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Sporulation and δ-endotoxin synthesis by *Bacillus thuringiensis*

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Abstract. *Bacillus thuringiensis* is distinguished from the very closely related *Bacillus cereus* and *Bacillus anthracis* by the presence of several plasmid-encoded δ-endotoxin genes. These δ-endotoxins, synthesized as protoxins, are produced in large quantities during sporulation and are packaged into intracellular inclusions. Ingestion of the inclusions by insect larvae leads to protoxin solubilization and conversion to toxins each specific for one of several orders of insects. The toxins form cation-selective channels in the membrane of cells lining the larval midgut with subsequent lethality. In most cases, δ-endotoxin synthesis and sporulation are closely coupled. The latter process in *B. thuringiensis* is probably virtually identical to that in *Bacillus subtilis* with the additional use of mother cell sporulation forms of RNA polymerase for the synthesis of the δ-endotoxins. There are other more subtle plasmid-encoded functions or plasmid interactions related to regulating protoxin synthesis. Consideration of both plasmid and chromosomal genes is thus critical for defining this organism.

Key words. Sporulation; δ-endotoxins; plasmids; insecticides; regulation.

Introduction

Bacillus thuringiensis (Bt) is a spore-forming bacterium with one of its most important features being the formation of intracellular inclusions comprised of protein protoxins (δ-endotoxins). These are very effective and highly specific biological insecticides [1–3]. Target insects include the larvae of lepidopteran and coleopteran plant pests as well as of dipteran mosquitoes and black flies, which are vectors for a variety of diseases including dengue and malaria. There are also less well characterized *Bt* subspecies active on other insect orders as well as nematodes [4].

Bt is very closely related to *Bacillus cereus* and *Bacillus anthracis* based on analyses of DNA restriction fragments and some sequencing [5]. There are thousands of *Bt* subspecies differentiated primarily by the array of plasmid-encoded δ-endotoxins (so-called *cry* genes), and thus the particular toxicity profile. A common pattern is the presence of several *cry* genes in an isolate which are most often transcribed during sporulation utilizing sporulation-specific forms of RNA polymerase [6, 7]. There is a very active synthesis of these protoxins, up to 20% of the protein synthesized during sporulation, with their deposition as intracellular, crystalline inclusions (fig. 1).

Upon completion of sporulation and mother cell lysis, the spore and inclusions are released. In a few cases, an inclusion is enclosed within the exosporium [8]. When inclusions are ingested by lepidopteran or dipteran larvae, the protoxins are solubilized in the alkaline midgut and converted to toxins, primarily by removal of the carboxyl halves by trypsin-like enzymes. The X-ray crystal structures of two toxins, one active on Coleoptera [9] and the other on Lepidoptera [10], are virtually superimposable despite only about 36% amino acid identity. Both have a three-domain structure with domain I comprised of a bundle of seven amphipathic α helices and domains II and III of β sheets but in different conformations. Domain I of the δ-endotoxins is related to other pore-forming toxins such as certain colicins and the B subunit of the diphtheria toxin [11]. Each contains a cluster of amphipathic helices which are believed to comprise the pore-forming region.

Loops within domains II and III are involved in the initial reversible binding of the toxin to receptors on larval midgut cells [3]. The best characterized of several such

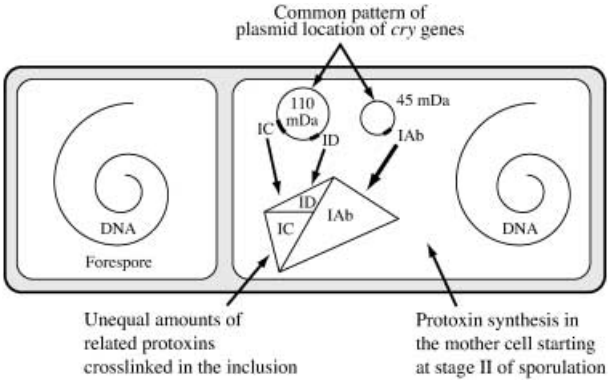

Figure 1. Schematic of the formation of a diamond-shaped inclusion comprised of unequal amounts of three related Cry1 protoxins.

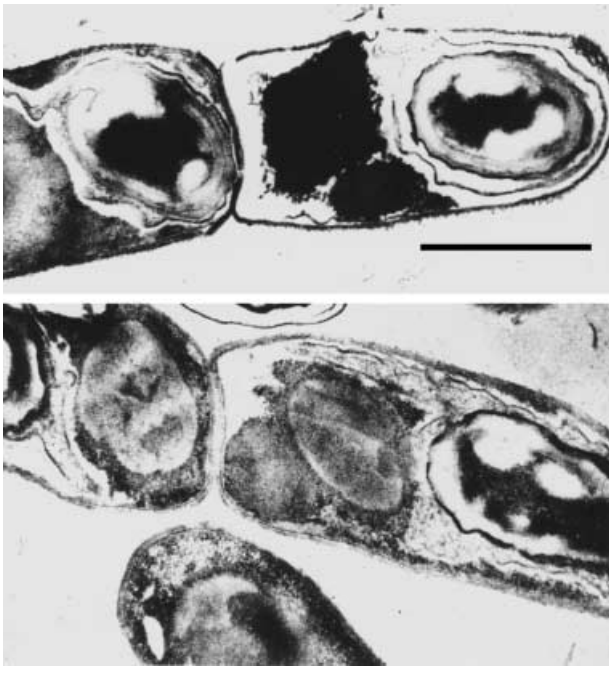

Figure 2. Thin section electron micrographs of late-sporulating *Bt* subsp. *kurstaki* HD1 (top) and *Bt* subsp. *israelensis* (bottom). In the top panel, the cell is beginning to lyse with the release the dormant endospore (on the right) and two darkly staining inclusions (on the left). The larger, diamond shaped inclusion contains a mixture of three Cry1A protoxins. The smaller inclusion is comprised of the Cry2A protoxin. In the bottom panel, the inclusion in the left part of the sporulating cell is multifaceted and is surrounded by a thin net. This inclusion contains four dipteran-active protoxins plus a cytolytic factor. Line is 1 μm.

receptors are aminopeptidase N and a cadhedrin-like molecule [3]. Following this reversible binding, the toxin subsequently associates tightly with the membrane (so-called irreversible binding) with two of the amphipathic α helices within domain I inserting into the membrane [12]. At some stage, there is an aggregation of the toxins (number unknown) either within the membrane or perhaps at the membrane surface prior to insertion [12]. Cation selective channels form resulting in osmotic swelling of the cells lining the larval midgut with subsequent lethality [13].

It is likely that insect larvae ingest spores along with the inclusions and in some cases, spores are synergistic with the toxin [14, 15]. Synergism is probably due to the germination of spores in the midgut with the production of a variety of pathogenic factors by the vegetative cells [16], including in some subspecies specific vegetative insecticidal proteins, the VIPs [17].

Most *Bt* isolates contain a large amount of plasmid DNA (up to 20% of the total cell DNA). Plasmids >50 Mda are common, but some subspecies have as many as 12 size classes ranging from 3–4 Mda to >150 Mda [18]. The *cry* genes are generally confined to just a few of the larger plasmids [19], often with two or more in close proximity on a plasmid of >100 Mda plus a rather prevalent gene, *cry1Ab*, on a plasmid of 40–50 Mda (fig. 2) [20]. In many if not all cases, *cry* genes are surrounded by IS and/or transposon-like sequences, usually within 1–2 kbp of the coding region [1, 21], suggesting a possible derivation from mobile genetic elements. For one of the genes, *cry1Ca1*, the region immediately upstream of the promoters encodes a transposase [22]. The significance of this close association is not evident. It may simply reflect a recent transposition event for this particular *cry* gene and perhaps the start of the evolution of the upstream sequence into a regulatory region.

Some of the cry genes are within operons which include open reading frames (*orf's*) encoding proteins with undefined functions [6]. One of the more extreme examples of such *cry* gene clustering is the presence of four such genes and one encoding a cytolytic toxin, all on a plasmid of 75 Mda in *Bt* subsp. *israelensis,* which is active on Diptera [23]. This subspecies produces a multifaceted inclusion (fig. 1) comprised of the four δ-endotoxins plus the cytolytic factor [24].

There is limited information about other functions for most of the plasmid DNA. The fact that the *cry* genes are invariably present on plasmids rather than the chromosome means that this bacterium must be considered in the context of the array of both plasmid and chromosomal genes and the contributions of each to cell growth, survival and toxicity.

One of the more intriguing questions is the origin and function of the *cry* genes and why they have found a home in this bacterium. It is very likely that a very close relative of *Bt*, such as *B. cereus*, has been around a lot longer than the plasmid-encoded *cry* genes. The plasmids or transposons containing *cry* genes were probably transmitted to the bacterium and became a stable part of its genetic repertoire some time after the evolution of insects. The regulation of *cry* gene expression has or is evolving to ensure a coordination with sporulation. This is in part achieved by the use of mother cell forms of sporulation

RNA polymerases [6, 7]. As discussed later, there are likely to be additional factors involved in the differential expression of the individual *cry* genes.

The *cry* genes presumably helped to establish novel ecological niches for these newly endowed bacilli. *Bt* strains have been isolated from a wide variety of environments (primarily dead insects from treated fields). One of the anomolies is that mixtures of spores and inclusions, which have been used as commercial sprays for crops for over 30 years, have relatively short lifetimes. There is a rapid loss of toxicity and of *Bt* spores from treated areas known to be infested with target insects [25]. This implies that cycles of infection, growth and reinfection are rare, i.e. epizootic spreading is unlikely to be the mechanism for sustaining *Bt* in nature. The natural habitat for this organism may involve subtle interactions in the phylloplane, perhaps with plants. In fact, *Bt* isolates can be readily obtained from the underside of leaves [26], and a mutually beneficial scenario can be imagined.

Bt has thus commanded considerable interest both because of its potential as a biological control agent and because of the regulatory interaction between the plasmid-encoded δ-endotoxins and sporulation. It is this latter intriguing relationship which we wish to explore more extensively in this review.

The process of sporulation in *Bt* is coupled to protoxin synthesis

The sporulation process in *Bt* is likely to be very similar to the one so well characterized in *Bacillus subtilis* [27, 28]. This is inferred from an analysis of the unannotated genome of *B. anthracis,* which is available (www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism=b_anthracis). The presence of most of the key *B. subtilis* sporulation regulatory and structural genes are there and well conserved (>50% amino acid identity throughout most of the sequence). These include the *spoO* genes for the initiation of sporulation and the various sigma factors required for sequential mother cell and forespore gene transcription. Other key stage II and III sporulation genes involved in forespore formation as well as stages IV and V regulatory genes are also present. The kinases (Kin A and Kin B) which sense sporulation initiation signals at stage 0 are present, but interestingly, whereas the sequences of regions which are involved in phosphorylation are conserved, other parts of these proteins, in particular possible sensing regions, are not [J. Hoch, personal communication]. The implication is that *B. anthracis* may respond to different cues for initiating sporulation than does *B. subtilis*.

Among the genes encoding spore structural proteins, all of the *B. subtilis* germination operons are present and well conserved, as are those for the small acid-soluble proteins (SASPs) involved in binding to forespore DNA and providing amino acids for outgrowth. Key spore coat regulatory genes required for proper assembly such as *cotE* and *spoIVB* [28] are present, as are many of the coat structural protein genes (table 1). However, several coat protein genes, in particular those involved in the assembly of a cross-linked outer coat structure, are probably absent. *B. anthracis* has some novel, rather prevalent coat proteins which may perform similar functions.

Table 1. Presence of homologues of *Bacillus subtilis* spore coat genes in the *Bacillus anthracis* genome.

Likely to be present*	
cot B *cot D* *cot E* *cot F* *cot H* *cot Z* *cot JA* *cot JC* *yaa H*	
Questionable[+]	
cot Y *yrb A* *yrbB*	
Unlikely to be present[‡]	
cotA *cotC* *cot G* *cot M* *cot S* *cot T* *cot V* *cot W* *cot X*	
Genes involved in regulation of spore coat synthesis	
well conserved: *ger E, spoIVA*	
conserved: *sod A*	
unlikely: *spoVID*	

* At least 35% identity and 50% positive throughout most (>80%) of the sequence. Probability score < –10.

[+] >40% identity but scattered throughout part (<50%) of the sequence. Probability score –6 to –10.

[‡] <35% identity and <50% positive spread throughout 10–50% of the sequence. Probability score > –5.

Since *B. anthracis* and *Bt* are very closely related by a number of criteria [5], it is likely that the spore genes (as well as the sporulation process) found in the former are also present in *Bt.* In particular, the mechanism of initiation of sporulation, forespore formation and the regulation of the sequential morphological changes are almost certainly the same as in *B. subtilis*. In fact, some genes encoding critical sporulation factors such as the SpoOA protein, sigma E and sigma K have been cloned from *Bt* [6]. As with *B. anthracis*, there may be unique features of the kinases involved in the initiation of sporulation and perhaps some different spore coat structural proteins both reflecting presumed differences in the ecology of *B. anthracis* and *Bt*.

Regulation of protoxin synthesis

As mentioned above, the *cry* genes have probably been superimposed on this sporulation process by the transfer of plasmids or transposons containing these genes into a *Bt* progenitor, most likely *B. cereus*. Subsequently, the regulation of expression of the *cry* genes was established in order to coordinate sporulation with the synthesis of the protoxins and their assembly into inclusions. Many *cry* genes are transcribed by both of the mother cell forms of RNA polymerase containing σ^E or σ^K [1, 6, 29]. Consequently, transcription begins at stage II of sporulation

by the σ^E form of RNA polymerase and continues into late sporulation by the σ^K form of the enzyme. This dual control ensures the prolonged synthesis and thus accumulation of large amounts of the protoxins. Other *cry* genes (and at least one operon) utilize only σ^E-RNA polymerase [6], thus confining protoxin synthesis to a shorter period during sporulation. In addition, transcription is initiated at a low level at the end of vegetative growth for at least two *cry* genes or operons by a σ^H- (encoded by the *spo0H* gene and thus probably a stage I function) RNA polymerase [6, 30]. It is not known why there are such variations in transcription patterns, but overall the synthesis of both spore components and of substantial quantities of the protoxins must be ensured by the sharing of these various forms of sporulation RNA polymerases.

An interesting variation on the use of the sporulation RNA polymerases is that the dual promoter regions for the σ^E and σ^K forms of the enzyme utilized by many *cry* genes overlap [6, 31]. Some sporulation genes contain these two promoters in tandem and there are two sporulation genes of unknown function with such an overlap, though their significance is not known. This rather rare promoter arrangement was found to be important for modulating the transcription of *cry1* protoxin genes [31]. There is approximately equal transcription from both promoters, and as mentioned above, this ensures a constant rate of protoxin synthesis over a prolonged time.

The promoter overlap means that the −10 region for the upstream σ^K-dependent promoter is within the spacer region for the σ^E-dependent promoter. Mutations within this −10 region which departed from the consensus sequence resulted in a four- to fivefold enhanced rate of transcription from the σ^E-dependent promoter as measured with *lacZ* fusions. Mutations to a consensus σ^E −10 region resulted in an inhibition of transcription. Surprisingly, when there was a 'promoter-up' mutation, the inclusions produced were smaller than those formed by the wild type because the excess protoxin was rapidly turned over. Apparently, one or more steps required for the assembly of the protoxins into inclusions was limiting, implying an orderly process regulated by one or more inclusion packaging factors.

Promoter overlap somehow provides a mechanism for controlling the rate of transcription from the σ^E-dependent promoter. One possibility is that the binding of RNA polymerase (probably the σ^E form) to this −10 region within the spacer modulates the initial rate of transcription. Promoter overlap is an important modification of the use of sporulation transcription factors for the specific needs of regulating *cry* gene expression and the assembly of protoxins into inclusions.

Not only is the overall rate of transcription of *cry1* genes controlled, but the individual genes are also transcribed at different rates, resulting in unequal amounts of the protoxins in inclusions (fig. 2) [20, 32]. Several factors appear to be involved in this regulation: (i) differences in plasmid and thus *cry* gene copy number; (ii) inherent differences in protoxin stability and/or the presence of factors (perhaps chaperones) which stabilize the protoxins for packaging; (iii) differing rates of transcription of *cry* genes [20] despite virtually identical overlapping promoter regions in many cases. These transcription differences may be due to upstream sequences which are unique even for closely related *cry* genes [33].

The evidence that a region upstream of the dual promoters is involved in regulation is based on gel retardation studies and mutations of the protein binding sites. A 300-bp oligonucleotide from the upstream region of the *cry1A* gene was retarded by a protein identified as the E2 subunit of pyruvate dehydrogenase (PDH) [33]. Binding was to specific sequences 200–300 bp upstream of the initiation codon and included an inverted repeat. Mutation of the E2 binding sites resulted in a modest decrease in expression of a *cry1A-lacZ* fusion, suggesting some regulatory role for this protein. Binding by E2 to the upstream regions of other *cry* genes was less efficient, implying some specificity. As mentioned previously, the region upstream of the dual promoters for the *cry1Ca1* gene encodes a transposase [22]. The presence of such an element in close proximity to a *cry* gene may be indicative of the ongoing evolution of the regulatory regions for these genes.

The finding that a catabolic enzyme subunit may also serve as a regulatory protein was unexpected. There is also evidence for a regulatory function for E2 in *B. subtilis,* since deletion of the *pdhC* gene encoding the E2 subunit resulted in a lower frequency of sporulation with a block at late stage II [H. Gao and A. Aronson, unpublished results]. In contrast, a deletion of the *pdhD* gene encoding the E3 subunit resulted in loss of PDH activity, but the cells sporulated almost as well as the wild type. The PDH complex is important in catabolism in aerobically growing cells but is no longer required by stage II of sporulation when both the major carbon source (usually glucose) and secondary carbon sources such as pyruvic acid have been exhausted. At that time, the PDH complex dissociates, releasing the E2 core as soluble components (probably stable trimers). In *Bt*, the availability and DNA-binding properties of E2 at stage II of sporulation may have been exploited for modulating *cry* gene transcription. The binding to the upstream regions of *cry* genes could be a mechanism for signaling a change in stage II gene expression including, for *Bt*, the synthesis of δ-endotoxins.

As mentioned above, it is also possible that the regulatory regions of these *cry* genes are still evolving and that the binding of E2 is not yet an essential feature of their regulation. Regulation attributable to the upstream sequences and proteins such as E2 would imply that some sort of fine tuning of the transcription of each gene is important.

The lack of knowledge of the ecology of *Bt* and thus the benefit of producing so much of a variety of insecticidal proteins makes it difficult to speculate on such regulation.

A major exception to transcription patterns of *cry* genes during sporulation is the expression of the *cry3A* gene [7]. Transcription of this gene is initiated in late logarithmically growing cells by a vegetative form of RNA polymerase. Transcription commences 558 bp upstream of the start codon, and the messenger RNA (mRNA) is processed to a shorter, more stable form. This substantial difference in transcription pattern may reflect differences in the properties of the Cry3 and Cry1 protoxins, in particular features of protoxin structure which are needed to package the proteins into inclusions so that they can be readily solubilized in the midgut of target insects.

As noted previously, the structure of the Cry3A toxin is almost identical to that of a lepidopteran-active toxin, Cry1Aa [9, 10], despite only about 36% amino acid identity. Both are synthesized as protoxins, but they differ in that the Cry3A protoxin lacks the extensive cysteine-rich carboxyl half present in the Cry1Aa protoxin. The high pH and reducing conditions found in the midguts of lepidopteran and dipteran larvae are important for reducing the intermolecular disulfide bonds cross-linking Cry1 protoxins in inclusions [34] and for protoxin solubilization. In contrast, the Cry3A protoxin is packaged probably primarily by ionic interactions into inclusions which can be readily solubilized at pH 7–8 in the absence of a mercaptan [35]. These solubilization conditions approximate those found in midguts of coleopteran larvae.

The difference in the time of synthesis of the Cry3A versus most other protoxins may reflect the changes in the physiological conditions within sporulating cells. Intermolecular disulfide bonds are rarely found in the cytoplasm of bacterial cells, which is generally reducing. During the mid to late stages of sporulation, however, the intracellular environment becomes more oxidative, as reflected by the preponderance of the oxidized rather than the reduced forms of low molecular weight thiols [36]. At that time, the disulfide bonds needed for intracellular inclusion formation could be formed, so the time of synthesis of most protoxins (stage II–V of sporulation) would coincide with this change. The absence of substantial disulfide bonds in Cry3A inclusions indicates that assembly would not be dependent upon a more oxidative environment so that synthesis of this protoxin could begin in late exponentially growing cells with completion by stage II. The timing of protoxin synthesis would thus reflect the physiological conditions within sporulating cells.

The location of protoxin genes on various plasmids rather than in the chromosome appears to be of importance to the toxicity profile in several ways. One would be gene copy number, which is about 3–5 for the large plasmids containing *cry* genes. Another is the mobilization and transfer of such plasmids by cell mating with either *B. cereus* or other *Bt* strains [18, 37].

A more subtle aspect is the instability of certain protoxin-encoding plasmids [38]. As mentioned previously, a prominent pattern among strains producing several lepidopteran-active protoxins is two or more of the *cry* genes in close proximity on a plasmid of ~120 Mda and a particular cry gene, *cry1Ab*, on a 40–50 Mda plasmid (fig. 2) [20, 38]. The 40–50 Mda plasmid is relatively unstable (1–10% of random colonies lack the *cry1Ab* gene) in many subspecies [38], probably due to plasmid incompatibility. As a result, a population of cells is mixed in terms of *cry* gene composition, with a small fraction lacking the *cry1Ab* gene. It should be noted that this unstable 40–50-mDa plasmid containing the *cry1Ab* gene can be readily transferred to other cells by mating [37]. This ensures that this gene, one of the more prevalent lepidopteran-active protoxin genes, is sustained in the population.

The Cry1Ab protoxin differs most significantly from other related protoxins in the absence from the carboxyl half of 28 amino acids, including four cysteines [39]. This deletion results in a less stable protoxin [40] and one that forms fewer disulfide bonds with other Cry1 protoxins in an inclusion. The absence of this protoxin not only changes the specificity profile, but the solubility properties of the inclusion [20, 41]. For example, inclusions containing the Cry1Ca1 and Cry1Da1 protoxins require a higher pH to solubilize than do inclusions comprised of these two protoxins plus the Cry1Ab3 protoxin. This shift in composition and solubility would alter the range of susceptible insects and thus the effectiveness of the *Bt* subspecies in a particular environment. While it is not apparent why such flexibility in protoxin composition is needed, these subtle properties presumably benefit the survival of various *Bt* subspecies in their niche.

Why all the plasmids in *Bt*?

Beyond the *cry* genes, little is known about other genes present in the extensive array of plasmids found in many subspecies. There is a protein of 20 kDa produced by *Bt* subsp. *israelensis* (encoded as part of a *cry* gene operon) which has been demonstrated to enhance the accumulation of certain protoxins [42, 43], presumably by a direct interaction. Some of the *cry* genes are present in operons with *orf*'s of unknown function [6]. At least one of the encoded orf's in the *cry2* operon, which is comprised largely 15–21 repeats of a 15–16-amino acid sequence, may also function to stabilize protoxins for packaging into inclusions [44]. The *cry2* operon has three *orf*'s, the one mentioned above, one encoding the Cry2 protoxin and a third of unknown function. Deletion of the latter did not

result in detectable effects on Cry2A protoxin accumulation, so it must have a more subtle function.

The Cry2 protoxin is packaged into an inclusion distinct from the bipyramidal inclusion containing the disulfide cross-linked Cry1 protoxins (fig. 1). The insect specificity profile for Cry2 toxins includes some active only on certain Diptera as well as those active on both Lepidoptera and Diptera. The Cry2 inclusion is often present in close proximity to the Cry1 inclusion, implying that there may be some factor to ensure such an association. The Orf's of unknown function could be involved in such interactions.

There is a plasmid-encoded small molecular weight insecticidal factor (β-exotoxin) produced by some subspecies, and the genes involved in its synthesis are on a large plasmid, probably the same one containing *cry* genes [45]. There is at least one plasmid-encoded bacteriocin, and the vegetative insecticidal proteins (VIPs) are also plasmid encoded [17]. Some of the very small plasmids are composed almost exclusively of transposon sequences [46].

There is also evidence that a plasmid factor is responsible for the localization of certain inclusions within the exosporium rather than free in the mother cell cytoplasm [47]. Such enclosure would ensure that the spore and inclusion are released together, perhaps to function synergistically in toxicity. This arrangement may also serve to exploit the hydrophobic properties of the exosporium. This thin coating over the spore coat is chemically complex and has no essential spore function, but it may aid in spore dispersal. Packaging an inclusion within the exosporium could be useful in an aqueous environment for keeping the inclusion at the water surface where target larvae may be actively feeding. Subspecies which produce exosporial-enclosed inclusions have no known target insects, however, so we can only speculate as to the purpose of such packaging.

There still remains considerable plasmid-encoding potential since most *Bt* subspecies contain > 10% of their total DNA as plasmids. There is the possibility that much of this DNA is in very small *orf*'s as found in *Borrelia* species and to some extent in the large plasmids present in *B. anthracis* [48]. These *orf*'s may not encode proteins (or functional RNAs) and may reflect the evolution of plasmid genes.

There may be spore-protoxin interactions

Given the extensive synthesis of protoxins in sporulating cells, it is not surprising that some would aggregate on the spore surface [49], probably on the exosporium. The association is fairly loose, and the protoxins can be removed by extensive washing or by purification of spores through density gradients. Their presence does confer toxicity to spores, which may or may not be of significance. It was also noted that the spore coat of *Bt* strains producing protoxins was somewhat thinner than that on spores from plasmid-cured acrystalliferous strains or from *B. cereus* [49]. This thinning may simply be due to the excess protoxin displacing spore coat proteins from the spore surface. Alternatively, the synthesis of certain outer spore coat proteins may be modulated to accommodate protoxins on the surface.

Another intriguing property of *Bt* spores is their ability to germinate in the alkaline environment of the midguts of lepidopteran larvae [50]. They may be able to respond more efficiently than spores from Cry-variants or from nontoxic *B. cereus*. If so, an adaptive germination property may be present in these spores, perhaps attributable to the protoxin on the surface. There is precedence for special germination properties of *B. anthracis* spores involving a *ger* operon encoded within the pathogenicity island on the large plasmid, pXO1 [51]. These Ger proteins apparently enhance germination within lung macrophages. By analogy, the protoxin-coated *Bt* spore may be structured to optimize germination in larval midguts.

It is known that spores can enhance the toxicity of δ-endotoxins for certain insect larvae [14, 15]. This synergism is almost certainly due to the vegetative cell rather than the spore per se and is directly attributable to the array of pathogenic factors produced by *Bt* cells [16, 52]. Deletion of a key regulatory gene, *plcR*, resulted in the inability to produce many pathogenic factors such as phospholipase C, enterotoxins and hemolysins and thus to enhance toxicity. The capacity of *Bt* spores to germinate rapidly in the larval midgut could thus contribute significantly to the overall effectiveness of a particular subspecies.

The process of inclusion assembly

Inclusions often have a crystalline array but vary considerably in shape and size. Many *Bt* subspecies produce more than one inclusion per cell each comprised of different protoxins. In *Bt* subsp. *israelensis* active on dipteran larvae, there is a multifaceted inclusion surrounded by a net (fig. 1) which contains four δ-endotoxins and a cytotoxin [24]. The latter is synergistic with one or more of the toxins [53, 54]. Such packaging ensures ingestion of all of the toxins by target insect larvae, thus reducing the probability of developing resistance [55]. Little or no resistance has been found in the field or in laboratory selections.

As previously discussed, the mixture of Cry1 protoxins in crystalline inclusions have highly homologous carboxyl halves, including 16–19 cysteines which are cross-linked by intermolecular disulfide bonds [34]. Other inclusions such as the those consisting of the Cry2 or Cry3 protoxins contain just a single species, and in the case of the latter,

ionic bonds rather than disulfide bonds are involved. Many subspecies produce both a Cry1 and Cry2 inclusion (fig. 1); those containing a Cry3 protoxin seem to have just the one δ-endotoxin gene and thus form a single inclusion.

Assembly begins with regulation of the rates of synthesis as exemplified by the overlapping promoters for many *cry1* genes. As previously noted, there is evidence for chaperone-like proteins or proteins which stabilize protoxins such as the 20-kDa protein produced by *Bt* subsp. *israelensis* [42]. For the Cry1 δ-endotoxins, there is extensive intermolecular disulfide bond formation, which is likely to be assisted by one or more protein disulfide isomerases [56]. In *Escherichia coli*, these enzymes function in the more oxidizing environment of the periplasm, but they could function intracellularly in late sporulating cells, which is more oxidative. To date, there is no evidence for the involvement of such enzymes in inclusion formation, but this process has not been extensively investigated.

Another function related to assembly is the location of the inclusion within the sporulating cell. A few *Bt* subspecies, *Bt* subsp. *finitimus* being the best studied, form an inclusion within the exosporium [8, 47]. This subspecies also produces at least one additional inclusion outside of the exosporium. The *cry* genes encoding the protoxins present either within or outside the exosporium are on different plasmids, so it was possible to isolate strains lacking one or the other of these protoxin-encoding plasmids. In each case, the inclusions formed at the expected site. Transfer of the large plasmid containing the *cry* gene for the enclosed inclusion to *B. cereus* resulted in transformants which formed an inclusion within the exosporium [47].

The gene(s) required for conferring such localization are not known, but they must reside on the same plasmid as the *cry* gene. One possible mechanism is coordination of the time of synthesis of this particular protoxin with that of the exosporium. This complex structure appears to be synthesized during a large part of sporulation. The *cry* gene encoding the enclosed protoxin contains dual overlapping promoters similar to those found in many *cry1* genes, so its synthesis is likely to start at stage II and continue until late stage V. Unless there are special upstream sequences involved in regulating the time of expression of this particular *cry* gene, it is difficult to visualize how such a timing mechanism would work. Another possibility is that there is a plasmid gene(s) encoding a protein which somehow binds this protoxin to the inner surface of the exosporium. Inclusion assembly would then be confined to such a site ensuring a location within the exosporium, for whatever reason.

The overall process of the synthesis of several protoxins in different amounts, their assembly into inclusions and the localization of these structures is complex. There are likely to be many more plasmid and chromosomal genes involved in these processes reflecting the extensive interactions between these two classes of genetic elements in this species.

Summary

1) The sporulation process in *Bt* is almost certainly very similar to that found in *B. subtilis*. Differences in spore coat structural proteins and in the properties of stage 0 kinases involved in sensing signals for the initiation of sporulation could reflect the unique ecological conditions of various spore-forming bacilli.

2) *Bt* is important because of the formation of inclusions comprised of protoxins active on insect larvae. These highly specific proteins are synthesized from plasmid-encoded genes, so the presence of such plasmids is an important feature of this species.

3) Most *Bt* subspecies contain multiple *cry* genes often flanked by transposon or insertion sequences. Sometimes the *cry* genes are parts of operons with other *orf*'s of unknown functions. Most protoxin synthesis occurs primarily during sporulation, exploiting various forms of sporulation RNA polymerases, but with unique promoter arrangements. The protoxins are packaged into inclusions, and this process involves regulation at the level of transcription, translation and inclusion assembly.

4) Other plasmid genes may function as chaperones, in packaging, or for inclusion localization. Certain plasmid properties, such as mobilization and incompatibility, also have roles in regulating δ-endotoxin synthesis or in the population dynamics of the array of expressed *cry* genes. These *Bt* plasmids are an important component of the genome of these bacteria and must be considered when defining this species.

5) There are fascinating outstanding questions about the origin of these *cry* genes and how their regulation became integrated with sporulation. Why these bacilli produce so many of these plasmid-encoded δ-endotoxins is another area for speculation.

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