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AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 7/13/87

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. C5-141

Attachment

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PD-AAW-283

C5-141

BIOTECHNOLOGICAL
APPLICATIONS LTD. (BAL)
יישום ביוטכנולוגי (י"ב) בע"מ

PROGRESS REPORT: AID-CDR-Project No. PDC-5544-G-SS-5098-01

entitled "Biological Control of Mosquitos by Bacillus Sphaericus".

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REPORT FOR AID

I. Introduction

The overall aim of the research is to clone the Bti toxin gene in B. sphaericus, in order to produce a persistant, broad spectrum mosquito larvicide. Due to the very limited history of B. sphaericus as a cloning host, our strategy has been to first clone the desired genetic material into E. coli, the most familiar host, and into B. subtilis, the most convenient Bacillus for use in cloning. After verifying that we have cloned the appropriate DNA fragment(s), in a form which is suitable for expression in Bacillus, we will proceed to the final stage of inserting the Bti DNA into B. sphaericus.

II. Cloning of the Bti toxin gene into E. coli

It had previously been reported that the Bti toxin gene was located on a 9.7 kb Hind III fragment of the 112 kb plasmid (Ward et al., 1984). To isolate this fragment, the 112 kb plasmid was cut with Hind III and run on a 1 % agarose gel in acetate buffer. The region containing the 9.7 kb fragment was electroeluted and ligated to Hind III-cut pUC 8 plasmid (Vieira and Messing, 1982). The recombinant plasmids were used to transform E. coli JM83.

The plasmid pUC 8 has a size of 2.7 kb and carries an ampicillin-resistance (amp^r) marker plus a portion of the ß-galactosidase gene allowing identification of recombinant clones by a color reaction when the colonies are grown in the presence of the inducer isopropyl-thio-galactoside (IPTG) and the chromogenic substrate X-gal (Maniatis et al., 1982). The Hind III site of pUC 8 is located in a polylinker region within the ß-galactosidase gene. When no inserted foreign DNA is

present, JM83 colonies grown on amp, IPTG, X-gal plates are a blue color. Those colonies containing recombinant plasmids are white.

After transformation, white ampr colonies were selected for further analysis. Small-scale "quick preps." of plasmids were prepared, digested with Hind III, and run on a 0.6 % agarose gel in acetate buffer. (Figure 1). The smears at the bottom are RNA. Hind III digestion removed the inserted DNA from each recombinant plasmid. In every case the pUC 8 portion of the recombinant can be seen runnin, at 2.7 kb while the inserted foreign DNAs vary in size. Several clones containing a 9.7 kb insert were isolated and two of them are shown in the gel in Figure 1 (lanes B and F).

Immunoassay results (not shown) on the colonies using antibodies against the 28 kDa and the 65 kDa Bti crystal proteins indicated that a very low level of 28 kDa protein was being produced in those colonies containing a 9.7 kb insert. Results with antibodies to the 65 kDa protein were unclear due to high background levels. Results of hemolysis of red blood cells by these clones were negative as were the results of the in vivo mosquito larvae assay.

Clone A34 (Figure 1, lane F) was chosen for all future work on the 9.7 kb Bti fragment in E. coli. Although the recombinant E. coli clone was not useful for killing mosquito larvae, it served as a stable source for the isolation of large quantities of purified 9.7 kb Bti fragment. (Figure 2). Clone A34 is still stable after several transfers over several months.

Figure 1: Detection of plasmids containing the 9.7 kb insert

Lanes A to D and F to L: Plasmids were prepared from quick preps. and cut with Hind III. The samples were not treated with RNase.

Lane E: λHind III markers (kb): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0.

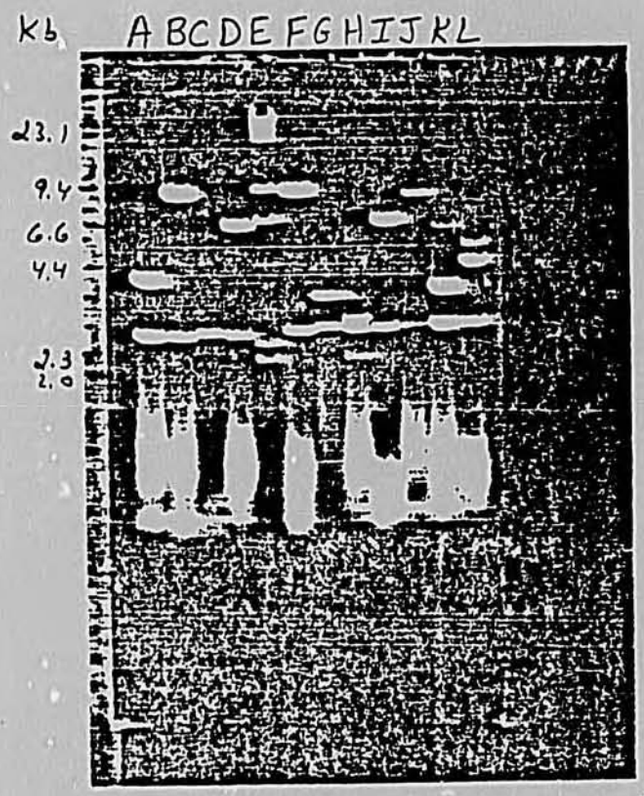
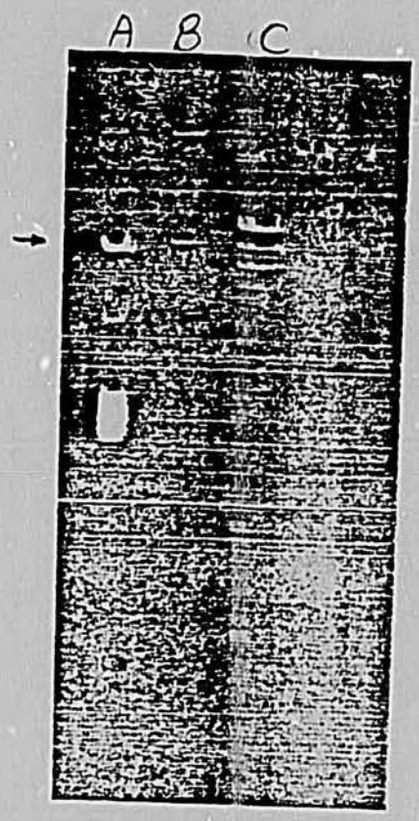


Figure 2: Purification of the 9.7 kb fragment

Lane A: Plasmid DNA isolated from clone A34, cut with Hind III but not treated with RNase.

Lane B: Purified 9.7 kb fragment after electroelution.

Lane C: λHind III markers (kb): 23.1, 9.4, 6.6 (Only the 3 largest marker fragements are visible).



III. Cloning the Bti toxin gene directly into Bacillus

The chosen cloning vector was pPL603E, a 5.1 kb plasmid coding for resistance to neomycin and cloramphenicol, and containing a single Hind III site within the cat gene (Mongkolsuk et al., 1984). Insertion of DNA at this site inactivates the cloramphenicol resistance, although not the inducibility of the downstream DNA expression by low concentrations of cloramphenicol (Duvall et al., 1984). pPL603E is derived from pUB110.

Bti DNA was prepared by a method which yields preferentially the DNA of large plasmids (Casse et al., 1979), and both vector and insert were digested with Hind III.

The host strain was B. subtilis IE20 which contains the plasmid pBD6, a derivative of pUB110 and pSA2100. This plasmid, which codes for resistance to Kanamycin and Streptomycin, segregates at temperatures above 35°C (Dubnau et al., 1980, and observation).

Competent IE20 was transformed by ligation mixture of digested plasmid vector and insert DNA, plated on Kanamycin (20µg/ml), and incubated at 42°C. The approximately 1000 resultant colonies were patched in parallel onto plates of nutrient agar containing 4% whole human blood and 0.1 µg/ml Cm, or 10 µg/ml Cm.

The rational for plating transformants on blood-containing agar was that the larvicidal Bti protein also has hemolytic activity (Yamamoto et al., 1983; Armstrong et al., 1985), and that colonies of the host strain were in general only weakly hemolytic if at all.

The screening revealed that 7% of the transformants were hemolytic, implying that they carried the inserted Bti DNA fragment in expressible form. Only 6% of the transformants were entirely lacking in inserted DNA, as indicated by resistance to Cm. The hemolytic activity of the colonies which were positive on blood-agar plates was

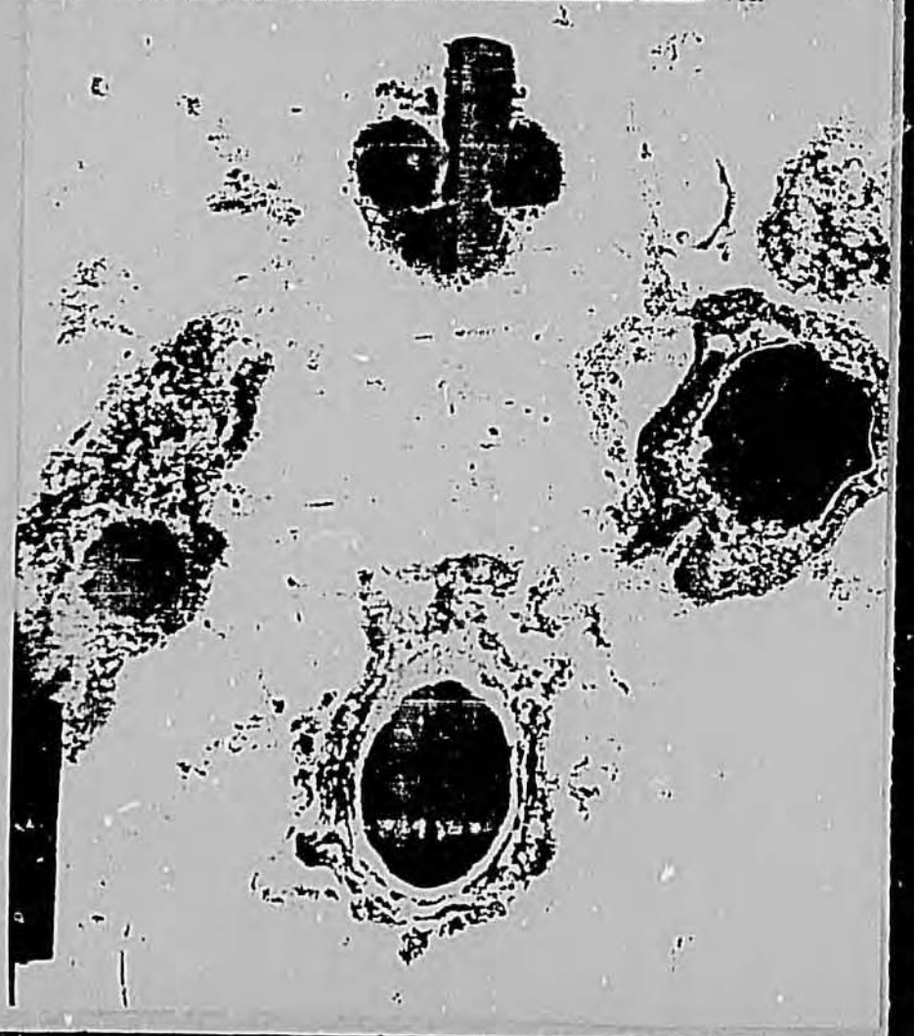
quantified by the assay of spore/crystal powders of the colonies as previously done in our laboratory (Sandler et al., 1985). The most active colonies were selected for further study.

Crystalline inclusions were noted in several of the hemolytic colonies of transformed B. subtilis. Electron microscopy showed that the inclusions resembled those of Bti (Figure 3). However, at least in one case, the presence of crystals was restricted to a limited time period during sporulation, and electron microscopy indicated that the inclusions disappeared completely between the time of one sampling, 22 hours after innoculation into medium not containing antibiotic, and a second sampling 4 hours later (not shown in the figures).

Several hemolytically active colonies were tested for the presence of Bti crystal proteins and for mosquito larvicidal activity. In addition, the 9.7 kb DNA fragment re-isolated from the transformed E. coli clone designated A34 was ligated to the Hind III site of plasmid pP1603E and used to transform B. subtilis strain IE20. Kn-resistant, Cm-sensitive colonies were isolated and tested for hemolytic activity. Several of these active colonies were tested further, together with the colonies arising from cloning of Bti DNA directly into B. subtilis. Figure 4 shows the results of immunoassay of several recombinant Bacillus subtilis clones derived from the two complementary experiments. Results of the mosquito assay are given in Tables 1 and 2.

Figure 3:

a) Sporangium of B. subtilis clone 57, grown in the absence of Kn. Both spore and amorphous inclusion are visible.
Total magnification: x 48,000.



b) Another field from the same sample as in a, above, the precise forms of the crystalline components of the inclusion are visible.
Total magnification: x 38,550.

Figure 3:

c) Additional field of the same sample as in Figure 3a and b.
Total magnification: x 28,550.

d) Bti sporangium with amorphous crystalline inclusion; individual components of the inclusion are also visible in lower right-hand section.
Total magnification: x 35,525.

Figure 4: Immunoassay of B. subtilis recombinants:
Spots were prepared as described in Experimental Procedures
Cm = grown in media containing chloramphenicol (0.1 μg/ml)
Kn = grown in kanamycin (5 μg/ml); N11 is Bti.
168 and IE20 are B. subtilis negative controls.
The other samples are B. subtilis recombinants.
All samples were spotted in duplicate.

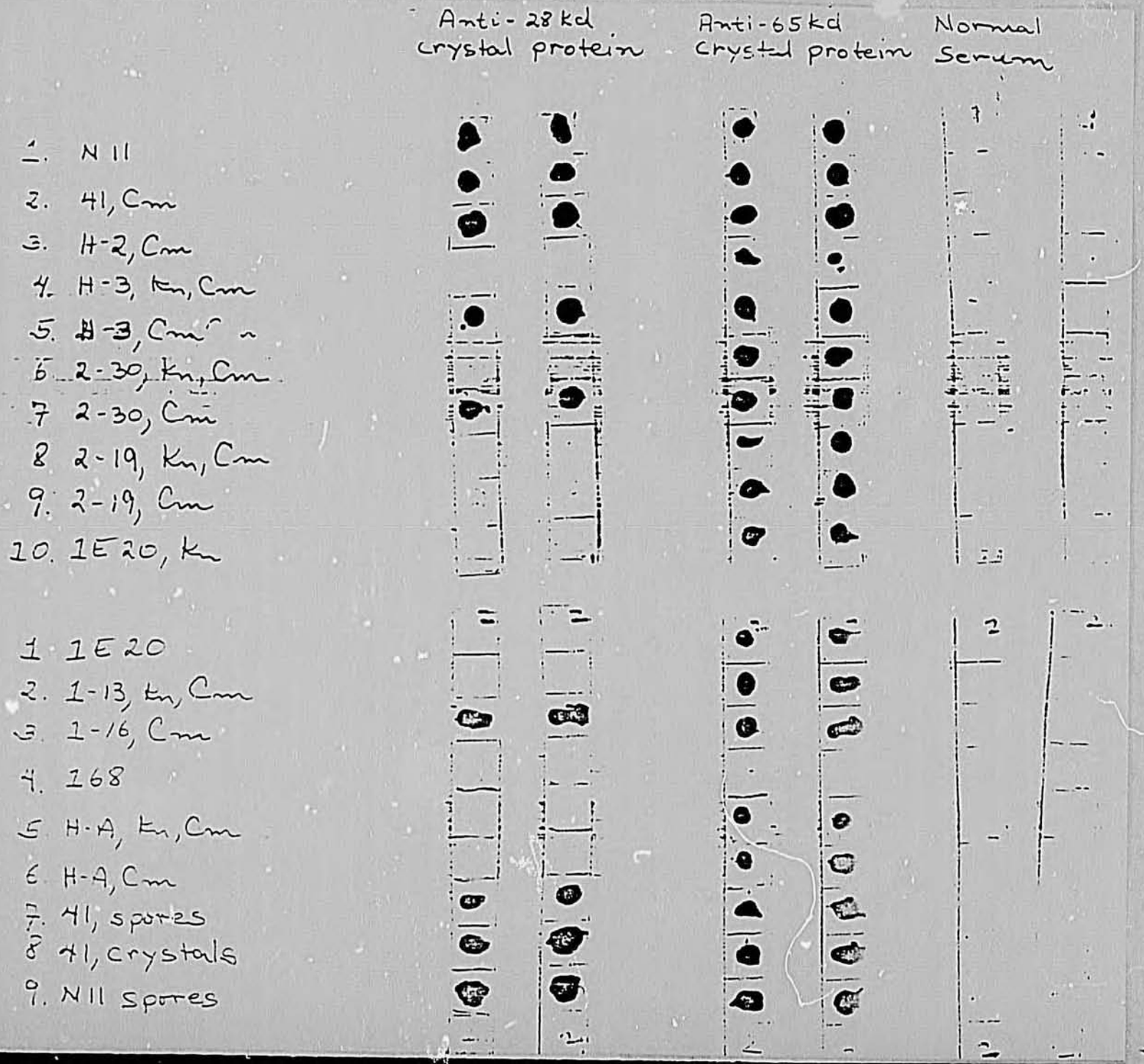


TABLE 1: *In vivo* Mosquito Larvae Assay of *B. subtilis* recombinants

SAMPLE	Final conc. in the mosquito larvae assay	Dead Larvae After 2 h	Dead Larvae After 24 h
Group A (Kn+Cm)			
H-3	60 µg/ml	0	0
1-13	"	0	0
2-19	"	0	0
2-30	"	0	1
H-A	"	0	0
Group B (Cm)			
1-16	60 µg/ml	10	10
2-19	"	1	10
2-30	"	10	10
H-A	"	0	8
H-2	"	10	10
H-3	"	10	10
41	"	10	10
Group C (Cm)			
1-16	6 µg/ml	10	10
2-19	"	0	2
2-30	"	0	10
H-A	"	1	2
H-2	"	10	10
H-3	"	9	10
41	"	10	10

cont.

Group D			
Controls:			
168(B.subtilis)	60 µg/ml	0	0
1E20(B.subtilis)	60 µg/ml	0	0
Bti	60 µg/ml	10	10
Bti	3 µg/ml	10	10

Kn = 5 µg/ml kanamycin

Cm = 0.1 µg/ml chloramphenicol

Samples in group A were grown in Kn and Cm while those in groups B and C were grown only in Cm.

TABLE 2: In vivo mosquito larvae assay to show chloramphenicol effect

SAMPLE	Final concentration (in mosquito larvae assay)	Dead Larvae	
		2 hours	24 hours
1-16			
Cm (0.1µg/ml)	0.4 µg/ml	10	10
		6	10
		10	10
1-16			
No Cm	0.4 µg/ml	0	0
		0	0
		0	0

Cm = chloramphenicol

The most striking result of these experiments was the precise correlation of the immunoassay results using the anti-28 kDa antibodies and the level of killing in the in vivo mosquito larvae assay (Figure 4 and Table 1). Growth of the bacteria in kanamycin inhibits sporulalation and the inhibitory effects of kanamycin can be seen in both the immunoassay and the larvicidal assay (Figure 4 and Table 1). Hemolysis results (not shwon) also showed this effect. Samples such as 1-16 (from the 9.7 kb cloning) and 41 (from the shotgun cloning) which showed very high levels of 28 kDa protein were also very highly toxic to mosquito larvae. Clone 2-19 had a low level of 28 kDa protein and showed a low level of larvicidal activity.

All recombinant bacteria in these experiments were grown in the presence of chloramphenicol (0.1 µg/ml). The induction of the cat gene of pPL603E by chloramphenicol was discussed previously. To illustrate this induction in our own system, Table 2 shows the effects of low levels of chloramphenicol (0.1 µg/ml) during the growth of the bacteria. Samples of 1-16, a B. subtilis recombinant which contained the 9.7 kb fragment within the cat gene, were tested in triplicate on mosquito larvae to compare activity after growth in media with or without chloramphenicol.

III. Conclusion

The reported results to date indicate that we have succesfully cloned a fragment of Bti DNA capable of causing toxin production in Bacillus. However, although we have produced highly toxic clones of Bacillus subtilis, we have seen that toxin production is apparently more sensitive to culture conditions than in Bti, and that toxicity is easily lost by these clones. We now intend to proceed to the second stage of our project, the insertion of our recombinant DNA into B. sphaericus. We will also attempt to overcome the problems which have arisen with

the B. subtilis clones. In addition to the possible utility of these clones, the experience thus acquired should help us to overcome similar problems which may arise in the production of active, stable, transformants of B. sphaericus.

IV. Experimental Procedures

Media:

E. coli were grown in LB (Luria-Bertani)-medium, prepared as described in Maniatis et al., (1982). Plates were prepared with 15 grams Difco bacto-agar per liter of LB. Plates containing ampicillin had a concentration of 50 µg ampicillin/ml LB. Amp, IPTG, X-gal plates contained amp (50 µg/ml), 0.1 mM IPTG, and 0.004% X-gal. IPTG is isopropylthiogalactoside and X-gal is 5-bromo-4-chloro-3-indolyl-ß-D-galactoside.

Ms media for B. subtilis was prepared as follows for 1 liter:
16 gm nutrient broth, 2 gm KCl, (plus 15 gm Bacto agar for plates). After autoclaving, the following salts and glucose were added to give a final concentration of: 0.05% $MgSO_4.7H_2O$, 1mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2$, 1 uM $FeSO_4$, and 0.5% glucose. When kanamycin was added, it was brought to a final concentration of 5 µg/ml.

Enzymes:

Restriction enzymes were purchased from New England Biolabs, Boehringer Mannheim and Anglian.

Preparation of lyophilized (freeze-dried) bacterial powders for mosquito larvae testing and immunoassay

Bacteria were grown in MS media until the sporulation stage. 10 ml of culture were centrifuged for 10 min at 4000-5000 rpm in a Sorvall

GLC-2B table top centrifuge at room temperature. The supernatant was discarded. The pellet was resuspended in 10 ml water and centrifuged again as described above. The supernatant was discarded and the pellet was resuspended in 1 ml water and frozen at -20°C overnight. It was then lyophilized until it formed a dry powder.

E. coli lyophilized powders were prepared from cultures grown overnight in LB which were then washed in a similar manner as described above with either water or saline (0.9% NaCl).
Powders were then weighed out and resuspended in water before being added to the cups containing the mosquito larvae.
For immunoassay, the lyophilized material was resuspended at 2 mg/ml in sodium carbonate (pH 10.5) and placed at 37°C for 30 minutes to dissolve the crystals. Then 5 µl of each sample were spotted on nitrocellulose strips.

Preparation of E. coli colonies for Immunoassay

Colonies growing on LB plates containing ampicillin (50 µg/ml) were transferred to sterile nitrocellulose filters. The filters were then placed on new amp plates with the colonies facing up and left to grow overnight.

The following day the filters were removed from the plates and dried with a blow dryer. Then the filters were placed on four 3MM (Whatman) sheets which had been soaked in the following solutions:

1. 0.5 N NaOH
2. 1.5 M Tris-HCl (pH 7.5)
3. 2 x SSC
4. 70% Ethanol

The filters were placed on each for 5 minutes and were dried briefly on 3MM paper between transfers. After drying at 60°C for 2 hours, the colonies were used for immunoassay.

Preparation of Antibodies

Antibodies to the specific Bti crystal proteins were prepared by Pnina Baschkin. Crystals were isolated from Bti and the disolved proteins were then chromatographed on a Sephacryl gel filtration S-200 column and on a Whatman DE-52 ion exchange column. The appropriate fractions were run on a preparative gel. Bands of the correct sizes were electroeluted. The proteins were then concentrated and injected into rabbits.

Immunoassay

The immunoassay was done on either colonies transferred directly to nitrocellulose filters or on extracts spotted on nitrocellulose strips. Preparation of the samples for immunoassay was described above.

Pre-incubation: The nitrocellulose filters wre placed in individual containers and incubated in 5% low-fat milk powder in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl for two hours at 37°C on a shaker. The volume used in each case was enough to cover the whole filter or strip.

Incubation with the first antibody: The pre-incubation buffer was discarded. Antibody was diluted 1:500 in the 5% milk solution (in Tris, NaCl) and pipetted onto the filter. It was then left at room temperature overnight without shaking.

Washes: The first wash was 0.2% triton X-100, 100 mM Tris-HCl (pH:7.5), 100 mM NaCl, 5 mM $MgCl_2$ for 10 minutes at room temperature on a shaker. The filters were then washed 5 times in $AP_{7.5}$ buffer (100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$) on a shaker. Each wash was for 10 minutes.

Incubation with the second antibody: The filters were then incubated for 2 hours on a shaker at room temperature in alkaline phosphatase anti-rabbit IgG (H+L) prepared in goat (from BioYeda) diluted at 1:1000 in $AP_{7.5}$ buffer.

Washes: They were then washed twice in $AP_{7.5}$ buffer and four times in $AP_{9.5}$ buffer (1000 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$). Each wash was for 10 minutes on a shaker at room temperature.
Color reaction: The following two solutions were prepared in a large enough volume to cover all the filters or strips.

(In a dark or covered bottle) 1 mg nitro blue tetrazolium/3 mls $AP_{9.5}$
(N B T)

0.5 mg 5-bromo-4-chloro-3-indolyl phosphate/ 15 µl dimethyl formamide
(B C I P) (D M F)

The two solutions were vortexed together and poured over the filters. The filters were left at room temperature (with slight shaking at intervals) and the color generally developed within several minutes.
Termination of color reaction: To stop the color reaction when the background started getting high, the filters were rinsed twice in a 1:5 dilution of $AP_{7.5}$. They were then left to dry between 3MM papers to protect them from the light.

In vivo Mosquito Assay:

For each test, 10 mosquito larvae (third instar stage) were placed in a cup containing 50 mls of water at room temperature. The freeze-dried bacterial samples which had been resuspended in water, at various concentrations for different experiments, were pipetted directly into the cups containing the mosquito larvae. The cups were left standing at 27°C and were checked after 2 hours, 24 hours and 48 hours for the presence of dead larvae.

Hemolytic Assay:

The hemolytic assay was done as described by Sandler et al., (1985).

Plasmid Quick Preps. (Holmes and Quigley procedure):

1.5 mls of an overnight culture of bacteria were transferred to a microfuge tube and centrifuged 1 to 2 minutes at 12000 x g in an Eppendorf centrifuge to pellet the bacteria. The supernatant was discarded, another 1.5 mls of the same culture were added to the tube and the centrifugation was repeated. After the second supernatant was discarded, 35 µl STET buffer {8% sucrose, 5% triton x-100, 50 mM EDTA, 50 mM Tris-HCl (pH 8)} were added and the pellet was resuspended by vortexing for two minutes. 6 µl of freshly prepared lysozyme solution (10 mg/ml) were added and the tube was vortexed briefly. It was then placed in a boiling water bath for 40 seconds and centrifuged at room temperature for 15 minutes to pellet the clot of genomic DNA and cell debris. The supernatant was transferred to a new tube and an equal volume of isopropanol was added. It was then placed at -20°C for at least 20 minutes to precipitate the plasmid DNA. After a 5-minute spin in the cold room, the supernatant was discarded and the pellet was dissolved in water. In most cases the plasmid was digested with restriction enzymes and RNAse before loading on a gel.

Agarose Gels:

Agarose gels for visualizing DNA were generally 0.6% agarose in acetate buffer, unless stated otherwise in the text, and contained 1µg ethidium bromide per ml of gel solution. Acetate buffer was prepared according to Maniatis et al., (1982) and was used for both the gel and the running buffer. Large gels were run overnight (approximately 18 hours) at a constant voltage of 35 volts. Minigels were run for 40 minutes at 100 volts. Upon completion of the run, photographs were taken using an ultraviolet (U.V.) light table.

Electroelution:

This procedure was used to elute specific restriction fragments from agarose gels. In the initial experiments involving electroelution of Hind III fragments from the 112 kb Bti plasmid, no ethidium bromide was added to the preparative gel. Lamda ( ) Hind III markers were run in both end lanes of the gel. These end lanes were later sliced off and stained in ethidium bromide in order to identify specific regions of the gel.

All future gels for the preparation of DNA fragments were prepared with ethidium bromide so that the extra steps of cutting and staining were not necessary. However, when the DNA fragment was in a gel containing ethidium bromide, visualization of the fragment was performed with a weaker overhead U.V. lamp rather than the stronger U.V. light table in order to prevent nicking and breakage of the DNA.

An electroelution gel of 1% agarose in acetate buffer (no ethidium bromide) was prepared and a small rectangular area of agarose was removed. Dialysis tubing was cut open and placed in the hole to form a "pocket".

The restriction fragment to be eluted was cut out of a preparative agarose gel and placed in the "pocket" with acetate buffer surrounding it. The current was turned on (100 volts) until the fragment ran out of the agarose into the buffer. (This could be observed with an overhead U.V. light). At that point the current was reversed for 15 seconds to remove any DNA that was on the dialysis tubing. The buffer containing the DNA was transferred to microfuge tubes and fresh buffer was added to the pocket. The current was run again in reverse for 15 seconds and this buffer was also collected. To precipitate the DNA that was in the buffer, sodium acetate (pH 5.2) was added to a final concentration of 0.25 M and then 2 volumes of ethanol were added. The tubes were placed at -20°C overnight.

The following day they were spun for 15 minutes in the cold room at 12000 x g in an Eppendorf centrifuge. Supernatants were discarded and the pellets were resuspended in water and combined into one tube. The DNA was then re-precipitated in 0.25 M sodium acetate (pH 5.2) and 2 volumes of ethanol and placed at -20°C overnight. After a 15-minute centrifugation in the cold room, the supernatant was discarded and the pellet was air-dried and resuspended in water.

Ligation:

After digestion with the appropriate enzymes and heat-inactivation of the enzymes, vector DNA and insert DNA were combined, brought to a total volume of 15 µl and heated to 65°C for 10 minutes. The solution was brought to a final concentration of 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 20 mM DTT (dithiothreitol), 1 mM ATP and 800 units of T4 DNA ligase (Biolabs) were added to give a total reaction volume of 20 µl. The tube was incubated at 4°C overnight and then 10 µl was used for transformation of competent cells.

Preparation of E. coli Competent Cells:

E. coli was grown overnight in 40 ml LB shaking at approximately 35°C. The following day 40 ml of pre-warmed LB were innoculated with 2 ml of the overnight culture. The new culture was allowed to grow on a shaker at 35°C until $O.D._{550}$ reached 0.6 to 0.7. At that point the culture was transferred to ice for 10 minutes. Then 20 ml of the culture were spun in the Sorvall small angle rotor for 2 minutes, 7000 RPM at 4°C. The supernatant was discarded and the pellet was resuspended in 10 ml cold 50 mM $CaCl_2$. It was left on ice for 20 minutes and then centrifuged again as described above. The supernatant was discarded. The pellet was resuspended in 2 ml cold 50 mM $CaCl_2$ and left at 4°C overnight before transformation.

Transformation of E. coli:

10 µl of ligated plasmid DNA (generally 50-100 ng), were added to 0.2 ml competent E. coli cells, mixed by hand and left on ice for 30 minutes. The tube was then transferred to 42°C for 3 minutes. Then 1 ml of LB was added and the tube was placed at 37°C for 1 hour without shaking. The transformed cells were spread on plates and placed at 33-35°C overnight.

B. subtilis Transformation:

Transformation of B. subtilis was done by the standard procedure as described in Gryczan et al., (1978).

Electron Microscopy:

Electron microscopy was done by Dr. Ezra Rahamim at the Electron Mircoscopy Unit of the Medical School, Hebrew University of Jerusalem.