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MOLECULAR BIOIOGY OF *BACILLUS THURINGIENSIS* AND POTENTIAL BENEFITS TO AGRICULTURE

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ABSTRACT

We have cloned several Lepidopteran specific delta-endotoxin (DET) genes and expressed them in *E. coli.* A CrylA(B) type gene, (according to the classification proposed by Hofte and Whiteley, 1989), cloned from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 was selected for random mutagenesis experiments in the hopes of isolating toxins with enhanced activities. Regions of the toxin gene were classified as conserved or variable based on their extent of amino acid sequence homology with other *B.t.* DET genes. For the first set of mutagenesis experiments we focused on highly conserved domains. These targets were individually exposed to chemical mutagens and sub-cloned back into wild type DET genes. The resultant DET clones contain point mutations in the defined regions of the toxin genes.

A bank of mutant DET genes having one to three amino acid substitutions per clone was screened for toxicity to *Heliothis virescens* larvae. DNA from isolates which showed a five-fold increase in toxicity was sequenced to identify which codon(s) was altered. Interestingly, the mutations in the up-mutant clones were clustered in two regions of the DET gene. This data allowed us to design site directed mutagenesis experiments which led to a further enhancement of toxic activity toward H. *virescens.*

INTRODUCTION

Bacillus thuringiensis, (B.t.) is a sporulating bacterium which produces a proteinaceous crystal at the end of vegetative growth. Upon ingestion by certain insect larvae and modification in the insect gut, these crystals produce toxic effects including the cessation of feeding, gastrointestinal dysfunction, dehydration and ultimately death. Despite these toxic effects, which are noted with a wide variety of Lepidopteran larvae species, the crystals are harmless to other living species including mammals, bird, fish, and non-susceptible insects. The active toxic factor in the crystals is a protein termed the delta-endotoxin (DET) which appears to act primarily and specifically on the Lepidop- teran gut (Bulla et al., 1975).

A large number of varieties of *B.t.* have been identified, most of which are specifically toxic to Lepidopteran larvae. Relative toxicities toward different Lepidopteran species appear to be quite variable between *B.t.* varieties suggesting the existence of closely related but phenotypically distinct delta-endotoxin genes. In addition to literally hundreds of Lepidopteran specific strains, several subvarieties have been shown to have specific toxicity to Dipteran larvae (Goldberg and Margalit, 1977) and Coleopteran larvae (Hofte et al., 1987).

The active portion of the delta-endotoxin has been localized to the 5' half of the gene. About 70,000 daltons (d) of protein, encoded by the 3' half of the gene, is removed by proteolysis from the

carboxyl terminus during the activation process in the insect gut. When the amino-terminal coding portion of the gene is expressed in *E. coli*, it produces an approximately 67,000d protein which has toxic activity (Witt, 1985; Wabiko et al., 1986).

The goal of the project was to increase the biological activity of the toxin in order to increase its potency to less susceptible Lepidopteran larvae. Protein engineering through DNA mutagenesis resulted in significant increases in toxicity of the recombinant toxin toward *Heliothis virescens* larvae. Bisulfite and formic acid were used to introduce random point mutations within specified target regions of the delta-endotoxin gene. Bisulfite is a single strand mutagen which converts cytosine to uracil by deamination (Shortle, 1983). This change results in a transition of cytosine (C) to thiamine (T), or guanine (G) to adenine (A) depending upon whether the sense or the antisense strand is mutagenized. Formic acid depurinates DNA by breaking N-glycosyl bonds (Meyers et al., 1985). Polymerization across this template using the "error prone" polymerase reverse transcriptase most commonly results in transversions as purines are usually inserted opposite the site of damage.

MATERIALS AND METHODS

Strains. *Bacillus thuringiensis* strains were obtained from Dr. Howard Dulmage, U.S.D.A., Brownsville, Texas. *E. coli* SG4044 was a gift from Dr. Carol Gross, Univ. of Wisconsin, Madison, WI.

Propagation and Isolation of Phage DNA. M13 derived recombinant phage stocks and isolation of phage DNA was prepared according to methods described by Messing (1983).

Preparation of Plasmid DNA was prepared according to methods described by Davis et al. (1980).

Transformation of competent *E. coli*. Competent *E. coli* cells were prepared according to the method of Cohen et al. (1972). 100 ng of DNA from a ligation reaction was added to 0.2 ml of competent JM103 and held on ice for 30 minutes. The cells were pulsed at 42°C for 2 minutes and returned to ice. Aliquots were plated on YT agar containing 50 ug/ml ampicillin and incubated overnight at 37°C.

Restriction Enzyme Digestion. All restriction enzymes were purchased from Bethesda Research Labs (Gaithersburg, MS) or New England Biolabs (Beverly, MA). Reactions were carried out according to the manufacturer's specifications.

DNA Ligations. DNA ligation reactions (20 ul) contained 60 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 uM ATP, 20 nM DNA termini and 20 units T4 DNA ligase (New England Biolabs). Incubation was at 14°C for 4 hours to ligate "sticky" ends or overnight for "blunt" ends. Three fragment ligations were set up with fragments present at a concentration of 0.04 pmole each in a 20 ul reaction volume.

Preparation of synthetic oligonucleotides. Synthetic oligonucleotides for use as primers for in vitro DNA polymerization were prepared by automated synthesis with an Applied Biosystems (Foster City, CA) 380A DNA synthesis machine according to the manufacturer's specifications.

Formation of Gapped Heteroduplex DNA. To prepare a template for bisulfite mutagenesis, plasmid DNA was linearized using a restriction enzyme site located away from the target region. In a separate reaction the plasmid was digested with restriction enzymes that cleaved upstream and downstream of the target region and the larger fragment was separated from the smaller fragment. Gapped duplex molecules were formed by mixing equimolar amounts of the single cut DNA with the gel isolated DNA, denaturing by boiling 10 minutes, and reannealing by gradual cooling to room temperature. Formation of heteroduplex circular molecules was assessed by their decreased mobility in 0.6% agarose gels, as compared to the migration of the homoduplex linear molecules.

Chemical Mutagenesis. Bisulfite mutagenesis was carried out as described by Shortle and Nathens (1983). General procedures for using formic acid as a mutagen have been described by Meyers et al. (1985).

Bioassays. Mutant phenotype was evaluated in a *Heliothis virescens*, tobacco budworm (TBW) in vivo toxicity assay according to methods described by Raulston and Lingren (1972) and Dulmage et al. (1976).

RESULTS

A recombinant DET clone designated prAK, encoding a truncated DET gene for expression in *E. coli* is diagrammed in Fig. 1. The toxin produced from this clone is ~82kd in size and encompasses the first 723 amino acids of a *B.t.* subsp. *kurstaki* HD-1 protoxin of the CrylA(b) type according to the classification proposed by Hofte and Whiteley (1989). This clone served as the basis for our first round of mutagenesis experiments.

In our approach to mutagenesis, the following guidelines were set: (i) mutagenize one defined section of the gene at a time, (ii) generate a limited number of changes (2→3) within a given target area in ≥ 90% of the clones, and (iii) use results from the region directed mutagenesis experiments to follow-up with a more site-specific approach.

Fig. 1 shows that the prAK DET gene can be divided into segments from 342bp to 683bp in length by digestion with different pairs of the restriction enzymes shown. Our first target, defined by Bam H1 and Xba 1, was selected based on its high degree of amino acid conservation in a number of different *B.t.* toxin genes.

Two mutagens were used to achieve a wide range of possible amino acid substitutions. By exposing single stranded DNA in heteroduplex molecules to bisulfite for 30 minutes, we obtained between 2 and 3 base changes per molecule for each target region. By altering the amount of time that the DNA was exposed to formic acid we were able to achieve a range of mutation densities as

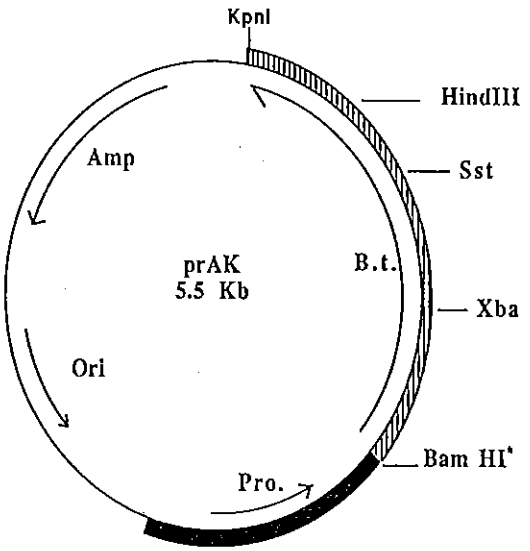

Fig. 1. prAK is an *E. coli* expression plasmid for a *B.t.* subsp. *kurstaki* delta-endotoxin gene. Solid bar represents an *E. coli* promoter; hatched bar indicates *B.t.k.* sequences coding for the first 723 amino acids of the protoxin; single line represents pBR322 sequences encoding ampicillin resistance and an origin of replication. Restriction enzymes shown were used to define targets for mutagenesis. *BamHI in an engineered site.

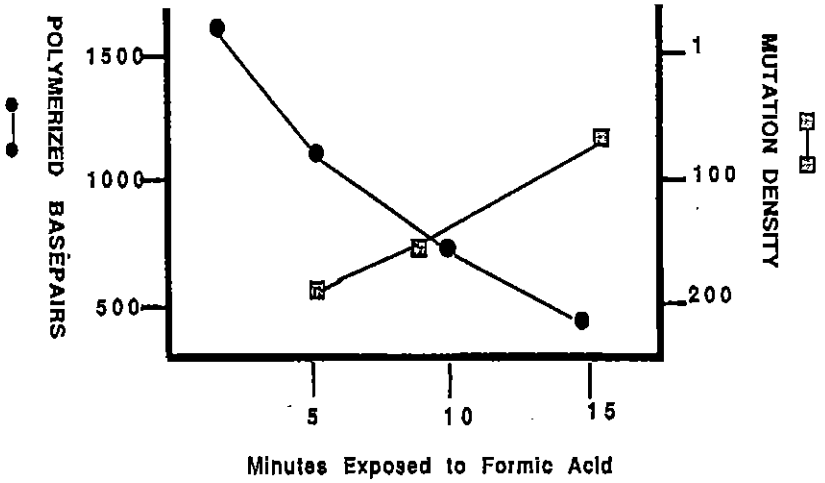

Fig. 2. Relationship of template mutation density to distance polymerized in vitro. Mutation density is expressed as the frequency of apurinic sites, (i.e. 200 = 1 in 200 sites, 1 means saturation, etc.). Polymerized basepairs represents the maximum distance we were able to polymerize across each damaged template. As can be seen from this graph, mutation density and polymerization distance are inversely proportional. Further, the mutation density is a direct result of the length of time the template is exposed to the mutagen.

illustrated in Fig. 2. A number of improvements were necessary in order to achieve these high mutation densities. The basic strategy we used is summarized in Fig. 3.

E. coli transformed with mutation containing prAK DNA were grown to stationary phase in YT media supplemented with 50 ug/ml ampicillin. Clones were selected at random for DNA sequence analysis. An average mutation frequency of 2 changes per target region in greater than or equal to 90% of the clones was achieved and was a prerequisite for proceeding to in vivo bioassays.

Mutant toxins were evaluated in a *Heliothis virescens*, tobacco budworm (TBW), diet assay. This primary screen was designed to highlight clones having 5 fold or greater increased toxicity to TBW than the wild type clone prAK. No attempt to calculate LC 50 values was made in this first assay given the large number of clones to be screened. Rather, a concentration of *E. coli* cells expressing prAK DET was chosen that when mixed into the insect diet would give 50% growth retardation. It was, therefore, easy to distinguish mutant clones having significantly (5 fold) increased or decreased toxicity from the parent clone. All bioassays of mutant clones were run with a wild type prAK positive control as well as an *E. coli* SG4044 negative control. Numerical scores were assigned in this preliminary screening based on larval size as shown in Table 1(A). A dose response for prAK toxicity to *Heliothis virescens* using this method of scoring is shown in Table 1(B).

For our primary screen the sample size of 1 ml of culture was chosen. The amount of toxin per culture was quantitated using SDS PAGE and gel scanning densitometry and was shown to be consistent at a level of approximately 2% total cellular protein (data not shown). Higher expression levels could be obtained with different promoters/host cells, however, often more variability was seen. An example of data generated from a primary screen is given in Table 2.

Dilution series with eleven mutant toxins expressed in *E. coli* versus the wild type clone (prAK) demonstrated that the mutants had greater than or equal to 5-fold enhanced toxicity towards *Heliothis virescens* larvae. Data for two representative clones, p36a65 and p48a14, are given in Table 3.

DNA was isolated from eleven different confirmed up-mutants and cloned into M13 vectors for sequence analysis. One to three amino acid substitutions per clone were found in the targeted region

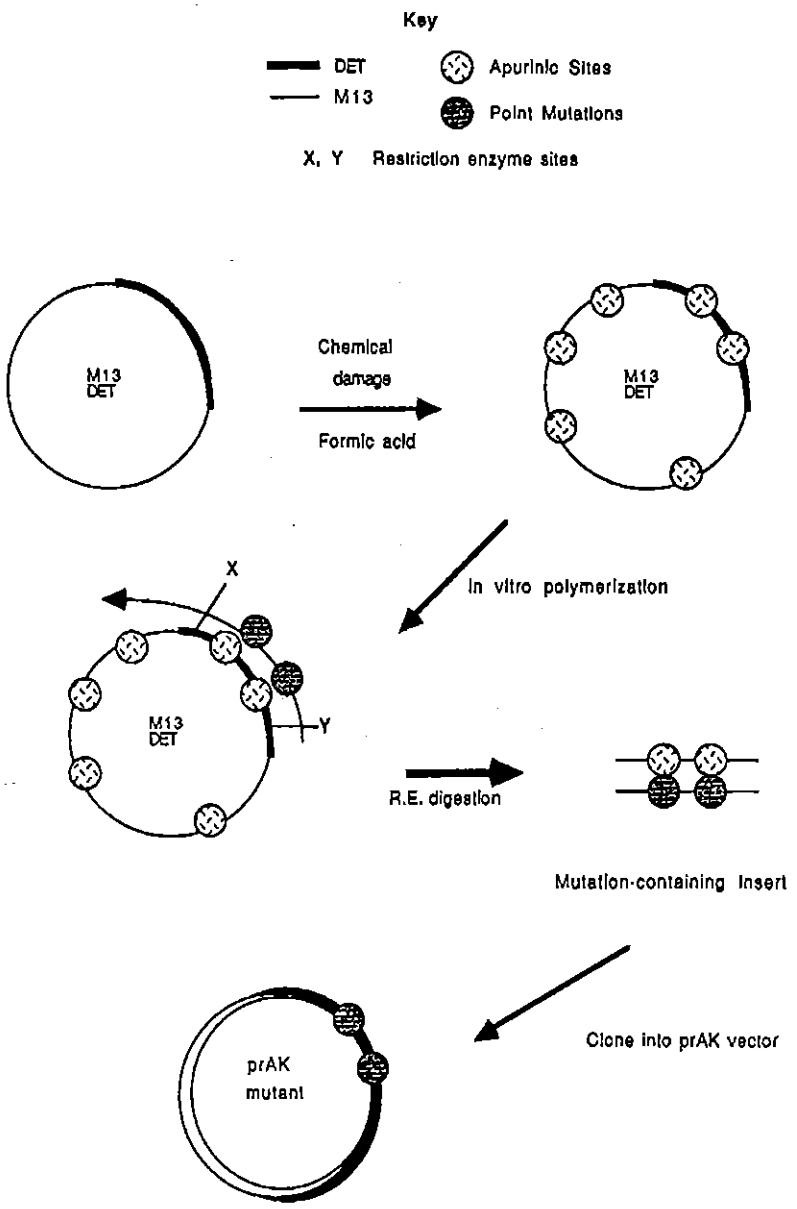

Fig. 3. Basic strategy for formic acid mutagenesis. Single strand DNA is obtained by cloning the DET gene into M13 phage. Apurinic sites are introduced by exposure to the mutagen and mutation containing fragments are obtained by annealling a complementary oligonucleotide, polymerization, and restriction enzyme digestion. The mutation-containing insert fragment is then cloned into a wild type prAK vector thus confining the mutations to a predetermined segment of DNA within the DET gene.

TABLE 1(A)

Toxicity score	Weight range (mg)	Average weight (mg)
1.0	1.3–1.9	1.6
1.5	2.7–3.1	2.8
2.0	5.5–6.3	5.8
2.5	11.7–12.3	12.0
3.0	17.3–22.2	19.6
3.5	30.8–34.2	32.8
4.0	50.1–52.4	51.1
4.5	76.6–94.3	84.8
5.0	111.9–114.7	113.5
6.0	119.8–134.3	125.1
7.0		>140.0

TABLE 1(B)
Dose response of prAK to *H. virescens* larvae

Amount of culture*	Toxicity scores**		
10 ml prAK	+	+1.5	+2.0
5 ml	1.5	2.5	2.5
2 ml	2.0	3.0	3.5
1 ml	3.0	3.5	4.0
0.5 ml	3.0	4.0	4.0
0.25 ml	3.5	5.5	6.0
5 ml *E. coli*	5.5		

*Culture was mixed in with the diet and divided between three larvae.
**Score correlates with size of larva as shown in Table 1(A). A plus (+) indicates larval death.

TABLE 2
Primary *H. virescens* toxicity scores of up-mutant p26-3

Sample*	Toxicity scores		
SG 4044	5	5	6
	5	6	6.5
prAK	3.5	4	4
	3	3.5	3.5
p26-3	1	1.5	3
	1	1	1.5
	1.5	1.5	1.5
	1	1.5	2
	1	1.5	2

*Samples evaluated in these screenings were overnight cultures of *E. coli* strain SG4044 itself (negative control), SG4044 transformed with prAK (wild-type control), and SG4044 transformed with mutant clone p26-3. Each set of three scores represents 1 ml of culture (sub-lethal dose) mixed in with insect diet and divided evenly between three larvae.
Assays were read after 5 days. The numerical scores correlate with larval size as shown in Table 1A.

TABLE 3
Dilution series for mutants p36a65 and p48a14

Sample	Concentration mg/ml*	Toxicity scores		
prAK	10 mg/ml	1	2	2
	5	2	2	2
	2.5	2.5	2.5	3
	1	2.5	3.5	4
	0.75	2.5	3	4
	0.5	3.5	3	3.5
	0.25	3.5	3.5	4
p36a65	10 mg/ml	+	+	1.5
	5	+	+	+
	2.5	+	+	1.5
	1	1	1.5	2
	0.75	1.5	2	2
	0.5	2	2.5	3
	0.25	2	2.5	3
p48a14	10 mg/ml	+	+	+
	5	+	+	+
	2.5	+	+	1.5
	1	1.5	2	2
	0.75	1	1.5	2
	0.25	2	2.5	3
	0.25	2	2.5	3
SG4044	10 mg/ml	5.5	5.5	6

*Concentration is expressed as mg of lyophilized *E. coli* cells added per ml of insect diet.
Samples evaluated in these screenings were overnight cultures of *E. coli* strain SG4044 itself (negative control), SG4044 transformed with prAK (wild-type control), and SG4044 transformed with mutants p36a65, and p48a14. Each set of three scores represents the indicated amount of lyophilized cells mixed in with insect diet and divided evenly between three larvae.
The numerical scores correlate with larval size as shown in Table 1(A). (+) indicates larval death.

of the delta-endotoxin gene as summarized in Table 4. In all cases the bisulfite generated mutants had C→T or G→A transitions nd the formic acid derived clones had A→ or G→T, T→A and C→A transversions due to the bias of reverse transcriptase to insert an adenine opposite the apurinic site.

In order to learn more about these mutations, we performed several rounds of subcloning individual mutations into wild type backgrounds, as well as combining point mutations from different clones. Restriction enzymes having four base-pair recognition sequences occurred frequently throughout the plasmid and could be used to separate multiple mutations in a given clone. We therefore gel isolated an Nsi I/Sst 1 restriction fragment digested it with an enzyme (Xho11 or FnuD11) that was unique to that fragment and which occurred between point mutations present in a given clone as shown in Fig. 4. Nsi 1/Xholl fragments were mixed with Xholl/Sstl fragments from the prAK wild type clone or another mutant clone and ligated into a prAK Nsi l/Sstl vector. Over twenty new clones were generated by this "mix and match" approach and screened in bioassays. The toxicity scores for these clones ranged from similar to wild type to greater than original up-mutants, indicating synergistic and/or additive effects of the multiple point mutations. This data, together with the data described below and presented in Fig. 5, allowed us to identify single point mutations which can give dramatic increases in toxicity to *H. virescens.*

TABLE 4

Mutant		Amino acid position	Nucleotide change	AA change
p26-3		119	GCA → ACA	Ala → Thr
		130	ATG → ATA	Met → Ile
		201	GGC → GAC	Gly → Asp
p48a14	Bisulfite	101	GAA → AAA	Glu → Lys
		116	GAG → AAG	Glu → Lys
		217	CGT → CAT	Arg → His
p48c5		116	GAG → AAG	Glu → Lys
		187	GCG → ACG	Ala → Thr
p36a65		122	ACT → ATT	Thr → Ile
		125	GCA → GTA	Ala → Val
p95a76		123	AAT → TAT	Asn → Tyr
p95a86		188	ACT → TCT	Thr → Ser
p98c1		188	ACT → TCT	Thr → Ser
p99c62	Formic acid	4	AAT → TAT	Asn → Tyr
		105	AAT → TAT	Asn → Tyr
		204	ACA → ACT	Thr → Thr
p107c22		94	AAC → AAA	Asn → Lys
		194	AAT → AAA	Asn → Lys
107c25		–11	TTG → TAG	
		184	TTT → AAT	Phe → Ile
114a30		–15	TAT → TAA	
		95	CAA → AAA	Gln → Lys

This table summarizes the up-mutants recovered directly as a result of the mutation experiments. The amino acid position is given relative to Met.

In a related set of experiments we discovered that our truncated wild type *B.t.* subsp. *kurstaki* DET was less active (as much as ten fold) than a full length gene (also of the crylA(b) type) we cloned from *B.t.* subsp. *wuhanensis*. We were interested in screening some of our mutants in the background of this clone to see if the increased activity could be transferred. Eleven "full-length" mutant DET genes were cloned for expression in *E. coli* and the results from bioassays with these toxins are shown in Fig. 5.

The amount of toxin in each fermentation was calculated by SDS PAGE and gel scanning densitometry of serial dilutions of lyophilized *E. coli* cells. Five concentrations of toxin were used with ten 2nd instar *H. virescens* larvae per concentration to determine LC 50 values. The wild type strain pBT301 was normalized to 100 and the mutant strains were assigned values indicating their relative potency (i.e. a mutant clone with an LC 50 half that of wild type would be assigned a potency of 200, etc.). As can be seen from these data, the activity of the 130 kd protoxin from *B.t.* subsp. *wuhanensis* could be enhanced 3 to 5 fold by subcloning selected point mutations from prAK into the coding sequence. Furthermore, several truncated mutant toxins were more active than the *B.t.* subsp. *wuhanensis* full length gene (data not shown).

DISCUSSION

By cloning *B.t.* delta-endotoxin genes for expression in *E. coli*, we were able to apply protein engineering techniques to obtain mutant toxins with increased activity towards *Heliothis virescens* larvae. Results from mutagenizing the N-terminal portion of the DET gene defined by Bam HI and Xba I restriction enzyme sites shown in Fig. 1 gave 11 mutant clones with enhanced toxicity to *H.*

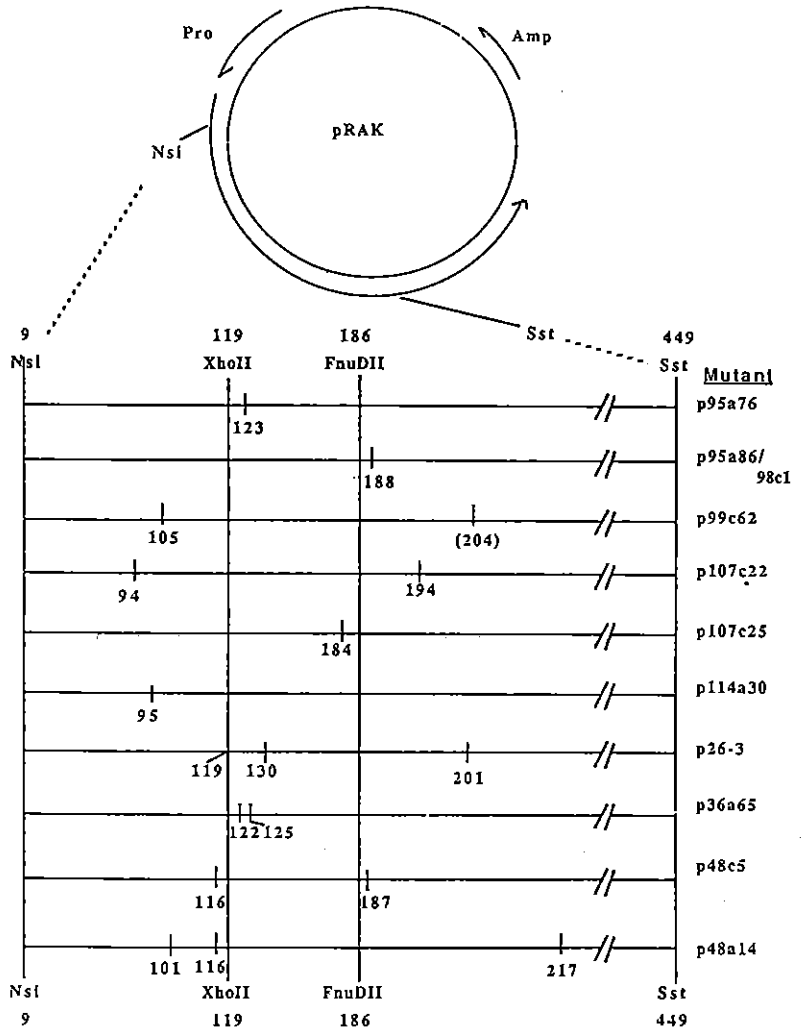

Fig. 4. Location of the amino acid substitutions present in the up-mutant clones relative to restriction enzyme sites shown. Numbers along the horizontal bars refer to amino acid numbers relative to Met as +1 in each mutant listed on the right. The positions of the restriction enzyme sites are also given as amino acid numbers for ease of comparison. As can be seen from this diagram, XhoII and FnuDII are useful for separating and combining multiple point mutations from each clone following the three-fragment approach described in the methods section.

virescens. These up-mutant toxins consistently could be used at five-fold less concentration than the parent clone to get the same degree of feeding inhibition in preliminary screening assays.

Selected mutant genes (derived from the original mutagenesis reactions and "mix and match" ligations described in the text) were subcloned into a gene coding for a 130 kd protoxin from *B.t.* subsp. *wuhanensis*. An enhancement of 3 to 5 fold in toxicity to *H. virescens* was observed for five of these clones. Because the genes isolated from *B.t.* subsp. *kurstaki* and *wuhanensis* are highly

Strain	* Relative Potency
BT210	119
BT301	100
BTA	297
BT66	349
BT36a65	261
BTP	161
BTS	412
BT67	171
BT106	105
BT26-3	489
BTC	319
BT98c1	497
BT114a30	103
CAG629	0

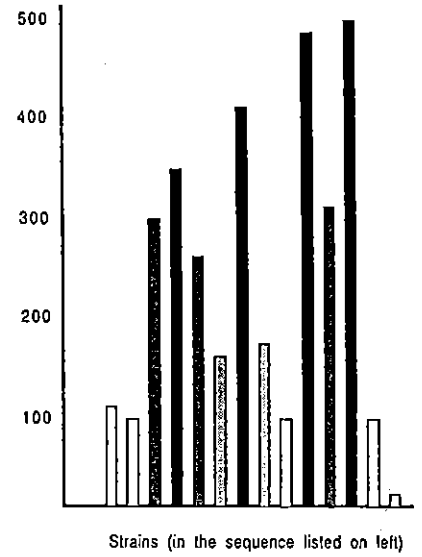

Fig. 5. Relative potency of the full length mutant *B.t.w.* toxin clones. The value for the wild type *B.t.w.* clone pBT210 was normalized to 100. Relative potencies were extrapolated from LC50 values for each strain as described in the text. Potency values are shown graphically in the same sequence as they are listed in the chart on the left.

The amino acid substitutions present in each of the full length clones are as follows *BTA* 101 Glu->Lys, 116 Glu->Lys; *BT66* 122 Thr->Ile, 125 Ala->Val, 201 Glyc->Asp; *BT36a65* 122 Thr->Ile, 125 Ala->Val; *BTP* 119 Ala->Thr, 122 Thr->Ile, 125 Ala->Val *BTS* 119 Ala->Thr, 188 Thr->Ser; *BT67* 119 Ala->Thr, 130 Met->Ile, 188 Thr->Ser; *BT106* 119 ala->Thr, 130 Met->Ile; *BT26-3* 119 Ala->Thr, 130 Met->Ile, 201 Gly->Asp; *BTC* 116 Glu->Lys; *BT98c1* 188 Thr->Ser; *BT114a30* 96 Gln->Lys.

*Relative potencies were extrapolated from LC50 values for each strain as described in the text.

**pBT210 is a wild type full length clone of the *B.t.* subsp. *wuhanensis* toxin gene expressed at higher levels behind a stronger promoter than in pbT301. It was included as a control for variability in the bioassay and the SDS PAGE quantification of toxin. In theory its value should be 100.

homolgous (differences lie outside of the toxic boundary defined by amino acid 608) this increased activity was not surprising. The question of why all of the "up-mutants" generated in prAK did not increase the baseline toxicity of the full length *B.t.* subsp. *wuhanensis* gene is an interesting one. One possible explanation is that there are differences in the folding of toxins when they are expressed in truncated vs. full length forms. Experiments to address this question are underway.

The results presented in Fig. 5, as well as toxicity data from truncated "mix and match" clones, enabled us to highlight codons for site directed mutagenesis experiments. These experiments led to the conclusion that certain positions within this highly conserved segment of the DET gene were tolerant to a number of different substitutions (manuscript in preparation).

Future research plans are to determine whether any of our mutant toxins have a broader spectrum of activity, to mutate other portions of the DET coding sequence (variable and/or conserved), and to express our current up-mutants in other hosts.

Additionally, because the mutations described in this paper occur in a conserved region of the DET gene, it is envisioned that they may be instrumental in raising the baseline toxicity of a number of other DET genes when subcloned into the appropriate positions.

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