12

Biochemical Genetics of the Bacterial Insect-Control Agent *Bacillus thuringiensis:* Basic Principles and Prospects for Genetic Engineering

DONALD H. DEAN

Molecular, Cellular and Developmental Biology Program, The Ohio State University, Columbus, Ohio 43210, USA

Introduction

A primary goal of agricultural genetic engineering concerns the control of insects. We are approaching a point where insect control is threatened by the increasing resistance of insects to chemical pesticides. At the same time the public is reacting to the use of new chemical pesticides for fear of toxicity or carcinogenicity. It is with these concerns that scientists are seeking new microbial pesticides or improvement of existing insecticidal bacteria through fermentation and genetic engineering. Indeed, some of the microbial insecticides are particularly suitable for molecular genetic engineering because of the copious toxin which can be coded by a single gene. Although insect viruses (*see* Carter (1984) in volume 1 of this Series) and a variety of bacteria have received considerable attention as biological pest-control agents, *Bacillus thuringiensis* appears to have the greatest potential as an effective agent, *vis-à-vis* genetic engineering.

The molecular biologist and geneticist can contribute to this effort by providing answers to basic questions about the biogenesis of the insect toxins, such as: when are they made and how are they regulated; what gene encodes the toxin, and what molecular mechanisms result in the apparent copious production of the toxin? Answers to these questions will assist not only the genetic engineer in manipulating the toxin gene but also the fermentation microbiologist in adjusting growth conditions to take advantage of the timing of toxin biogenesis. This review will address these questions by application of information gleaned from studies on sporulation of *B. thuringiensis*, one of the most-studied microbial insect-control agents, and *B. subtilis*, the best-

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characterized *Bacillus* with respect to sporulation physiology and genetics (*see also* Chapter 5).

At present, the use of recombinant DNA techniques is most applicable if the genetic system to be manipulated is coded for by a single gene. Manipulation is even easier if the gene product is made in abundant amounts so that it can easily be assayed. The economic requirement is that the gene product should be of commercial importance. For *B. thuringiensis*, all of these criteria are met. The study of genetic engineering of *B. thuringiensis* is, indeed, a model for the genetic engineering of other systems such as overproduction of proteins, mechanisms of regulation of secondary products, deposition of proteins into stable crystals and activation of toxins by proteolytic cleavage.

General properties of the *B. thuringiensis* group of bacteria

Bacillus thuringiensis may be defined as a Gram-positive spore-forming group of bacteria, which forms a parasporal crystal and is usually pathogenic to insects, particularly Lepidoptera (moths and butterflies). The crystal is estimated to comprise about 30% of the cellular protein. As it is currently classified, *B. thuringiensis* is a complex with over 20 subspecies (*Table 1*). It is

Table 1. Classification of *Bacillus thuringiensis*

H-serotype	Variety name	References
1	*thuringiensis*	Berliner (1911)
2	*finitimus*	Heimpel and Angus (1958)
3a	*alesti*	Toumanoff and Vago (1951)
3a, 3b	*kurstaki*	Kurstak (1964)
4a, 4b	*sotto*	Ishiwata (1901)
4a, 4b	*dendrolimus*	Talalaev (1956)
4a, 4c	*kenyae*	Norris and Burges (1963)
5a, 5b	*galleriae*	Isakova (1958)
5a, 5c	*canadensis*	de Barjac and Bonnefoi (1972)
6	*subtoxicus*	Heimpel and Angus (1958)
6	*entomocidus*	Steinhaus (1951)
7	*aizawai*	Bonnefoi and de Barjac (1963)
8a, 8b	*morrisoni*	Norris (1964)
8a, 8c	*ostriniae*	Ren *et al.*, (1975)
9	*tolworthi*	Norris (1964)
10	*darmstadiensis*	Kreig, de Barjac and Bonnefoi (1968)
11a, 11b	*toumanoffi*	Kreig (1969)
11a, 11c	*kyushuensis*	Ohba and Aizawa (1979)
12	*thompsoni*	de Barjac and Thompson (1970)
13	*pakistani*	de Barjac *et al.* (1977)
14	*israelensis*	Goldberg and Margalit (1977)
15	*indiana*	Delucca, Simonson and Larson (1979)
16	*dakota*	Delucca, Simonson and Larson (1979)
17	*tohokuensis*	Iizuka *et al.* (1982)
18	*kumaotoensis*	Iizuka *et al.* (1982)
19	*tochigiensis*	Ikzuka *et al.* (1982)
20	*yunnanensis*	Yu, Z. Huazhong Agri. Coll. China (1984)
21	*colmeri*	Delucca, Palmgren and de Barjac (1984)
—*	*wuhanensis*	Hubei Institute, China (1976)
—*	*fowleri*	Kreig and Langenbruch (1981)
—†	*galechae*	Heierson, Landen and Boman (1983)

* No flagella — cannot be serotyped.
† Recently classified as H-serotype 1.

very closely related to *B. cereus* and except for the properties of crystal formation and entomocidal activity, cannot be distinguished from *B. cereus* by routine bacteriological tests (de Barjac, 1981). DNA–DNA hybridization among *B. thuringiensis, B. cereus* and *B. anthracis* has shown an 80–100% homology (Kaneko, Nozaki and Aizawa, 1978; Seki *et al.*, 1978), certainly justifying the taxonomic union of these three bacteria. The presence of toxin genes on plasmids (hence unstable) in *B. thuringiensis* (*see below*) and *B. anthracis* (Mikesell *et al.*, 1983) makes a single phenotypic characteristic such as toxicity a poor reason for separate classification. None the less, for practical and economic reasons *B. thuringiensis* remains a separate species. The most satisfactory means of distinguishing *B. thuringiensis* from *B. cereus*, or for distinguishing between the individual varieties of *B. thuringiensis* is by serology (de Barjac, 1981). *Table 1* lists the known varieties and their serotypes.

The varieties of *B. thuringiensis* differ considerably in their toxicity and insect host range. For example, the varieties *finitimus* and *fowleri* produce crystals but are not entomocidal, even to the insects from which they were isolated (Kreig and Langenbruch, 1981). The recently isolated varieties *dakota, indiana, tohokuensis, kumanotoensis, tochigiensis* and *colmeri* have relatively weak insecticidal activity (A.J. Delucca, personal communication). An extensive bibliographic review identifies varieties (from H-1 to H-14) which are particularly toxic to certain insect pests but weakly active to others (Krieg and Langenbruch, 1981). Other varieties such as *kyushuensis, darmstadiensis* and *israelensis*, are toxic to mosquito larvae, but are not pathogenic to the Lepidoptera. In all, over 130 species of Lepidoptera, Diptera and Coleoptera are controlled by *B. thuringiensis* varieties, including the most serious crop pests (e.g. cotton bollworm (*Heliothis zea*), cabbageworm (*Pieris rapae*), European corn borer (*Ostrinia nubilalis*)) and vectors of the most devastating human diseases (malaria, schistosomiasis, filariasis and yellow fever). As a group, these bacteria offer a variety of insecticidal proteins which vary in specificity and potency.

Not only do the biological activities of *B. thuringiensis* vary according to their insect host, but considerable variability in toxicity has been reported within the serotypes (Dulmage and Co-operators, 1981). The original commercial variety of *B. thuringiensis* was a strain of the variety *thuringiensis* which was rather weak and thus unfortunately generated considerable antipathy to *B. thuringiensis* as a control agent. Varieties *thuringiensis* and *galleriae* are still widely used in Europe. The current industrial standard in the United States is strain HD-1 variety *kurstaki* (Dulmage, 1970). Dulmage and Co-operators (1981) have identified other *kurstaki* isolates which have even greater toxicity than HD-1 against the particular test organisms *Heliothis virescens* (tobacco budworm) and *Trichoplusia ni* (cabbage looper). *Table 2* shows examples of this variability in toxicity and raises the question of what biochemical differences might result in such wide variation. This comparison also demonstrates that some aspects of the potency of this entomocidal bacterium may be improved. Our understanding of how these proteins, and the genes which encode them, dictate such entomocidal specificity should lead to improved biological pest control.

Table 2. Variable potency of strains of the same serotype

Strain	Potency *(IU/mg)	Percentage increase in potency over HD–1	H Serotype	Crystal type
HD–1	15 400	—	3a3b	k–1
HD–244	70 600	360	3a3b	k–73
HD–191	61 000	296	3a3b	k–73
HD–263	54 600	255	3a3b	k–1
HD–164	35 000	127	3a3b	k–1
HD–243	34 000	120	3a3b	k–1

* The international unit (IU) is defined as:

$$\frac{LC_{50}\ \text{Standard}}{LC_{50}\ \text{Test Sample}} \times \text{Potency of Standard, IU/mg} = \text{Potency of test sample, IU/mg.}$$

Biochemical properties of the delta-endotoxins

The biochemistry and mode of action of the *B. thuringiensis* crystal protein (or delta-endotoxin) has been the subject of earlier reviews (Fast, 1981; Huber and Lüthy, 1981; Lüthy and Ebersold, 1981). The general properties of the crystal protein are described here to provide a focus for further discussion and speculation on the nature of the gene which encodes it and how it is regulated.

In most of the known cases the crystal is composed of a single polypeptide chain which is called delta-endotoxin. Comparative electrophoresis demonstrates that crystal proteins from various serotypes (Huber *et al.*, 1981) have a similar molecular weight of about 130 000 daltons. It was first shown in the serotype H-3a/b (*kurstaki*) (Bulla, Kramer and Davidson, 1977) and later in several other serotypes (H-1, H-2, H-3, H-4, H-6, H-7 and H-14; Zalunin, Chestukhina and Stepanov, 1979; Huber *et al.*, 1981) that a predominant polypeptide exists upon solubilization of the crystal. Exceptions to this are found in the case of *B. thuringiensis* var. *thuringiensis berliner* 1715 (H-1) (Lüthy *et al.*, 1983) and H-5 (Chestukhina *et al.*, 1980) each of which have two proteins associated with the crystal. *B. thuringiensis* var. *israelensis* (serotype H-14) apparently has three associated proteins weighing 25 000, 65 000 and 130 000 daltons (Huber *et al.*, 1981; Thomas and Ellar, 1983a,b). For other varieties, the crystal may contain other proteins but delta-endotoxin is the major component and is present as a dimer in the crystal (Bulla, Kramer and Davidson, 1977; Fast, 1981; Huber *et al.*, 1981). It is important to realize that even gradient 'purified' crystals are not free of contaminating cellular proteins, such as spore coat proteins and proteases. It is therefore possible for crystal preparations to contain antigens other than the toxins. This should be kept in mind when evaluating the data.

An outstanding property of delta-endotoxin is that it is not toxic as a crystal subunit but must be solubilized and proteolytically cleaved to a toxic peptide. For most serotypes the crystal protein is a 130 000 dalton protein, whereas the toxic peptide is half that size (65 000 daltons). This indicates that only a portion of the delta-endotoxin gene codes for the active peptide. Such activation is apparently not the case for H-14 because reduction of its crystal protein(s) to an active peptide has not been shown to be necessary. On the contrary, it has been

shown to be active in the soluble form without proteolytic cleavage, although solubilization reduces its activity by one hundredfold (Thomas and Ellar, 1983a,b).

The observation that the delta-endotoxin is a single polypeptide chain allows the prediction that it is encoded by a single gene (Martin and Dean, 1981). This prediction may hold at least in the varieties which have one protein per crystal. This hypothesis is supported by plasmid studies described below and is almost axiomatic from the protein work. The assumption that the delta-endotoxin gene is a single gene suggests that its manipulation, either by classic mutagenesis or recombinant DNA techniques, will be much easier than it would be if several genes were involved.

Serological analyses of crystal proteins (Krywienczyk, Dulmage and Fast, 1978) have revealed that, in most cases, the antigenicity of crystal proteins permits assignment of strains to their proper H-serotype. It may be assumed that differences in crystal antigenicity bear some correlation to the host (insect) specificity of the toxin. Such analyses were extended to crystal proteins isolated from the same H-serotype which do not share antigenicity. Normally, the variety *kurstaki* (flagellar serotype H-3a/b) has a different crystal protein serotype (k-73) (Krywienczyk, Dulmage and Fast, 1978). More recent work (Krywienczyk *et al.*, 1981; Yamamoto, Garcia and Dulmage, 1983) has revealed that a significant number of isolates (10/49) of the serotype H-1 (variety *thuringiensis*) have a crystal protein serology (k-1) usually found in H-3a/b (variety *kurstaki*). Seven of the isolates had crystal serologies corresponding to a mixture of k-1 and *thuringiensis* crystal antigens. These results may indicate that there is a fairly high exchange of delta-endotoxin genes between strains of different flagellar type. The above finding, that a mixture of crystal serotypes may be produced by the same cells, indicates that the same cell may harbour both delta-endotoxin genes. The production of more than one toxin in the same cell has indeed been accomplished artificially for the Lepidoptera toxin of var. *thuringiensis* and the mosquito toxin of var. *israelensis* (Klier, Bourgouin and Rapoport, 1983) and for toxins from two different varieties (González, Brown and Carlton, 1982).

Monoclonal antibodies directed against proteolytically cleaved delta-endotoxin from *B. thuringiensis* var. *thuringiensis* have been described recently (Huber-Lukac, Lüthy and Braun, 1983). Several antibodies were obtained which reacted with at least four different antigenic sites on the delta-endotoxin. Three types of interactions between the antigens and antibodies could be observed: (1) some antibodies did not block toxicity (feeding inhibition); (2) one antibody partially inhibited toxicity, and (3) one antibody completely neutralized toxicity. Huber-Lukac, Lüthy and Braun (1983) speculate that the inactivating antibodies may either bind to the active site (assuming that there is only one) or bind to another part of the protein and change its conformation. It is noteworthy that the antibody in class (2) not only partially neutralized toxicity but also blocked the binding of certain antibodies in class (1). Class (1) antibodies do not, however, affect the action of the class (2) antibody. Class (3) antibodies do not affect the binding of either of the other classes. It is possible that class (2) antibody alters the conformation of the antigen affecting both

binding of antibodies and toxicity. The idea that altered conformation is a part of the activation process is also presented by Huber-Lukac, Lüthy and Braun (1983). Proteolytic cleavage may alter the conformation or otherwise unmask the active site of delta-endotoxin.

The crystal proteins from most serotypes share a number of common features. They are active against Lepidoptera, they have similar molecular weights (*c.* 130 000) and they are activated by proteolytic cleavage. The genes which encode them even share DNA homology (*see below*). From this we may conclude that the delta-endotoxin genes evolved from a common ancestor.

The toxins of serotype H-14 (var. *israelensis*), active against Diptera, however, differ radically in entomocidal behaviour, biochemical and serological properties, DNA homology and physical appearance of the crystal in electron micrographs. One must conclude that it is genetically non-homologous to the other *B. thuringiensis* serotypes. If the term delta-endotoxin, which has been applied to both of these classes of diverse toxins, is a valid terminology, then we may say that there are two types of delta-endotoxins. Type I is active against Lepidoptera and Type II is active against Diptera. However, the vast differences between these types support the proposal that the toxic crystal protein(s) of the variety *israelensis* should not be named delta-endotoxin. A summary of properties of delta-endotoxins is given in *Table 3*.

Other toxins are present in *B. thuringiensis*. A mosquito-toxic protein of molecular weight 65 000 has been identified in certain strains of the variety *kurstaki* (Yamamoto and McLaughlin, 1981). This 'mosquito factor' is about four orders of magnitude less toxic than that of variety *israelensis*, and has been located in the cell as a 'bump' or cuboidal structure associated with the larger delta-endotoxin crystal (Yamamoto and Iizuka, 1983).

Table 3. Properties of endotoxins from different serotypes of *B. thuringiensis*

Property	Description	Reference
1. A single polypeptide	Approx. 130 000 daltons in most serotypes	Bulla, Kramer and Davidson (1977); Huber *et al.* (1981)
Exception	25 000, 65 000 and 130 000 daltons in serotype 14	Huber *et al.* (1981); Clark and Dean (unpublished results)
2. Product of a single gene	Hypothesis	Martin and Dean (1981)
3. There are a variety of delta-endotoxin proteins	a. spectrum of action from flagellar serotypes	Dulmage and Co-operators (1981)
	b. Crystal serotyping	Krywienczyk *et al.* (1978)
	c. Difference within the same flagellar serotype	Krywienczyk *et al.* (1981)
4. Small region of delta-endotoxin is active toxin	a. Tryptic digest releases a smaller toxic peptide	Fast (1981); Huber and Luthy (1931)
	b. Active region is on *N*-terminal region of the protein	Chestukhina *et al.* (1982)

Another toxin that has been extensively studied is the beta-exotoxin 'fly toxin' or 'thuringiensin' (Lecadet and de Barjac, 1981; Šebesta, *et al.*, 1981). This heat-stable toxin has moderate toxicity to house flies and has been employed in the Soviet Union. It has been identified as an ATP analogue and is teratogenic, mutagenic and mitogenic (Lecadet and de Barjac, 1981). Its use in the United States is prohibited by the Environmental Protection Agency. Most varieties, including the commercial varieties, do not produce beta-exotoxin.

Other toxins have been reported in earlier literature including alpha-endotoxin, gamma-exotoxin, 'labile'-exotoxin, 'water-soluble'-toxin and 'mouse factor' (for summary *see* Faust, 1975). However, these toxins are produced in only a few strains and have been little studied. At present genetic engineering approaches are not applicable to these toxins.

Regulation of delta-endotoxin biogenesis

The life cycle of *B. thuringiensis* is more complex than that of non-sporulating bacteria. When vegetative cells are growing in a rich medium, the cells do not form spores or crystals. However, both sporulation and crystal formation are induced at the end of logarithmic growth when the cells are derepressed by the exhaustion of carbon and nitrogen sources in the medium. The first appearance of crystal antigen is at about two hours after the beginning of stationary phase but deposition into a crystal does not occur until eight hours (Bechtel and Bulla, 1976). Delta-endotoxin continues to be deposited for several hours, developing a predominant crystal. It is clear from mutually exclusive mutants (spore$^-$, crystal$^+$; and spore$^+$, crystal$^-$) that the two processes of sporulation and crystal formation are not dependent upon one another. We may infer, however, that they are both developmentally regulated and perhaps by the same molecular mechanisms.

The genetic regulation of delta-endotoxin is central to its genetic manipulation. Therefore, let us consider the known regulatory mechanisms for sporulation in *B. subtilis* (*see* Chapter 5). The guiding model has been the transcriptional regulation observed in bacteriophage infection. In this model, bacteriophage directs the host core RNA polymerase to the phage genes by synthesizing a special RNA polymerase-binding protein called a sigma factor.

The expression of a gene is regulated by the interaction of RNA polymerase with the initiation sequences of the gene. These sequences are called 'promoters' and actually consist of two regions on the DNA, at -35 and -10 bases 'upstream' from the starting point of the messenger RNA on the template (Losick and Pero, 1981). Specific sigma factors are responsible for directing RNA polymerase to specific promoters. Sigma factors are identified by their molecular weight. Thus, sigma 55 is the sigma factor of 55 000 daltons. During sporulation there is a 'cascade' of different sigma factors which sequentially replace the 'vegetative sigma' (sigma 55) and one another (Losick and Pero, 1981). The promoter regions are therefore of key importance in transcription developmental regulation. *Table 4* lists promoters (only the coding strand is given) of a number of vegetative *B. subtilis* genes such as '*veg*' (an unknown vegetative gene), penicillinase and '*tms*' (a temperature-sensitive gene). These

Table 4. Transcriptional regulatory sequence of cloned *Bacillus subtilis* genes

Gene	DNA Sequence of Promoter		References
	−35 region	−10 region	
E. coli	...TTGACA...†	...TATAAT...†	Rosenberg and Court (1979)
Veg. gene	...TTGACA...	...TACAAT...	Losick and Pero (1981)
penicillinase	...TTGCAT...	..TAATACT...	Kroyer and Chang (1981)
Alpha-amylase	...TTGTTATTA.	...TAAAAT...	Palva *et al.* (1981)
		or...TATAAT...	
tms	...TTGAAA...	...TATATT...	Losick and Pero (1981)
Erm	...TTCATA...	...TATAAT...	Horinouchi and Weisblum (1982a)
Cam	...TTGATT...	...TAAATT...	Horinouchi and Weisblum (1982b)
spoOH	...TTGACG...	...TATAAC...	Losick and Pero (1981)
spoVC	...AGGTTTAAA..	...GGTATTGTTTG...	Moran *et al.* (1981)
spoVG	...TATTTTTTCAAAAAATATTTTAAAA....		
		...CTAATTGATA...	
			Moran *et al.* (1981)

† Underlined bases are particularly 'invariant'

promoters are similar to each other, differing only by a few bases. The *E. coli* 'consensus' promoter, which is given as comparison, is also similar to vegetative *Bacillus* promoters. All of the *Bacillus* vegetative genes are transcribed *in vitro* by the sigma 55 which is the predominant RNA polymerase-binding protein isolated from vegetative cells. Analysis of developmentally regulated genes shows that 'early' sporulation genes such as *spoOH*, which is expressed before the end of logarithmic growth, have a similar promoter and are initiated *in vitro* by the same sigma factor as vegetative genes. Late sporulation genes (such as *spoVG* and *spoVC*) have quite different promoters and are initiated by two different sigma factors. Sigma 29 is found in the late logarithmic growth stage and sigma 37 is found in the late stationary phase (Losick and Pero, 1981).

By analogy, delta-endotoxin transcription may be initiated by a sigma factor which becomes active in stage II when delta-endotoxin is expressed. Unfortunately, no stage II sporulation genes or stage II sigma factors have been reported yet. Other regulatory mechanisms are known, as in the *E. coli lac* repressor but these are more likely to apply to metabolic processes than to developmental ones. Inducible or positive regulatory mechanisms are certainly not out of the question but, at the present time, transcriptional regulation by sigma factor exchange would be favoured as the leading model for regulation of delta-endotoxin biosynthesis. The DNA sequence of crystal gene promoters and their relationship to this model will be described below.

Expression may also be regulated at the translational step, either at the initiation of translation (binding of the *Bacillus* ribosome to the mRNA), or during the elongation process. It has been observed from *in vitro* studies that *B. subtilis* ribosomes recognize and bind less tightly to *E. coli* mRNAs than do *E. coli* ribosomes. Furthermore, *E. coli* ribosomes require initiation factor IF3 which has relatively less effect on Gram-positive ribosomes (McLaughlin, Murray and Rabinowitz, 1981a). It has been proposed (McLaughlin, Murray and Rabinowitz, 1981b) that ribosomes of *B. subtilis* and other Gram-positive

organisms require a more extensive ribosomal binding (Shine–Dalgarno) sequence (Shine and Dalgarno, 1975). Inspection of a number of sequenced genes from Gram-positive organisms (*Table 5*) shows that the vegetative genes tend to have more guanine-rich 16S RNA complementing sites than is usual for *E. coli* (*see trp* sequences, *Table 5*). Four bacteriophage sequences which follow this trend are also cited by McLaughlin, Murray and Rabinowitz (1981b). The 16S ribosomal RNAs which bind to the translational start site are:

E. coli 16S RNA 3′OH AUUCCUCCACUAG....5′ (Shine–Dalgarno sequence)

B. subtilis 16S RNA 3′OH UCUUUCCUCCACUAG....5′

The *B. subtilis* 16S rRNA sequence is from C. Woese and H. Noller cited in other published works (Kroyer and Chang, 1981; McLaughlin, Murray and Rabinowitz, 1981b). It is also observed (*Table 5*) that the two sporulation genes

Table 5. Ribosomal binding sites on cloned DNA which are expressed in *Bacillus subtilis*; comparison with *E. coli* sites

Gene	Nucleotide sequence of ribosomal binding sites	References
*amy**	...GAGAGGGAGGAAAC[ATG]...	Palva *et al.* (1981)
*pen***	...GAGGGAGACGATTTTG[ATG]...	Gray and Chang (1981)
pen†	...TATCGGAGGGTTTATT[TTG]...	McLaughlin *et al.* (1981a)
Erm†	...ATAAGGAGGAAAAAAT[ATG]...	Horinouchi and Weisblum (1982a)
Cam†	...TTAGGAGGCATATCAA[ATG]...	Horinouchi and Weisblum (1982b)
spoVG‡	...AAAGGTGGTGAACTACT[GTG]...	Moran *et al.* (1982)
trpL§	....AAAGGGTATCGACA[ATG]...	Yanofski *et al.* (1981)
trpE§	....ATTAGAGAATAACA[ATG]...	Yanofski *et al.* (1981)
trpD§	...CAGGAGCTTTCTG[ATG]...	Yanofski *et al.* (1981)

* *B. amyloliquifaciens;* ** *B. licheniformis;* † *S. aureus;* ‡ *B. subtilis;* § *E. coli*

which have had their ribosomal binding sites sequenced (Moran *et al.*, 1981) conform to the model described by McLaughlin, Murray and Rabinowitz, 1981b). The relationship of this model to the sequence of delta-endotoxin is discussed below.

Nature of the delta-endotoxin gene

Neither the nature of the delta-endotoxin itself nor its biogenesis suggests a location for its gene. The occurrence of crystal-deficient cells at high frequency, segregating from a cell line of *B. thuringiensis*, has been observed by several researchers (Toumanoff, 1955; Vaňková, 1957; Fitz-James and Young, 1959). The fact that these crystal-deficient cells did not revert to crystal formers gave strong suspicion that the delta-endotoxin gene is on a plasmid (for reviews *see* Martin and Dean, 1981; Clark *et al.*, 1984). The first study suggesting that

Table 6. Plasmids associated with crystal-protein production

Serotype	Strain	Plasmid (molecular weight)	Method of detection	References
thuringiensis	1(HD–2)	75×10^6	Curing	González, Dulmage and Carlton (1981)
	'F'	*c.*8×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
		and 50×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
	(HD–120)	*c.* 40, 50 and 100×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
	(HD–290	*c.* 45, 52 and 100×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
	berliner 1715	42×10^6	Hybridization*	Klier *et al.* (1982)
alesti	3a(HD–4)	105×10^6	Curing	González, Dulmage and Carlton (1981)
		c. 100×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
kurstaki	3a/b(HD–1)	105×10^6	Mating transfer	González and Carlton (1982)
		c. 50×10^6 and 100×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
	(HD–1 'Dipel')	*c.* 150×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
	3a/b,(HD–73)	50×10^6	Curing	González and Carlton (1982)
		50×10^6	Mating transfer	González and Carlton (1982)
		c. 50×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
	(HD–244)	*c.* 50×10^6 and 100×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)

plasmids have a role in coding for the biogenesis of the crystal protein was by Zakharyan *et al.* (1976). This group later isolated three plasmids from *B. thuringiensis* var. *caucasisus* (Zakharyan *et al.*, 1979). Numerous authors have isolated plasmids from a number of serotypes of *B. thuringiensis* and as I have recently reviewed the general subject of plasmids in *B. thuringiensis* (Martin and Dean, 1981; Clark *et al.*, 1984) I will not do so here. Suffice it to say that plasmids have been found in all serotypes.

The strongest direct evidence that the delta-endotoxin gene resides on plasmids in several serotypes has been reported in the last few years. The Eckhardt (1978) plasmid visualization technique has been used to analyse numerous crystal-deficient strains, which have been treated to cause loss of plasmids ('cured'). In this way, several plasmids have been associated with crystal-protein production (*Table 6*). The use of this technique is extremely important because, for most serotypes, the delta-endotoxin-bearing plasmid has a very high molecular weight and is not seen when other methods are used (González and Carlton, 1980).

Table 6 *(continued)*

Serotype	Strain	Plasmid (molecular weight)	Method of detection	References
kurstaki (contd)	(HD–252)	*c* 100×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
sotto	4a/b	40×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
galleriae	5a/b(HD–8)	130×10^6	Curing	González, Dulmage and Carlton (1981)
	(HD–8)	*c*. 150×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
morrisoni	8a/b	*c*. $150+ \times 10^6$	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
tolworthi	9	*c*. 45×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
darmstadiensis	10	*c*. 50×10^6	Hybridization*	Kronstad, Schnepf and Whiteley (1983)
israelensis	14(HD–567, HD–500)	72×10^6	Curing, mating transfer	González and Carlton (1984)
wuhanensis	—	Chromosome	Hybridization*	Kronstad, Schnepf and Whiteley (1983)

* Hybridized to the cloned crystal-protein gene.

Note: apparently erroneous values for the toxic plasmids have been published. For example: 3a/b k–1 (HD–1); 29×10^6, by curing (González, Dulmage and Carlton, 1981); 3a/b('Dipel'), 3×10^6 and 47×10^6, hybridization to cloned DNA (Schnepf and Whiteley, 1981)

A second important technique for correlating plasmids with toxin production is the discovery of a conjugation-like plasmid transfer 'mating' system which occurs between *B. thuringiensis* cells (González and Carlton, 1982; González, Brown and Carlton, 1982). Through the use of this method, several plasmids have been associated with crystal-protein production (*Table 6*) (González, Brown and Carlton, 1982). The toxin-coding plasmid of the variety *israelensis* (H-14) has recently been associated with a high-molecular-weight plasmid (Kamdar and Jayaraman, 1983) identified as 72×10^6 molecular weight by examination of cured strains (Clark and Dean, 1983; Ward and Ellar, 1983) and by mating (González and Carlton, 1984).

The delta-endotoxin gene from *B. thuringiensis* var. *kurstaki* HD-1 'Dipel' (serotype H-3a/b) has recently been cloned into *E. coli* and demonstrated to be expressed there at a low level (Schnepf and Whiteley, 1981). These workers originally reported hybridization between the cloned gene and two plasmids (Schnepf and Whiteley, 1981) but have more recently found that the coding region of the crystal-protein gene hybridizes to the 150 megadalton plasmid in that strain (Kronstad, Schnepf and Whiteley, 1983).

The coding portion of this gene has been identified by insertional inactivation with the transposon Tn5 (Kronstad, Schnepf and Whiteley, 1983). Fragments from within the coding region were used as a probe to hybridize to plasmids of 14 of the known varieties of *B. thuringiensis*. These studies revealed hybridization to single plasmids of the following eight strains: *sotto, darmstadiensis, toumanoffi, alesti, kurstaki* (HD-73), *galleriae, thuringiensis* F, and *thuringiensis* HD-2. In five strains (*tolworthi, kurstaki* HD-244, *kurstaki* HD-1, *thuringiensis* HD-120, and *thuringiensis* HD-290) hybridization was found to more than one plasmid. The authors concluded that there are multiple genes in these strains. The possibility that the plasmids containing the crystal gene are merely multimers of one another was negated by further fragmentation of total plasmids with a different restriction enzyme. Multimers would produce a limited fragment pattern the same as the monomer and only one or two, at most, of the fragments would hybridize the probe. In fact, three fragments hybridized the probe, suggesting that the fragments came from different crystal genes within the same cell. Not all of the varieties of *B. thuringiensis* hybridize to the aforementioned fragment probes (Kronstad, Schnepf and Whiteley, 1983). Those that did not were varieties *dakota, indiana, israelensis*, and *kyushuensis*. The latter two display insecticidal activity which is very different to that of the *kurstaki* strain used as a probe. The former two varieties, which were isolated from soil samples (Delucca, Simonson and Larson, 1979), are not confirmed insect pathogens.

Two other research groups have also reported cloning of crystal genes. One of these (Held *et al.*, 1982) used the same strain (*kurstaki*-HD-1 'Dipel') as Schnepf and Whiteley (1981) and the other employed *B. thuringiensis* var. *berliner* 1715 (Klier *et al.*, 1982). In both cases the authors conclude that a copy of the crystal gene (albeit non-functional) is present on the chromosome.

In the cloning of HD-1 'Dipel' by Held *et al.* (1982) a 'shotgun' of total (plasmid and chromosomal) DNA was cloned in bacteriophage Charon 4a. The clones were screened using an enzyme-linked immunosorbant assay (ELISA) with antibody against whole crystal. The positive clones thus were making antigen which reacted with antibody against the crystal. Extracts of the ELISA-positive clones were shown to kill *Manduca sexta* (tobacco hornworm). When the cloned DNA, the putative crystal gene, was subcloned into a plasmid, pHV33, and the whole cloned DNA fragment was used as a hybridization probe, it hybridized to the chromosomal DNA of a strain cured of all plasmids (non-toxic and acrystalliferous). In fact, certain subfractions of the original cloned DNA did not hybridize to plasmids, showing that it did not arise from plasmid DNA but implying that it came from the chromosome. This work is in conflict with that of Schnepf and Whiteley (1981) and Kronstad, Schnepf and Whiteley (1983) who cloned the crystal gene from the same strain. Kronstad, Schnepf and Whiteley (1983) demonstrated quite conclusively that the coding region of the crystal gene is not homologous with the chromosome in any of the strains tested (with the possible exception of var. *wuhanensis*). Furthermore, portions of the cloned DNA used by Held *et al.* (1982) hybridized back to a plasmid of 45 kb (30×10^6) whereas the coding portion of the gene studied by Kronstad, Schnepf and Whiteley (1983) hybridized to a plasmid of

150 Md (225 kb). One is left to conclude that Held *et al.* (1982) had cloned another gene from the chromosome which produced a protein which was toxic to the moderately sensitive insect *M. sexta*. As the crystal protein has been reported to be homologous to the spore proteins (Aronson *et al.*, 1982), perhaps the chromosomal sequences are coding for spore coat protein. The cross hybridization of chromosomal DNA to plasmids is best explained by detailed studies in the laboratories of Rapoport, Klier, Lecadet and Dedonder at the Pasteur Institute.

Klier, Lecadet and Rapoport (1978) have studied the strain *B. thuringiensis* var. *thuringiensis berliner* 1715. Stable messenger RNA has been found in this strain (Klier and Lecadet, 1976) which appears to code for the crystal protein (Glatron and Rapoport, 1972). Unlike other strains of *B. thuringiensis*, this strain does not lose its ability to form crystals spontaneously (Lecadet *et al.*, 1981).

The stable mRNA was used as a probe to detect clones of *berliner* 1715 total DNA (plasmid and chromosome) cloned into *E. coli*. The cloned DNA hybridized to plasmids from *berliner* 1715 and *kurstaki* HD-1 but was not expressed in *E. coli*. The genes were expressed as mRNA but not as protein in *B. subtilis*. Using this cloned DNA as a probe, new clones were prepared starting with plasmid DNA from *berliner* 1715. These clones were expressed in both *E. coli* and *B. subtilis* (Klier *et al.*, 1982). The new hybrid plasmid was transferred from *B. subtilis* to *B. thuringiensis* 'Cry⁻B'* (a crystal-lacking strain) (Klier, Bourgouin, and Rapoport, 1983) using a relatively new mating method of González, Brown and Carlton (1982). In the *B. thuringiensis* cellular background, the plasmid gene made copious amounts of crystal which cross-reacted with anti-crystal antisera and was toxic to *Ephestia kuehniella* (European flour moth). The cloned chromosomal gene was also transferred by mating into the 'Cry⁻B' strain but again it was not expressed (Klier *et al.*, 1982).

How may one account for the chromosomal sequences (it is better to avoid the word 'gene') hybridizing with the crystal-protein gene or plasmids which carry it? Hybridization among plasmids and between plasmids and chromosomal DNA shows that some plasmids of high molecular weight from *B. thuringiensis* will hybridize with chromosomal DNA (Lereclus *et al.* 1982), demonstrating that there is some sequence homology. How this homology relates to the crystal protein gene is uncertain.

It is clear that there has been confusion in the location and cloning of the crystal-protein genes. The weight of the evidence based on recombinant DNA cloning and curing supports the conclusion that the functional crystal-protein gene resides on plasmids in most cases (Schnepf and Whiteley, 1981; Klier, Bourgouin and Rapoport, 1983). The chromosomal sequences which have been identified have some DNA homology to the crystal gene sequences or to the sequences very near to it, but the significance of these sequences is not clear at present.

* In a strict sense the 'Cry⁻B' strain (Stahly *et al.*, 1978) is not *B. thuringiensis* because it does not cross-react with H-flagellar antisera of any known *B. thuringiensis* serotype (H. de Barjac and H. Dulmage, personal communication) yet it has flagella (is motile). This finding does not affect the results of Klier, Bourgouin and Rapoport (1983) but does affect the conclusions of Stahly *et al.* (1978), and Aronson *et al.* (1982) based on the 'Cry⁻B' strain.

Transcriptional studies and DNA sequencing have been performed on the crystal-protein gene (Wong, Schnepf and Whiteley, 1983) and on the chromosomal sequences (Klier, Parsot and Rapoport, 1983). The promoter sequences are given in *Table 7*, with sporulation sequences as comparison. *Table 8* shows the 'Shine–Dalgarno' sequences (i.e. sequences used by ribosomes to initiate protein synthesis) and the first sequences of the coding region with their resultant amino acids. It is clear from these tables that there is no homology between the plasmid crystal-protein gene and the chromosomal sequences in their regulatory regions or their early coding regions. Remembering that the early coding region (*N*-terminal region of the delta-endotoxin gene) is the toxic peptide region (Chestukhina *et al.* 1982), we must avoid concluding that the toxin gene is silently hidden in the chromosome.

Comparison between the promoter of the plasmid-encoded crystal-protein gene (Wong, Schnepf and Whiteley, 1983) and the promoter of the *spoVG* sporulation gene (Moran *et al.*, 1981) shows considerable homology (*Table 7*). Like the *spoVG* gene there seems to be two separate transcriptional start sites from the same regulatory area. This may, in part, account for the copious production of crystal protein. The promoters of the crystal-protein gene are very different from those of vegetative genes indicating, as proposed above, that the toxin gene is developmentally regulated at the level of transcription.

Inspection of the translational start signals, or so-called Shine–Dalgarno sequences (*Table 8*), reveals, as with other Gram-positive organisms, a strong binding between the homologous sequence as it appears on the mRNA and the 16S rRNA of the ribosome. Application of the rules of Tinoco *et al.* (1973) (a measure of the strength of binding between mRNA and 16S rRNA) shows a ΔG of -15 kcal/mol which is in the range of other Gram-positive ribosomal binding regions.

Summary and prospects for genetic engineering of *B. thuringiensis* crystal-protein genes

In summary, it is clear that the location of the crystal-protein gene is on plasmids in most of the important strains of *B. thuringiensis*. This statement is based on recombinant DNA cloning (Schnepf and Whiteley, 1981; Klier, Bourgouin and Rapoport, 1983), curing (González, Dulmage and Carlton, 1981) and mating (González, Brown and Carlton, 1982). Sequences on the chromosome will hybridize to the plasmids (Lereclus *et al.*, 1982) at or near the crystal gene (Klier *et al.*, 1982; Held *et al.*, 1982; Lereclus, Menou and Lecadet, 1983), but the function of the chromosomal sequences is not clear at present.

The sequencing of the regulatory region and the first part of the coding portion of the gene (Wong, Schnepf and Whiteley, 1983) has provided useful information about the regulation of the crystal protein. The sequence of the crystal-protein gene promoter reveals strong similarities to the late sporulation promoters. This might provide an explanation for the restriction of the crystal protein synthesis to the stationary phase. That is to say, both crystal protein and sporulation are apparently regulated by transcriptional mechanisms at the level of sigma factors and RNA polymerase.

Table 7. Comparison of the transcriptional regulatory sequences between the crystal-protein gene, chromosomal sequences and the Stage V spore genes

	−75	−70	−65	−60	−55	−50	−45	−40	−35	−30	−25	−20	−15	−10	−5	−1
A.	----------								TTGACA	----------				TATAAT	----------	
B.	[TCAAAAATTGATATTTAGTAAAATTA] GTTGCACTTTGTGCATTTTTCATAAGATGAGTCATATGTTTTAAATT															
C.	----------								GCCTACGCTTCTACTACAAATTTACAATTCCATACAT							
D.	[TATTTTTTCAAAAAATATTTTAAAAA] CGAGCAGGATTTCAGAAAAAATCGTGGAATTGATACACTAATGCTTTTA															

A: Consensus sequence of *B. subtilis* vegetative promoter; B: Sequence of crystal-protein gene; C: Sequence of chromosomal sequences; D: Sequence of *spoVG* gene.

Table 8. Comparison of the translational regulatory sequences of the crystal-protein gene and chromosomal sequences

Crystal-protein gene (Wong, Schnepf and Whiteley, 1983):

```
                  U                C   G    16S  rRNA
HO—UCU  U—CC UC CA . U–A
        : : :  :  : : : : : :  :  :
CTTAATAAAAGAGATGGAGGTAACTT ATG GAT AAC AAT CCG AAC ATC AAT GAA TGC ATT CCT TAT
          --------SD*-------      Met  Asp  Asn  Asn  Pro  Asn  Ile  Asn  Gly  Cys  Ile  Pro  Tyr
```

Chromosomal sequences (Klier, Parsot and Rapoport, 1983):

```
                    AC–UAG   16S  rRNA
HO–UCUUUCCUCC
    : :  : : :  : : :
ACTATTAATCAGGGTAATTTTTCAGCAACT ATG AGT AGT GGG AGT AAT TTA CAG TCC GGA GCT T
        -----SD*---                    Met  Ser  Ser  Gly  Ser  Asn  Leu  Gln  Ser  Gly  Ser
```

* SD: Shine–Dalgarno sequence.

A reason for the apparent overproduction of the crystal protein (the crystal represents about 30% of the cell protein) is not yet available. It is fair to point out, however, that the crystal protein is a very large polypeptide and the high percentage of total cell protein that it represents may be attributable, in large part, to its size rather than to an overproduction mechanism. Nevertheless, even if the delta-endotoxin were a smaller protein (as in H-14) the magnitude of its biogenesis is still considerable. The reason for this might be that the delta-endotoxin gene may have a particularly strong promoter, so that it binds RNA polymerase at an increased rate. The sequence data does not tell us how strong the promoter is, only how it is regulated. The delta-endotoxin gene may have particularly stable mRNAs which result in an amplified production of protein. This seems to be the case in *B. thuringiensis* var. *thuringiensis berliner* 1715 (Klier and Lecadet, 1976). Amplification of the delta-endotoxin may be related to increased copy number of the gene due to the fact that the gene is on a plasmid. However, in all cases studied, the plasmids are large and of low copy number (*c.* 1–2 per cell). Finally, the delta-endotoxin gene may initiate protein synthesis more efficiently by virtue of stronger ribosomal binding sites (Shine–Dalgarno sequences). We have seen from the sequences that the ribosomal binding site is strong, as are other known ribosomal binding sites of Gram-positive organisms, but is not especially strong within that group of organisms. At present we are not sure of the real reason for the high production of the delta-endotoxin genes. There are certain to be factors which regulate protein synthesis about which we know nothing at all.

To date there have been no reports of enhanced entomocidal activity through the use of genetic engineering. One may justly assume that there is considerable research on *B. thuringiensis* by genetic engineering companies and other companies which market, or have an interest in, insecticides. Such research does not spring forth into the literature as does that of academic institutions and we may not see the results of such efforts until products begin to appear. As Director of the *Bacillus* Genetic Stock Center, I am in a special position to view the flurry of activity in those companies which have an interest in *B. thuringiensis*. Of course detailed information is confidential, but it is fair to say that there is intense activity in the industrial sector.

The manipulations that may be made upon a purified crystal protein gene are limited only by the imagination of the workers. Simply transferring a low copy number gene, such as alpha-amylase, to a high copy number plasmid results in a considerable overproduction of 2000-fold more than wild type (Sibakov, Sarvas and Palva, 1983). New promoters or ribosomal binding sites may be substituted, as have been done with *B. subtilis* genes (Goldfarb, Doi and Rodriguez, 1981; Schoner, Williams and Lovett, 1983; *see also* Chapter 5) also with the effect of overproduction. The substitution of promoters must be done with a degree of caution, however. We have observed that the crystal-protein gene is under developmental control and is expressed only in the stationary phase. Unfortunately, little is known about the physiology and energy of the stationary phase compared with logarithmic growth (Maaløe and Kjeldgaard, 1966) but we know that protein turnover accounts for much of the *de novo* protein synthesis (Loshon, Swerdlow and Setlow, 1982). It may be that copious

production of proteins such as the crystal protein and spore coat protein can occur only in the stationary phase, or are harmful to the cell in the vegetative phase.

Directed mutagenesis may be applied (Shortle and Nathans, 1978) in order to enhance one of the various natural properties of the crystal-protein gene (i.e. the promoter, ribosomal binding site or active site). Secretion-regulatory sequences (Kroyer and Chang, 1981) may be attached in order to investigate the effect of turning an endotoxin into an exotoxin. This simple notion ignores the rather complex process of protein excretion.

It has been suggested that the delta-endotoxin gene be inserted into plants by way of methods already available (Barton and Brill, 1983) to create a systemic and highly specific toxin. This would not be harmful to the plant or to man and might only add additional protein value to the plant as far as human nutrition is concerned. This is but one example of a novel way to utilize the insect toxin of *B. thuringiensis*. Another example is the transfer of toxin genes to other bacteria which have better survival in nature. For example, the mosquito-toxic *B. thuringiensis* var. *israelensis* (H-14) survives only two days in nature. Other bacteria such as the natural pond microflora, would make more suitable hosts.

The benefit to man is great. Considering the expense of the crop damage by insects and health costs of insect-borne diseases, the impetus to find better (and safer) insecticides is strong. Clearly, by increasing either the amount of toxin per cell made by *B. thuringiensis* or by increasing its toxicity, one would lower the cost of usage and extend the range of vector control. There have been no reported attempts to utilize genetic manipulation (or genetic engineering) to enhance toxin production, but studies in numerous laboratories make these prospects highly possible in the near future.

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