. cereus*, and contains the HBL enterotoxin, it is further specifically envisioned that a mutation of the type disclosed herein can be introduced into *B. thuringiensis* in the same manner as is described herein.

In a further aspect, the invention is a method for
20 biological control of plant pathogens, where the method comprises applying an inoculum that includes as an active agent a novel enterotoxin-deficient mutant of a strain in the *Bacillus cereus* group. The active agent is preferably an enterotoxin-deficient *B. cereus* strain. The mutants of
25 the present invention can be used in a method for biological control in the same ways as UW85 and other such mutants, the methods for use being fully described, for example, in U.S. Patent No. 4,877,738, previously incorporated herein by reference in its entirety. A
30 suitable assay for monitoring the biocontrol activity of an enterotoxin-deficient strain of the present invention is a tomato emergence biocontrol assay as described by Smith, K.P., Handelsman, J., and Goodman, R.M., Modeling dose-response relationships in biological control:
35 Partitioning host responses to the pathogen and biocontrol agent, Phytopathology 87:720-729 (1997), incorporated herein by reference in its entirety.

The invention will be better understood upon consideration of the following examples which are considered to be exemplary and non-limiting on the scope of the invention.

5

EXAMPLES

Materials and Methods

Bacterial strains, plasmids, and media. *E. coli* DH5 α was used for cloning. *Bacillus thuringiensis* strain EG10368
10 (Deposited under accession number NRRL-B-21125 in the permanent collection of the Agricultural Research Service Culture Collection, Northern Regional Research Laboratory, U.S. Dept. of Agriculture, 1815 N. University Street, Peoria IL 61604, see U.S. Patent No. 5204237) was obtained
15 from Ecogen Inc., Langhorne, PA. *Bacillus cereus* strain AS4-12 (Deposited under accession number 55609 with the American Type Culture Collection, now located at 10801 University Boulevard, Manassas, VA 20110-2209) was isolated from soil from an alfalfa field in Arlington, Wisconsin
20 (Stabb et al., 1994) and is described in published International Patent Application No. PCT/US96/09496, incorporated herein by reference in its entirety. *E. coli* was grown in Luria-Bertani liquid or solid media. *B. cereus* and *B. thuringiensis* were grown in 50% tryptic soy
25 broth (TSB) or on 50% tryptic soy agar (TSA) plates (Difco Laboratories, Detroit, MI). Antibiotics were used at the following concentrations: for *E. coli*, ampicillin at 100 μ g/ml; for *B. cereus* and *B. thuringiensis*, chloramphenicol at 5 μ g/ml.
30 The *hblA* gene cloned from *B. cereus* F837/76 was provided by Amy Wong at the Food Research Institute in Madison, WI. It was previously cloned on a 2.1-kb *Eco*R1 fragment into pUC18 (Heinrichs et al., 1993, incorporated herein by reference in its entirety, sequence available in
35 GenBank at accession number L20441).

DNA isolation and manipulation. Plasmid DNA was isolated from *E. coli* using Qiagen plasmid prep kits (Qiagen Inc., Chatsworth, CA). Plasmid DNA was isolated from *B. cereus* and *B. thuringiensis* by the alkaline lysis method described
5 by Bron, S., "Plasmids" in Hardwood, C.R. and S.M. Cutting (eds.), Molecular Biological Methods for Bacillus, p. 75-174, John Wiley and Sons, Ltd., New York (1990), incorporated herein by reference in its entirety, except Solution A was supplemented with 8 µg/ml of RNase A.
10 Ligation reactions were performed by standard methods (Sambrook et al., 1989). When necessary, DNA restriction fragments were purified from agarose gels using the QIAEX II Gel Extraction Kit (Qiagen Inc., Chatsworth, CA).

Internal deletions in *hblA* were constructed with the
15 Erase-a-Base deletion generation system (Promega Corp., Madison, WI). pUC18::*hblA* was cleaved with *BsrBRI*, a unique restriction site near the center of *hblA*, a portion was removed by exonuclease III digestion, and the plasmid was religated (Fig. 1). The mutant allele, designated
20 *hblA1* (presented in SEQ ID NO:1), was sequenced across the deletion with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corp., Foster City, CA), and the sequencing gel was run by the University of Wisconsin Biotechnology Center. Both strands of *hblA1* were
25 sequenced, using the following primers which are based on the published sequence of *hblA* (Genbank accession number L20441): *hblA1*-F, 5'-GAAACAATGGTAGAGGCA-3' (SEQ ID NO:3); *hblA1*-R, 5'-GCAACTCCAACCTACACGA-3' (SEQ ID NO:4). Mutant allele *hblA1* is characterized as having a 123 bp in-frame
30 deletion spanning nucleotides 568-690 of the coding region identified by Heinrichs, *supra*. The amino acid sequence of the component encoded by *hblA1* is depicted at SEQ ID NO:2.

Transformations. Competent cells of *E. coli* (50 µl) were electroporated in 0.2-cm cuvettes with a Gene Pulser
35 apparatus (Bio-Rad, Richmond, CA) set at 25 µF, 200 Ω and

1.8 kV. Cells were transferred to 2 ml of LB and allowed to recover for 1 hr at 37°C with shaking before plating on selective media.

- Preparation of competent cells and electroporation of *B. cereus* and *B. thuringiensis* were performed as described previously (Silo-Suh et al., 1994), except the transformed cells were incubated for 2 hours at 28°C before plating. *B. cereus* apparently restricts methylated DNA from *E. coli* (Blackson and Handelsman, unpublished data), as was reported for *Bacillus thuringiensis* (Macaluso, A. and Mettus, A.-M., 1991). Therefore, recombinant plasmids isolated from *E. coli* were passed through *B. thuringiensis* EG10368, a highly transformable strain, before being introduced into *B. cereus* AS4-12 by electroporation.
- 15 **Southern hybridization analysis.** Total genomic DNA from *B. cereus* was obtained using the Easy-DNA Kit (Invitrogen Corp., Carlsbad, CA). To enhance cell lysis, the resuspended cells were vortexed for 4 min. with 50-75 µl of 0.1 mm zirconia/silica beads. Probe labeling and Southern hybridization analysis of *B. cereus* genomic DNA were performed as described previously (Milner et al., 1996). Genomic DNA was digested with *Xba*I and electrophoresed on a 0.8% agarose gel. Blots were probed with either pKM10 or a fragment of *hblA*. For the *hblA* probe, a 1022-bp internal fragment of the coding region was amplified by PCR using the following primer set: *hblA*-F, 5'-TTACTCGCTGTATCGACG-3' (SEQ ID NO:5); *hblA*-R, 5'-CCAACTATCTTTTCGCTGC-3' (SEQ ID NO:6).

- Western immunoblot analysis.** Culture supernatant for Western blot analysis was prepared essentially as described previously (Beecher and Wong, 1994). Stationary cultures of the strains were grown in BHIG at room temperature overnight. Flasks (250 ml) containing 50 ml of BHIG were inoculated with 0.5% (vol/vol) of the overnight cultures

and grown on a rotary shaker (200 rpm) for 5-6 hr at 30°C to 32°C. EDTA was added to each culture to a final concentration of 10 mM. The cultures were centrifuged at 4°C for 15 min at 14,600 x g and the culture supernatants were filter sterilized with 0.22- μ m pore-sized filters and, if necessary, concentrated by ultrafiltration (Centricon-10 concentrators, Amicon, Inc., Beverly, MA; Ultrafree-4 centrifugal filtration devices, Millipore Corp., Bedford, MA). The supernatants were stored at -20°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by standard methods (Laemmli, 1970). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane in a Trans-Blot electrophoretic transfer cell (Bio-Rad) overnight at 35 V and 4°C. Blotted proteins were probed with either preimmune rabbit serum or polyclonal antiserum prepared against purified B component from *B. cereus* F837/76 as described previously (Beecher et al., 1995). Immune complexes were detected with horseradish peroxidase-labeled goat anti-rabbit antibodies (Sigma), and visualized by chemiluminescence following treatment with ECL kit reagents (Amersham Life Sciences, Buckinghamshire, England). The molecular weights of the proteins are only approximate because they were referenced to prestained protein standards, whose apparent molecular weights can vary significantly relative to unstained protein standards.

PCR analysis. Genomic DNA for PCR analysis was prepared by the rapid cell-lysis method as described previously (Raffel et al., 1996), or with the Easy-DNA Kit. PCR reactions were performed with the hblA-F/hblA-R primer set in a thermocycler (RoboCycler 40, Stratagene, La Jolla, CA) with the following program: 40 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C; and a final 5 min extension at 72°C.

Construction of a *Ts* plasmid for gene replacement. A temperature-sensitive (*Ts*) shuttle vector, pKM10, capable

of replication in *B. cereus* and *E. coli* was constructed for performing the gene replacement. The shuttle/suicide plasmid was derived from the *E. coli* cloning vector, pGEM-3Zf(+), and the Gram-positive plasmid, pTV32, which
5 contains the naturally thermosensitive replicon from pE194 (Fig. 2). The ≈ 6.4 kb *Bam*H1 fragment of pTV32, containing the Ts replicon and a chloramphenicol resistance marker (*cat*), was ligated into the *Bam*H1 site of pGEM-3Zf(+). The smaller *Pst*I fragment of the resulting construct was
10 dropped out and the larger fragment religated. pKM10 can be maintained in *B. cereus* at 28°C and cured at 41°C.

The *Eco*RI fragment from pUC18 containing the mutant allele, *hblA1*, was subcloned into the unique *Eco*RI site of pKM10, and transformed into *E. coli*. The recombinant
15 plasmid was confirmed by restriction digest analysis. pKM10::*hblA1* was introduced into *B. cereus* AS4-12 by electroporation. Restriction digest analysis of plasmid DNA isolated from Cm^r transformants verified the presence of the recombinant plasmid.

20 **Gene replacement. Selection for plasmid integrants.**

AS4-12 containing pKM10::*hblA1* was grown overnight at the permissive temperature (28°C) in 50% TSB containing chloramphenicol to achieve a large population of plasmid-carrying cells. This culture was diluted 1:100
25 into nonselective broth and grown at 28°C with shaking for two hours and then shifted to the nonpermissive temperature (41°C) for 4-6 hours to eliminate or reduce the copy number of freely-replicating plasmid. The culture was plated at 41°C on media containing chloramphenicol to select for
30 cells in which the plasmid had integrated into the chromosome via a single crossover event between homologous regions of the wild-type and mutant *hblA* genes. The integration frequency was calculated as the ratio of colonies on selective plates to colonies on nonselective
35 plates. PCR analysis with *hblA* primers served as an

initial screen for the presence of the integrated plasmid, which was confirmed by Southern hybridization analysis of genomic DNA, probing with *hblA*.

Screen for second crossover event. A culture of the
5 plasmid integrant was grown overnight at the restrictive temperature with chloramphenicol to obtain a large population of the integrant. The culture was diluted 100-fold into fresh broth and grown at 40°C for 11 hr with or without Cm selection to allow for a second
10 recombinational event to occur between the wild-type and mutant *hblA* genes. To cure any freely-replicating plasmid that had excised from the chromosome, the culture was diluted 1:100 into selective or nonselective broth and grown overnight (~12 hours) at 40°C. The outgrowth from
15 the overnight culture was plated at 28°C on nonselective media. Individual colonies were screened for loss of chloramphenicol resistance by patching onto TSA + Cm. The frequency of the second crossover event was calculated as the ratio of Cm^r colonies to the total number of colonies
20 screened. PCR and Southern blot analyses were performed to distinguish which of the Cm^r colonies had replaced the wild-type gene with the mutant allele.

A first mutant clone ultimately selected for Western analysis and the hemolysis assay resulted from a culture
25 that was subjected to selection until plating. To summarize, the mutant derivative of AS4-12, considered by the inventors to be a preferred embodiment of the invention, was constructed by a two-step gene replacement. In the first step, clones in which the plasmid had
30 integrated into the chromosome via homologous recombination between *hblA1* and *hblA*^{*} were selected following growth at the nonpermissive temperature. In the second step, the plasmid integrant was screened for loss of chloramphenicol-resistance, resulting from a second crossover event and
35 plasmid curing. The Cm^r isolates were analyzed by PCR and Southern blot analysis to identify those which had

undergone a successful gene replacement.

A second mutant has been identified. The mutant is characterized as having a deletion in *hblA* that corresponds to nucleotides 460 - 823 of the published *hblA* sequence (Heinrich, 1993). The second mutant was obtained using a process similar to that described in connection with the first mutant except that when the screen for the second crossover event was performed, the integrant was permitted to grow at 28°C for several hours after establishing the large population of the integrant, because there is some evidence that growth at a permissive temperature can stimulate recombination between duplicate regions flanking an integrated plasmid in *Bacillus subtilis* and *Lactococcus lactis* (Noirot et al., 1987; Biswas et al., 1993). It is anticipated that the second mutant will be HBL enterotoxin-deficient.

Hemolysis assay. Culture supernatants of AS4-12 and the *hblA* mutants, prepared as described for Western blot analysis, were examined for the discontinuous pattern of hemolysis in a gel diffusion assay essentially as described previously (Beecher and Wong, 1994). Ten μ l of culture supernatant were placed in a 4-mm-diameter well in HBL blood agar. The plates were incubated at room temperature, 28°C or 37°C, and monitored periodically for hemolysis.

Complementation of hemolysis by *hblA*⁺ in trans. An *hblA*⁺ expression vector, pAD*hblA*, derived from pAD123(43-25) was constructed for complementation analysis of the mutants. This is not a true test of complementation because the *hblA* mutants are not Rec⁻ strains, and therefore, *hblA*⁺ could hypothetically recombine into the chromosome and re-form the wild-type genotype. Plasmid pAD123(43-25) is an *E. coli*/*B. cereus* shuttle vector with an ampicillin resistance gene for selection in *E. coli*, a chloramphenicol resistance gene for selection in *B. cereus*, and a chromosomal insert from *B. cereus* UW85 cloned upstream from a promoterless *gfp*

gene (Dunn and Handelsman, 1999). UW85 carrying pAD123(43-25) exhibits constitutive *gfp* expression during log-phase growth in BHIG. To construct the *hblA* expression vector, the *gfp* gene in pAD123(43-25) was removed and
5 replaced with *hblA*⁺. The *Xba*I-*Hind*III fragment containing *gfp* was removed from pAD123(43-25). The *Xba*I end of the plasmid was made blunt with Klenow fragment of DNA polymerase prior to digestion with *Hind*III. This fragment was ligated with the 1.7-kb *Sma*I-*Hind*III fragment from
10 pUC18 containing *hblA*⁺. Similar plasmids, containing either *hblA1* (pAD*hblA1*) or *hblA2* (pAD*hblA2*) were constructed as controls. The recombinant plasmids, verified by restriction digest analysis, were introduced into the appropriate *B. cereus* mutants. The resulting
15 strains were assayed for the discontinuous pattern of hemolysis.

Vascular permeability (VP) assay. Beecher and Wong (1994) showed that HBL causes a vascular permeability response in rabbit skin, including necrosis at sufficient
20 concentrations. An increase in vascular permeability allows leakage of serum proteins into the surrounding tissues. The reaction is quantitated by Evans blue dye, administered intravenously, which binds to the serum proteins, resulting in an area of bluing around the
25 injection site. A strong reaction is characterized by an area of necrosis at the center of the zone of bluing. Culture supernatants were prepared and concentrated by ultrafiltration as described for Western blot analysis. The assay was performed essentially as described by Glatz
30 et al. (1974). Briefly, 50- μ l samples of culture supernatant were injected intradermally into the back of a shaved New Zealand White rabbit. Three hours after the injections, 1% Evans blue dye (1 ml/kg) was administered intravenously, and the diameters of bluing and necrosis
35 were measured one hour later. Some rabbits were sedated with acepromazine (4 mg/kg) 10-30 min prior to the sample

and dye injections.

For the neutralization experiments, 62.5 μ l or 66.7 μ l of 12x-concentrated culture supernatant was mixed with 30 μ l of either preimmune, anti-B, or anti-L₂ rabbit serum, and then diluted with sterile distilled water to a supernatant concentration of 3x or 4x. The antisera were prepared against purified B or purified L₂ component from *B. cereus* F837/76 (Beecher et al., 1995).

Biocontrol assay. The biocontrol activity of AS4-12 and the mutants was examined in a growth-chamber tomato emergence assay (Smith et al., 1997). Bacterial strains were tested for the ability to suppress damping-off of tomato seedlings caused by *Pythium torulosum*. Inserts of a 72-cell bedding plant container that had been filled with autoclaved vermiculite (medium vermiculite premium grade, Strong-Lite Horticultural Products, Seneca, IL) were planted with seeds, 10 per cell, of tomato cultivar Wisconsin 55. A layer of vermiculite was added to cover the seeds. Four liters of deionized water either inoculated with 4 zoospores per seed or uninoculated were added to each planting tray flat. One ml of diluted bacterial culture that had been grown in 50% TSB for four days at 28°C was distributed over ten seeds. The planting trays received supplemental water every 12 hr to maintain a constant level throughout the experiment. The assay was conducted at 24°C, 40% relative humidity, and with a 12-hour photoperiod. Emergence was scored twelve days after planting.

Cultures of the *B. cereus* strains diluted 1:2, 1:8, and 1:16 (culture: sterile MilliQ water), and untreated seeds were evaluated at pathogen doses of 0 and 4 zoospores per seed. The treatments were arranged in a randomized split plot design where the *P. torulosum* dose (0 or 4 zoospores per seed) comprised the main plots and the bacterial doses comprised the subplots. Each treatment consisted of 3 cells per plot, for a total of 30 seeds. Plots that

received water only were replicated three times, while the plots that were inoculated with the pathogen were replicated five times to accommodate greater variability. The experiment was run two times. Fraction emergence was transformed by arcsine square-root before analysis. Mean emergence data from the two experiments was combined and analyzed by analysis of variance (Minitab release 10extra, Minitab, Inc., State College, PA). ANOVA was conducted on each culture dilution and untreated control. Mean separation tests were performed according to Fisher's protected LSD (Snedecor and Cochran, 1980).

Results

Western immunoblot analysis. To determine whether mutants 41a and 10e2 secreted detectable quantities of the B component of HBL, immunoblot analysis was performed on culture supernatants with polyclonal antiserum to the B protein. Culture supernatant of AS4-12 contained at least two proteins that reacted with the antiserum, indicated by a strong ~37-kDa band that migrated to approximately the same position as purified B protein (37.8 kDa) isolated from *B. cereus* F837/76, and a weaker ~39-kDa band. Sequence heterogeneity between the *hblA* genes from F837/76 and AS4-12 likely accounts for the slight difference in mobility exhibited between the B component purified from F837/76 and that expressed from AS4-12. Culture supernatants of both mutants exhibited the 39-kDa band, and a faint ~37-kDa band. It is likely that this 37-kDa band is also present in AS4-12, and that its presence has been unmasked in the *hblA* mutants. This band may correspond to another protein that is antigenically related to the B component, or it may represent a proteolytic degradation product of the 39-kDa protein. Strains 41a⁺ and 10e2⁺, carrying the F837/76 *hblA*⁺ gene *in trans*, accumulated the 37.8-kDa B component and the 39-kDa protein. Blots probed with preimmune rabbit serum did not display any reaction. The results suggest that mutants 41a and 10e2 do not

accumulate the B component of HBL in culture supernatant, but that they may express, in addition to the 39-kDa protein, another protein that is immunologically related to the B component.

5 **Hemolytic activity of mutants.** Strains 41a and 10e2 were tested for the discontinuous pattern of hemolysis characteristic of HBL, in which lysis of erythrocytes in blood agar typically begins several millimeters away from a colony or well containing culture supernatant, resulting in
10 a ring pattern (Beecher and Macmillan, 1990). Neither mutant exhibited the discontinuous hemolysis displayed by AS4-12, either around colonies or wells filled with concentrated culture supernatant. Introduction of *hblA*⁺ *in trans* on a low-copy plasmid, pAD*hblA*, restored the
15 wild-type phenotype to both mutants. The results indicate that deletions of 123 or 364 bases in *hblA* abolishes activity by AS4-12 in the discontinuous hemolysis assay.

Vascular permeability assay. The toxicity of mutants 41a and 10e2 was compared to that of AS4-12 in the vascular
20 permeability (VP) assay. Culture supernatants from both mutant strains produced reactions similar to AS4-12 at several doses. Unconcentrated supernatant from all three strains produced bluing, but no obvious necrosis, a result observed in several independent experiments. All three
25 strains exhibited necrosis with 3x- and 6x-concentrated supernatants. Six-fold-concentrated BHIG culture medium did not cause a VP response.

To determine whether an alternative B protein(s) contributed to the VP activities displayed by the mutants,
30 culture supernatant treated with antiserum to the B component was tested. Neutralization with anti-B serum did not attenuate the reactions caused by AS4-12 or 41a. However, it did appear to diminish the severity of the response elicited by mutant 10e2; i.e., the necrosis was
35 eliminated, although the size of the bluing zone increased.

In a second experiment, antiserum to B decreased the bluing caused by 10e2, and the necrosis caused by AS4-12. While these results are not conclusive, they suggest that a protein(s) that is antigenically related to the B component contributes to the VP activity in AS4-12 and the mutants. The contradictory results obtained with AS4-12, and the failure to detect any effect of anti-B serum on the VP activity elicited by mutant 41a, may indicate that a threshold level of neutralization is necessary to cause a detectable reduction in the VP response.

Neutralization with antiserum to the L₂ component appeared to reduce both the bluing and necrosis caused by all three strains, indicating that the L₂ component and/or an alternate L₂ protein contributes to the VP activity in AS4-12 and both *hblA* mutants. This result indirectly implicates the participation of a B-like protein in the VP activity exhibited by the *hblA* mutants, as Beecher and Wong (1994) found that the combination of purified L₁ and L₂ components failed to cause any dermal reaction in the VP assay.

Biocontrol ability of *hblA* mutants. Mutants 41a and 10e2 were tested for their ability to suppress damping-off of tomato seedlings caused by *P. torulosum* in a growth chamber assay. Tomato seeds were coinoculated with zoospores of the pathogen and sporulated cultures of AS4-12, 41a, or 10e2. Culture dilutions of 1:2, 1:8 and 1:16 were tested for biocontrol ability. All three strains significantly increased seedling emergence compared to the untreated control ($\alpha=0.05$), and neither mutant differed significantly from the parental strain at any of the bacterial doses ($\alpha=0.05$). In the absence of pathogen, none of the bacterial treatments influenced emergence compared to the untreated control (data not shown). These results suggest that the B component of HBL does not contribute to biocontrol by AS4-12 in the lab.

Grain yield. Preliminary field data support the growth chamber assay results. AS4-12 and 41a were evaluated for soybean yield-enhancing ability at two sites in Wisconsin during the 1998 field season (Oplinger et al., unpublished data). Soybean seeds coated with 41a and AS4-12 exhibited a significant increase in yield over untreated seeds at both sites ($P = 0.001$). AS4-12 and 41a increased soybean yield by 6.2% and 8.0%, respectively, at one site, and by 8.8% and 14.7% at a second site. Preliminary analysis suggests that the difference between the AS4-12 and 41a treatments at the second site was significant ($LSD = 10\%$). The superior performance of the *hblA* mutant at this site may be an anomalous result, although removal of this gene could might actually enhance biocontrol ability. Alternatively, another genetic difference between 41a and the parent strain could account for the yield difference.

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CLAIMS

We claim:

1. A method for obtaining a mutant *Bacillus*, the method comprising the steps of:
 - 5 introducing into a *Bacillus* a mutation that inactivates an active Hemolysin BL enterotoxin component selected from the group consisting of binding component B, lytic component L1 and lytic component L2; and
 - 10 selecting a mutant *Bacillus* comprising fewer than three active Hemolysin BL enterotoxin subunit components.
2. A method as claimed in Claim 1 wherein the introduced mutation is selected from the group consisting of a point mutation, a deletion, an insertion, and a rearrangement in a polynucleotide that encodes a Hemolysin
15 BL enterotoxin subunit component.
3. A method as claimed in Claim 2 wherein the polynucleotide is selected from *hblA*, *hblC*, and *hblD*.
4. A method as claimed in Claim 1 wherein the mutation is in a sequence that encodes a protein or peptide
20 that acts *in trans* to modify a Hemolysin BL component.
5. A method as claimed in Claim 1 wherein the mutation is a deletion.
6. A method as claimed in Claim 5 wherein the mutation is a deletion in *hblA*.
- 25 7. A method as claimed in Claim 1 wherein the *Bacillus* is selected from the group consisting of a *Bacillus cereus*, a *Bacillus thuringiensis*, and a *Bacillus anthracis*.

8. A method as claimed in Claim 7 wherein the *Bacillus* and the mutant *Bacillus* have biocontrol activity.

9. A method as claimed in Claim 8 wherein the *Bacillus* produces zwittermicin A.

5 10. A method as claimed in Claim 8 wherein the *Bacillus* is a *Bacillus cereus* selected from the group consisting of UW85, AS4-12, M1-9 and DGA34.

11. A method for obtaining a mutant *Bacillus cereus* AS4-12, the method comprising the steps of:

10 introducing into strain AS4-12 a mutation that inactivates an active Hemolysin BL enterotoxin component selected from the group consisting of binding component B, lytic component L1 and lytic component L2; and
15 selecting a mutant of strain AS4-12 that has fewer than three active Hemolysin BL enterotoxin subunit components.

12. A method as claimed in Claim 11 wherein the mutant comprises a deletion in *hblA* relative to *Bacillus cereus hblA*.

13. A *Bacillus* bacterium that does not produce an
20 active Hemolysin BL enterotoxin, the bacterium comprising fewer than three active Hemolysin BL enterotoxin subunit components.

14. A *Bacillus* bacterium as claimed in Claim 13 wherein the *Bacillus* is selected from the group consisting
25 of a *Bacillus cereus*, a *Bacillus thuringiensis*, and a *Bacillus anthracis*.

15. A *Bacillus* bacterium as claimed in Claim 14 wherein the *Bacillus* has biocontrol activity.

16. A *Bacillus* bacterium as claimed in Claim 15
wherein the *Bacillus* produces zwittermicin A.

17. A *Bacillus* bacterium as claimed in Claim 14
wherein the *Bacillus* is a *Bacillus cereus* selected from the
5 group consisting of a mutant of UW85, a mutant of AS4-12, a
mutant of M1-9 and a mutant of DGA34.

18. A *Bacillus* bacterium as claimed in Claim 17
wherein the *Bacillus* is a mutant of AS4-12 having a
deletion in *hblA* relative to strain AS4-12.

10 19. A *Bacillus* bacterium as claimed in Claim 18
wherein nucleotides 568 to 690 of *Bacillus cereus hblA* are
deleted.

20. A *Bacillus* bacterium as claimed in Claim 18
wherein nucleotides 460 to 823 of *Bacillus cereus hblA* are
15 deleted.

1/4

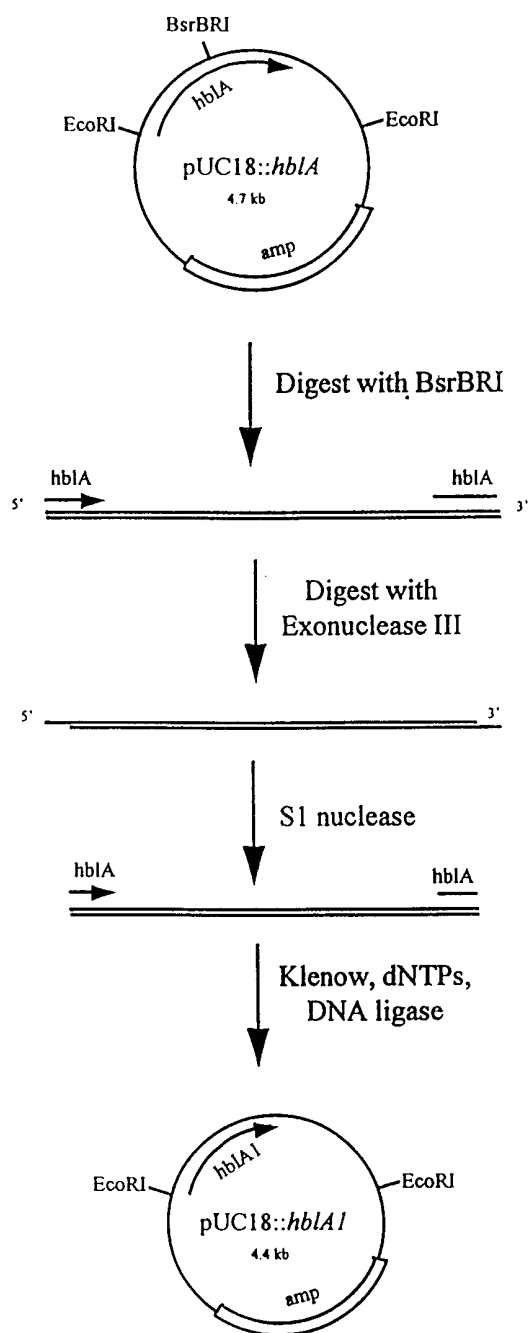


FIG 1

hblA sequence*

1 atg ata aaa aaa atc cct tac aaa tta ctc gct gta tcg acg tta tta act att aca acc
61 gct aat gta gtt tca cct gta gca act ttt gca agt gaa att gaa caa acg aac aat gga
121 gat acg gct ctt tct gca aat gaa gcg aag atg aaa gaa act ttg caa aag gct gga tta
181 ttt gca aaa tct atg aat gcc tat tct tat atg tta att aaa aat cct gat gtg aat ttt
241 gag gga att act att aat gga tat gta gat tta cct ggt aga atc gta caa gat caa aag
301 aat gca aga gca cat gct gtt act tgg gat acg aaa gtg aaa aaa cag ctt tta gat aca
361 ttg act ggt att gtt gaa tat gat acg acg ttt gac aat tat tat gaa aca atg gta gag
421 gca att aat aca ggg gat gga gaa act tta aaa gaa ggg att aca gat ttg cga ggt gaa
481 att caa caa aat caa aag tat gca caa caa cta ata gaa gaa tta act aaa tta aga gac
541 tct att gga cac gat gtt aga gca ttt gga agt aat aaa gag ctc ttg caq tca att tta
601 aaa aat caa ggt qca qat qtt qat gcc qat caa aag cgt cta gaa gaa gta tta qqa tca
661 gta aac tat tat aaa caa tta gaa tct gat ggg ttt aat gta atg aag ggt gct att ttg
721 ggt cta cca ata att ggc ggt att ata gtg gga gta gca agg gat aat tta ggt aag tta
781 gag cct tta tta gca gaa tta cgt cag acc gtg gat tat aaa gta acc tta aat cgt gta
841 gtt gga gtt gct tac agt aat att aat gaa atc gac aag gcg ctt gat gat gct att aac
901 gct ctt act tat atg tcc acg cag tgg cat gat tta gat tct caa tat tcg ggc gtt cta
961 ggg cat att gag aat gca gct caa aaa gcc gat caa aat aaa ttt aaa ttc tta aaa cct
1021 aat tta aat gca gcg aaa gat agt tgg aaa aca tta cga aca gat gct gtt aca tta aaa
1081 gaa gga ata aag gag tta aaa gta gaa act gtt act cca caa aaa tag

* The underlined segment is deleted from *hblA1*

FIG 2

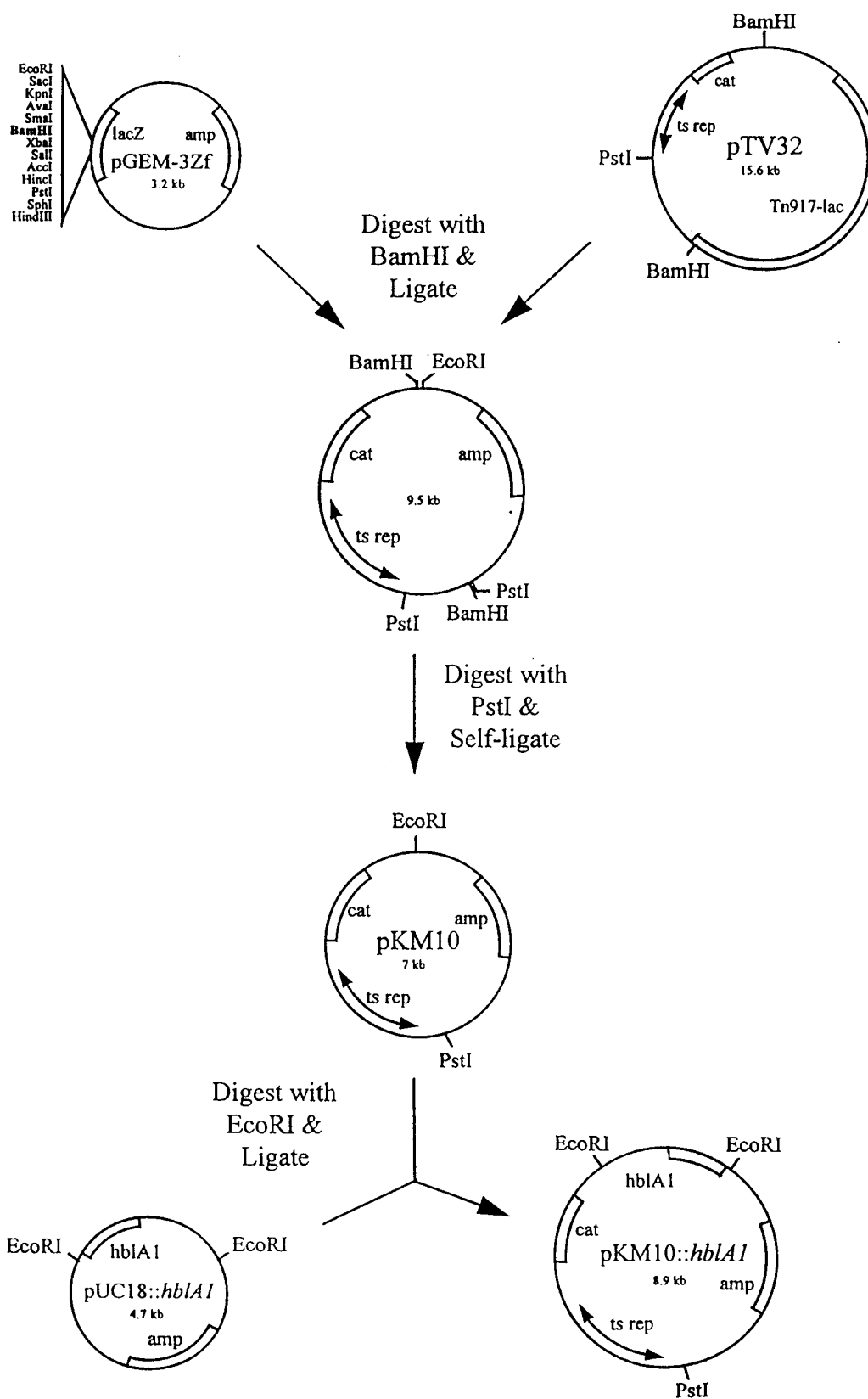


FIG 3

4 / 4

X=XbaI

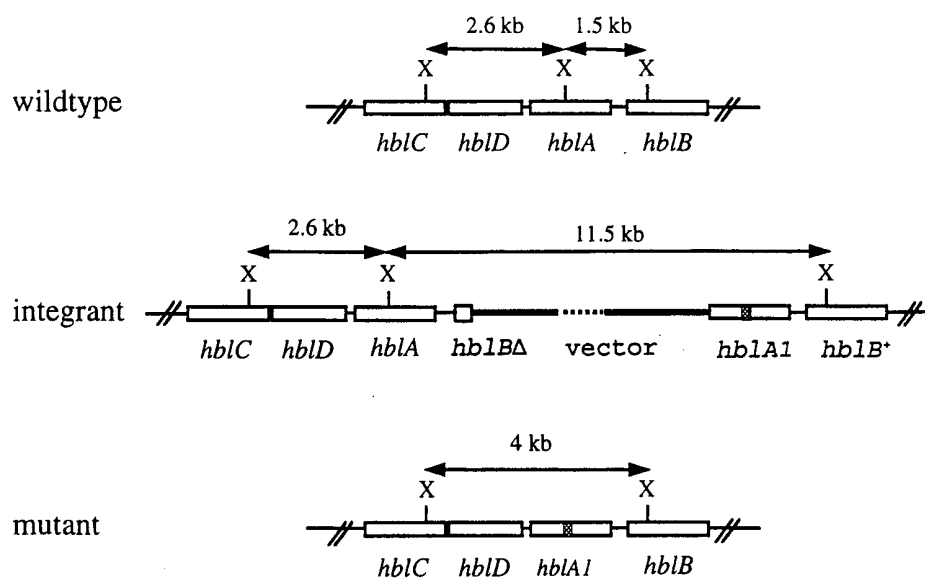


FIG 4

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Handelsman, Jo
Klimowicz, Amy K.
- 5 (ii) TITLE OF INVENTION: Enterotoxin-Deficient Bacillus
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Quarles & Brady
 - 10 (B) STREET: 1 South Pinckney Street
 - (C) CITY: Madison
 - (D) STATE: WI
 - (E) COUNTRY: US
 - (F) ZIP: 53703
- 15 (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- 20 (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - 25 (A) NAME: Berson, Bennett J
 - (B) REGISTRATION NUMBER: 37094
 - (C) REFERENCE/DOCKET NUMBER: 960296
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 608-251-5000
 - 30 (B) TELEFAX: 608-251-9166

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1005 base pairs
 - (B) TYPE: nucleic acid
 - 35 (C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS

5 (B) LOCATION: 1..1005

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

	ATG ATA AAA AAA ATC CCT TAC AAA TTA CTC GCT GTA TCG ACG TTA TTA	48
	Met Ile Lys Lys Ile Pro Tyr Lys Leu Leu Ala Val Ser Thr Leu Leu	
	1 5 10 15	
10	ACT ATT ACA ACC GCT AAT GTA GTT TCA CCT GTA GCA ACT TTT GCA AGT	96
	Thr Ile Thr Thr Ala Asn Val Val Ser Pro Val Ala Thr Phe Ala Ser	
	20 25 30	
	GAA ATT GAA CAA ACG AAC AAT GGA GAT ACG GCT CTT TCT GCA AAT GAA	144
	Glu Ile Glu Gln Thr Asn Asn Gly Asp Thr Ala Leu Ser Ala Asn Glu	
15	35 40 45	
	GCG AAG ATG AAA GAA ACT TTG CAA AAG GCT GGA TTA TTT GCA AAA TCT	192
	Ala Lys Met Lys Glu Thr Leu Gln Lys Ala Gly Leu Phe Ala Lys Ser	
	50 55 60	
	ATG AAT GCC TAT TCT TAT ATG TTA ATT AAA AAT CCT GAT GTG AAT TTT	240
20	Met Asn Ala Tyr Ser Tyr Met Leu Ile Lys Asn Pro Asp Val Asn Phe	
	65 70 75 80	
	GAG GGA ATT ACT ATT AAT GGA TAT GTA GAT TTA CCT GGT AGA ATC GTA	288
	Glu Gly Ile Thr Ile Asn Gly Tyr Val Asp Leu Pro Gly Arg Ile Val	
	85 90 95	
25	CAA GAT CAA AAG AAT GCA AGA GCA CAT GCT GTT ACT TGG GAT ACG AAA	336
	Gln Asp Gln Lys Asn Ala Arg Ala His Ala Val Thr Trp Asp Thr Lys	
	100 105 110	
	GTG AAA AAA CAG CTT TTA GAT ACA TTG ACT GGT ATT GTT GAA TAT GAT	384
	Val Lys Lys Gln Leu Leu Asp Thr Leu Thr Gly Ile Val Glu Tyr Asp	
30	115 120 125	
	ACG ACG TTT GAC AAT TAT TAT GAA ACA ATG GTA GAG GCA ATT AAT ACA	432
	Thr Thr Phe Asp Asn Tyr Tyr Glu Thr Met Val Glu Ala Ile Asn Thr	
	130 135 140	

	GGG GAT GGA GAA ACT TTA AAA GAA GGG ATT ACA GAT TTG CGA GGT GAA	480
	Gly Asp Gly Glu Thr Leu Lys Glu Gly Ile Thr Asp Leu Arg Gly Glu	
	145 150 155 160	
	ATT CAA CAA AAT CAA AAG TAT GCA CAA CAA CTA ATA GAA GAA TTA ACT	528
5	Ile Gln Gln Asn Gln Lys Tyr Ala Gln Gln Leu Ile Glu Glu Leu Thr	
	165 170 175	
	AAA TTA AGA GAC TCT ATT GGA CAC GAT GTT AGA GCA TTT GGG TTT AAT	576
	Lys Leu Arg Asp Ser Ile Gly His Asp Val Arg Ala Phe Gly Phe Asn	
	180 185 190	
10	GTA ATG AAG GGT GCT ATT TTG GGT CTA CCA ATA ATT GGC GGT ATT ATA	624
	Val Met Lys Gly Ala Ile Leu Gly Leu Pro Ile Ile Gly Gly Ile Ile	
	195 200 205	
	GTG GGA GTA GCA AGG GAT AAT TTA GGT AAG TTA GAG CCT TTA TTA GCA	672
	Val Gly Val Ala Arg Asp Asn Leu Gly Lys Leu Glu Pro Leu Leu Ala	
15	210 215 220	
	GAA TTA CGT CAG ACC GTG GAT TAT AAA GTA ACC TTA AAT CGT GTA GTT	720
	Glu Leu Arg Gln Thr Val Asp Tyr Lys Val Thr Leu Asn Arg Val Val	
	225 230 235 240	
	GGA GTT GCT TAC AGT AAT ATT AAT GAA ATC GAC AAG GCG CTT GAT GAT	768
20	Gly Val Ala Tyr Ser Asn Ile Asn Glu Ile Asp Lys Ala Leu Asp Asp	
	245 250 255	
	GCT ATT AAC GCT CTT ACT TAT ATG TCC ACG CAG TGG CAT GAT TTA GAT	816
	Ala Ile Asn Ala Leu Thr Tyr Met Ser Thr Gln Trp His Asp Leu Asp	
	260 265 270	
25	TCT CAA TAT TCG GGC GTT CTA GGG CAT ATT GAG AAT GCA GCT CAA AAA	864
	Ser Gln Tyr Ser Gly Val Leu Gly His Ile Glu Asn Ala Ala Gln Lys	
	275 280 285	
	GCC GAT CAA AAT AAA TTT AAA TTC TTA AAA CCT AAT TTA AAT GCA GCG	912
	Ala Asp Gln Asn Lys Phe Lys Phe Leu Lys Pro Asn Leu Asn Ala Ala	
30	290 295 300	
	AAA GAT AGT TGG AAA ACA TTA CGA ACA GAT GCT GTT ACA TTA AAA GAA	960
	Lys Asp Ser Trp Lys Thr Leu Arg Thr Asp Ala Val Thr Leu Lys Glu	
	305 310 315 320	

GGA ATA AAG GAG TTA AAA GTA GAA ACT GTT ACT CCA CAA AAA TAG 1005
 Gly Ile Lys Glu Leu Lys Val Glu Thr Val Thr Pro Gln Lys *
 325 330 335

(2) INFORMATION FOR SEQ ID NO:2:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 335 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Met Ile Lys Lys Ile Pro Tyr Lys Leu Leu Ala Val Ser Thr Leu Leu
 1 5 10 15
 Thr Ile Thr Thr Ala Asn Val Val Ser Pro Val Ala Thr Phe Ala Ser
 20 25 30
 15 Glu Ile Glu Gln Thr Asn Asn Gly Asp Thr Ala Leu Ser Ala Asn Glu
 35 40 45
 Ala Lys Met Lys Glu Thr Leu Gln Lys Ala Gly Leu Phe Ala Lys Ser
 50 55 60
 Met Asn Ala Tyr Ser Tyr Met Leu Ile Lys Asn Pro Asp Val Asn Phe
 20 65 70 75 80
 Glu Gly Ile Thr Ile Asn Gly Tyr Val Asp Leu Pro Gly Arg Ile Val
 85 90 95
 Gln Asp Gln Lys Asn Ala Arg Ala His Ala Val Thr Trp Asp Thr Lys
 100 105 110
 25 Val Lys Lys Gln Leu Leu Asp Thr Leu Thr Gly Ile Val Glu Tyr Asp
 115 120 125
 Thr Thr Phe Asp Asn Tyr Tyr Glu Thr Met Val Glu Ala Ile Asn Thr
 130 135 140
 Gly Asp Gly Glu Thr Leu Lys Glu Gly Ile Thr Asp Leu Arg Gly Glu
 30 145 150 155 160

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Ile Gln Gln Asn Gln Lys Tyr Ala Gln Gln Leu Ile Glu Glu Leu Thr
      165                      170                      175

Lys Leu Arg Asp Ser Ile Gly His Asp Val Arg Ala Phe Gly Phe Asn
      180                      185                      190

5 Val Met Lys Gly Ala Ile Leu Gly Leu Pro Ile Ile Gly Gly Ile Ile
      195                      200                      205

Val Gly Val Ala Arg Asp Asn Leu Gly Lys Leu Glu Pro Leu Leu Ala
      210                      215                      220

Glu Leu Arg Gln Thr Val Asp Tyr Lys Val Thr Leu Asn Arg Val Val
10 225                      230                      235                      240

Gly Val Ala Tyr Ser Asn Ile Asn Glu Ile Asp Lys Ala Leu Asp Asp
      245                      250                      255

Ala Ile Asn Ala Leu Thr Tyr Met Ser Thr Gln Trp His Asp Leu Asp
      260                      265                      270

15 Ser Gln Tyr Ser Gly Val Leu Gly His Ile Glu Asn Ala Ala Gln Lys
      275                      280                      285

Ala Asp Gln Asn Lys Phe Lys Phe Leu Lys Pro Asn Leu Asn Ala Ala
      290                      295                      300

Lys Asp Ser Trp Lys Thr Leu Arg Thr Asp Ala Val Thr Leu Lys Glu
20 305                      310                      315                      320

Gly Ile Lys Glu Leu Lys Val Glu Thr Val Thr Pro Gln Lys  *
      325                      330                      335

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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- 25 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- 30 (A) DESCRIPTION: /desc = "primer"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAAACAATGG TAGAGGCA

18

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "primer"

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

GCAACTCCAA CTACACGA

18

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "primer"

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

TTACTCGCTG TATCGACG

18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "primer"

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

CCAAGTATCT TTCGCTGC

18