THE STRUCTURE AND FUNCTION OF BACILLUS THURINGIENSIS δ-ENDOTOXINS AND PROSPECTS FOR BIOPESTICIDE IMPROVEMENT

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ABSTRACT

The spore-forming bacterium *Bacillus thuringiensis* produces novel and highly specific insecticidal proteins (δ-endotoxins) grouped into two main families - Cry and Cyt toxins - by amino acid sequence similarity. Both types of δ-endotoxin bind to insect-specific receptors on the surface of gut epithelial cells and in a second irreversible step insert into the cell membrane to form leakage channels that result in cell death by colloid osmotic lysis. The protein nature of these toxins coupled with genetic engineering offers great potential for pesticide improvement and resistance management and has allowed them to be expressed in plants as systemic biopesticides. The X-ray structure of the first Cry toxin revealed putative membrane insertion and receptor binding domains whose functions are being explored by intensive mutagenesis and domain swapping. The first Cyt toxin structure has now been described and is entirely different from the Cry toxins - despite their similar toxic mechanism. Current biochemical and genetic attempts to define structure-activity relationships for these toxins will be reviewed. The potential of these pesticides has been further enhanced by the recent cloning and sequencing of Cry toxin receptors. These receptors are transmembrane proteins exposed on the lumen surface of midgut epithelial cells. The structure of these receptors, their role in toxin recognition, membrane pore formation and toxin resistance will be discussed. Although the best Bt toxins are insecticidal at concentrations typical of potent chemical pesticides, many of them are ten or even 1,000 times less potent. Recent work suggests that additional virulence factors may contribute to the insecticidal potency of *Bacillus thuringiensis*. Strategies to enhance toxin potency for improved spray formulations and to allow them to be expressed at realistic levels in transgenic plants will be discussed.

INTRODUCTION

The most promising insecticidal biopesticide has proved to be *Bacillus thuringiensis* (Bt), an aerobic gram-positive spore-former first reported in 1901 by the Japanese scientist Ishiwata (Dulmage and Aizawa, 1982) as the causative agent of the "sotto" disease of the silkworm (*Bombyx mori*). During sporulation this bacterium produces cytoplasmic protein crystals which are released into the environment together with the mature spore at the end of sporulation. The proteins in these crystals (δ-endotoxins) are insecticidal and therefore of commercial interest as novel biopesticides, both as spray preparations and more recently as 'systemic' biopesticides through the introduction of endotoxin genes into transgenic plants. Insecticidal activity has been shown towards dipteran (mosquito and fly) larvae and coleopteran (beetle) larvae in addition to the lepidopteran larvae to which the strain was first shown to be active. Extensive screening for strains with novel activities has resulted in the discovery of toxicities to insects of an additional three orders, the hymenoptera (ants), the homoptera (aphids) and the phthiroptera/mallophaga (lice) (Drummond *et al.*, 1992; Feitelson, 1993). Some strains of Bt are reportedly toxic to non-insect pests such as the nematode parasites of mammals and plants (Edwards *et al.*, 1990; Bone, 1989), the trematoda (animal parasitic liver flukes), the acari (mites) and some protozoan pathogens (Feitelson, 1993).The recent identification of a Cry toxin in *Clostridium bifermentans* subsp. *malaysia* (Barloy *et al.*, 1996) suggests that these toxins may be more widely distributed.

Bt isolates have traditionally been classified using the serological diversity of their flagellar or "H" antigens (de Barjac and Frachon, 1990). 45 serotypes that have been identified in this way

and subspecies names assigned to them (Lecadet *et al.*, 1994). However since activity towards a target insect is defined by the number and type of δ-endotoxins in the crystalline inclusion, classification systems for the endotoxins are needed (Höfte and Whiteley, 1989; Crickmore *et al.*, 1995). In this paper Cry toxins are named according to the revised Cry toxin nomenclature on WWW site:http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html.

BACILLUS THURINGIENSIS PATHOGENESIS

While the δ-endotoxins are the most important factors in insect pathogenesis, Bt produces several other toxic factors including α and β-exotoxins, a 'louse' factor with activity against certain mammalian biting lice (Gingrich *et al.*, 1974) and various exoenzymes and immune inhibitors. The α-exotoxin is a heat-labile protein active against mice and several lepidopteran insects (Krieg, 1971). The heat-stable β-exotoxins are nucleotide analogues produced during vegetative growth (
Levinson *et al.*, 1990).Because they are broadly inhibitory to DNA-directed RNA polymerases, preparations of Bt-based insecticides in most countries utilise strains that do not produce the β-exotoxin. Estruch *et al.* (1996) have recently identified novel insecticidal toxins produced by several strains of Bt that are active against lepidopteran insects. These insecticidal proteins are expressed in the vegetative stage of growth starting at mid-log phase as well as during sporulation. The production of exoenzymes by vegetative Bt such as chitinase (Lüthy, 1980), phospholipase C (Taguchi *et al.*, 1980) and haemolysin (Pendleton *et al.*, 1973) may play a secondary role in the overall pathogenicity of Bt. Bt is equipped to evade the insect immune system by production of two immune inhibitors, InA and InB (Lövgren *et al.*, 1990; Edlund *et al.*, 1976). Since the biological role of these toxins is to convert the insect into a food source for the bacterium (Ellar, 1990) the role of the spore in overall pathogenesis should not be neglected

BT δ-ENDOTOXINS: THE PROTEINS, GENES AND CLASSIFICATION

SDS/PAGE analysis of the crystalline inclusions produced by Bt subspecies reveals that generally, they are composed of one or more polypeptides with molecular masses ranging from 25-140 kDa .Höfte and Whiteley (1989) proposed a uniform nomenclature and classification scheme based on amino acid sequence similarity (deduced from DNA) and also host range . At that time 42 δ-endotoxin genes had been cloned, and discounting identical or nearly identical sequences fourteen unique genes were identified. Thirteen of these genes encoded a family of related proteins (Cry proteins). These Cry proteins were further subdivided into four major classes (revised names according to Crickmore *et al.*, 1995 in parentheses.) The *cry*I (*cry*1) genes encode 130-140 kDa polypeptides which are primarily active against lepidopteran larvae. The *cry*II (*cry*2) genes encode proteins of a predicted molecular weight of 71 kDa and show only limited homology to the other Cry toxins. CryIIA (Cry2A) displays dual activity to both lepidopteran and dipteran insects, whereas CryIIB (Cry2B) is toxic only to lepidopteran larvae. CryIII (Cry3) proteins are 71 kDa (with the exception of CryIIIC (Cry3C); Lambert *et al.*, 1992a) and are toxic towards coleoptera. The diptericidal proteins encoded by the *cry*IV(*cry*4, *cry*10A, *cry*11A) genes are the most diverse; 135, 128, 78, 67 kDa termed CryIVA (Cry4A), CryIVB (Cry4B), CryIVC (Cry10A) and CryIVD (Cry11A) respectively (Höfte and Whiteley, 1989). The *cry*V (*cry*5Aa) gene encodes an 81 kDa protein and is active against both lepidoptera and coleoptera. This toxin is considered to be a naturally truncated CryI (Cry1) toxin, its specificity and sequence similarity most resembles CryIB(Cry1Ba). The fourteenth unique gene identified in the Höfte and Whiteley (1989) classification scheme was *cyt*A (*cyt*1A) ((27 kDa). The protein encoded by this gene is diptericidal and broadly cytolytic *in vitro*. and displays no sequence similarity to Cry toxins. CytA has been joined in the classification scheme by CytB (Cyt2A), a related protein with the same toxic properties (Koni and Ellar, 1993). The X-ray structure of Cyt2A (Li *et al.*, 1996) has shown that the Cyt toxins have a very different three dimensional structure to the Cry toxins as will be described later.

Problems with the Höfte and Whiteley (1989) classification scheme in attempting to

accommodate the ever increasing number of new δ-endotoxins resulted from the mixed criteria used to construct the classification. For example, CryI toxins were designated lepidopteran specific. However, CryIA(b) (Cry1Ab7) from Bt ssp *aizawai* IC1 (Haider and Ellar, 1987) and CryIC (Cry1Ca1) (Smith *et al.*, 1996) are now known to exhibit dual activity against lepidoptera and diptera. CryIB (Cry1Ba1) has also been shown to exhibit toxicity to three orders of insects (coleoptera, lepidoptera and diptera), (C. Zhong, 1996). The cloning of other toxin genes which display no sequence similarity to the *cry* or *cyt* genes also demands a new system of classification (Brown and Whiteley, 1992). For these reasons a nomenclature has been devised which relies solely on amino acid similarity between the full length toxin sequences (Crickmore *et al.*, 1995). The scheme is to be published and is currently available on the WorldWideWeb http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html.

δ-ENDOTOXINS: GENE LOCATION AND EXPRESSION

The cloning of the *cry*1Aa1 gene from a Bt ssp *kurstaki* HD1 Dipel plasmid provided the first direct evidence that these genes were located on plasmids (Schnepf and Whiteley, 1981). Some Bt plasmids are also capable of conferring crystal production on recipient acrystalliferous Bt or *B. cereus* (Gonzalez and Carlton, 1982) by conjugal transfer. Despite difficulties in discriminating between large (>150 MDa) plasmids and sheared chromosomal DNA fragments there have been suggestions that some δ-endotoxin genes may reside on chromosomal DNA. The gene for the recently identified Cry toxin in *Clostridium bifermentans* subsp. *malaysia* (Barloy *et al.*, 1996) is chromosomally located. Bt δ-endotoxins are also often found in close association with mobile genetic elements such as Insertion Sequences and transposons (Mahillon *et al.*, 1994). Such regions may well facilitate the transfer of δ-endotoxin genes not only between plasmid and chromosomal DNA, but also between plasmids (Whiteley and Schnepf, 1986). The existence of Bt δ-endotoxin genes as part of composite transposons on single conjugative plasmids may be an important mechanism whereby Bt can create new strains with novel activities and host spectra.

With the exception of *cry*3A from Bt ssp *tenebrionis* (Sekar, 1988), δ-endotoxin gene expression is restricted to the stationary or sporulation phase of the growth cycle . Transcripts of *cry*3A and crystal protein antigens could be detected in vegetatively growing cells (Sekar, 1988). A *cry*5 gene has been cloned which is expressed from early stationary phase (Kostichka *et al.*, 1996). It was also found that the protein product was exported into the supernatant. S1 nuclease mapping was used to identify two adjacent transcriptional start sites (BtI and BtII) for a ssp *kurstaki* HD1 Dipel gene (Wong *et al.*, 1983). These were found to be used sequentially during sporulation; promoter BtI was active during stages II and III of sporulation, whereas BtII was active from Stage III onwards. The high level of δ-endotoxin synthesis during sporulation has been partly attributed to sporulation-produced mRNA which is approximately six times more stable than mRNA in vegetative cells (Petit-Glatron and Rapoport, 1976). There is some evidence that this stability arises from the presence of potential stem-loop structures at the 3' end of the transcript (Whiteley and Schnepf, 1986; Ward and Ellar, 1986; Chungjatupornchai *et al.*, 1988; Widner and Whiteley, 1989) which protect it from exonuclease degradation.

Toxin gene expression has also been shown to be regulated post-translationally. In *E. coli*, expression of cloned *cyt*1A increased substantially, provided a region of DNA located 4 kb upstream of the *cyt*1A promoter was present (McLean and Whiteley, 1987). This region contains a gene encoding a 20 kDa protein which appeared to enhance the production of Cyt1A (Adams *et al.*, 1989). The 20 kDa polypeptide acted post-translationally, occurred in small amounts in the CytA inclusion and possibly protected the δ-endotoxin from proteolysis (Visick and Whiteley, 1991). The related Cyt2A however, shows no requirement for the 20 kDa protein for efficient expression, probably reflecting its greater resistance to proteolysis (Koni and Ellar, 1993).A similar observation has been noted for Cry2Aa. The *cry*2Aa gene occurs in a three gene operon (*orf*1, *orf*2, *cry*2Aa) (Widner and Whiteley, 1989). Expression of *cry*2Aa

was dramatically reduced in the absence of *orf*2 and no Cry2Aa inclusions were observed (Crickmore and Ellar, 1992). It has been suggested that Orf2 acts as a molecular chaperone, either assisting in Cry2Aa crystal formation or protecting the δ-endotoxin from proteolytic degradation (Crickmore and Ellar, 1992). Disruption of *orf*1 had no obvious effect on *cry*2Aa expression, but its role remains intriguing since it has been identified as part of the *cry*2A, *cry*2C, *cry*11A and *cry*9C operons (Widner and Whiteley, 1989; Wu *et al.*, 1991, Dervyn *et al.*, 1995 and B. Lambert, unpublished).

δ-ENDOTOXIN MECHANISM: SOLUBILISATION AND PROTEOLYTIC ACTIVATION

Bacillus thuringiensis δ-endotoxins are synthesised as inactive protoxins which are insoluble in water but solubilise in the insect midgut where the protoxin is converted to the active form by 'trimming' with gut proteases. The activated toxin then binds to and inserts into the membrane of midgut epithelial cells via insect-specific receptors, creating transmembrane leakage pores that cause cell swelling and disruption by colloid osmotic lysis (Knowles and Ellar, 1987). The insect gut consists of three regions, the fore, mid and hind gut. Since the foregut and hindgut are protected by a cuticle layer, the first area of exposed tissue encountered by the activated toxin is the mid-gut region and this is therefore likely to be the site of action of the δ-endotoxin. Studies of the mechanism of action of these toxins, principally in lepidopteran insects, (Knowles and Dow, 1993) have highlighted four main factors that appear to govern the action of these toxins in target insect midguts: crystal solubilization, protoxin activation, receptor binding and pore formation (Knowles, 1994).

The processes of solubilization and activation in the midgut of susceptible Lepidoptera and Diptera are quite well understood. These insects have an alkaline-reducing midgut environment containing digestive enzymes suitable for the dissolution and activation of δ-endotoxins active against these insect species (Dow, 1986). The third major insect target group susceptible to Bt is the Coleoptera. Coleopteran larval midguts are generally neutral to acidic. Cry3A, the first δ-endotoxin discovered with coleopteran activity (Krieg et al., 1983) is most active against the beetle family Chrysomelidae (leaf beetles). The larvae of these beetles appear to have a midgut pH range of 4.5-6.6 (Grayson, 1958; Koller et al., 1992).

Evidence for the requirement for proteolytic activation was demonstrated by the lack of toxicity displayed by solubilised protoxins (compared to solubilised and protease treated protoxins) injected into the haemocoel of lepidopteran larvae (Lecadet and Martouret, 1967), or exposed to cultured insect cell lines (Johnson, 1981). Undoubtedly, the process of inclusion solubilisation and proteolytic processing occurs simultaneously. The midgut lumen of lepidopteran and dipteran larvae contains a variety of proteases including serine proteases which are optimally active in the alkaline pH of the insect midgut. Dipteran larvae have been shown to be rich in chymotrypsin-like and trypsin-like enzymes (Yang and Davies, 1971; Kunz, 1978) which have been demonstrated to activate protoxin molecules *in vitro*. Midguts of susceptible coleopteran larvae contain mainly cysteine proteases which are active under the mildly acidic coleopteran gut environment (Thie and Houseman, 1990; Purcell *et al.*, 1992).

Proteolytic processing of the δ-endotoxins can occur at the C-terminus, the N-terminus or both, depending on the δ-endotoxin class. Lepidopteran-specific Cry1 toxins (120-140 kDa) are processed to toxic fragments with molecular weights of 60-70 kDa. This active moiety is derived from the amino terminal half of the protoxin with the removal of 500 to 600 amino acid residues from the C-terminus and the first 27 to 29 residues at the N-terminus (Schnepf and Whiteley, 1985; Höfte *et al.*, 1986; Adang *et al.*, 1985; Sanchis *et al.*, 1989). In contrast to the lepidopteran toxins the 72 kDa Cry2A proteins undergo little or no proteolysis at their C-terminus but are more extensively degraded at their N-terminus (Nicholls *et al.*, 1989). The diptericidal Cry4A and Cry4B δ-endotoxins appear to be activated in a similar way to the lepidopteran Cry1 toxins; the activity being located in the N-terminus (Angsuthanasombat *et al.*, 1991; Chilcott and Ellar, 1988; Chungjatupornchai *et al.*, 1988). Cry11A appears to be cleaved into two halves of 30 and 35 kDa (Chilcott and Ellar, 1988), but it is not known if one

or both fragments are required for toxicity. *In vitro*, Cry3A does not readily solubilize in the neutral to acidic gut pH conditions reported for susceptible beetle larvae (Koller *et al.*, 1992). Carroll et al (1997) investigated this paradox by examining the properties of the Cry3A toxin after various proteolytic treatments. In many cases the toxin was cleaved into polypeptides that remained associated in non-denaturing conditions. Interestingly a chymotrypsinized Cry3A product was soluble at neutral pH at 3 mg/ml, retained full activity against susceptible beetle larvae and exhibited specific binding to *Leptinotarsa decemlineata* midgut membranes. A recent report (Novillo-Almendros *et al.*, 1996) indicates that chymotrypsin-like enzyme activity is present in the midgut of *L. decemlineata*.. SDS-PAGE and protein sequencing demonstrated that chymotrypsin cleaves the toxin into three polypeptides of 49, 11 and 6 kDa, nicking at the beginning of α-helix 4 in Domain I and at the end of β-sheet 19 in Domain III (Fig 1), although the 11 kDa polypeptide may be further processed. Remarkably these polypeptides remain associated in solution presumably held together by non-covalent interactions within the Cry3A structure. The introduction of two nicks into the primary structure will increase the charge on the protein and may account for the increased solubility, although associated conformational changes cannot be ruled out. This result raises the question of whether Cry3A *in vivo* activity requires all three of the polypeptides produced by chymotrypsin. This is currently being studied, but reports that deletion mutagenesis (Höfte and Whiteley, 1989) resulting in N- and C-terminal truncations within analogous regions of other Cry proteins yielded non-toxic proteins, would tend to support a role for all the Cry3A segments.

Cry3A domain I inter-helical proteolytic nicking may introduce the flexibility into the Cry toxin structure needed to allow unfolding and penetration of all or part of the toxin structure into target insect membranes (Li *et al.*, 1991). We have also found that a Cry1B protein is proteolyzed between putative helices 3 and 4 when treated with gut extract from *P. brassicae*, a susceptible insect species (Carroll & Ellar, unpublished). Cry1Ba is reported to exhibit dual lepidopteran and coleopteran toxicity, with the coleopteran activity being enhanced by prior solubilization and trypsin treatment (Bradley *et al.*, 1995). Conceivably inter-helical processing in domain I is also important for its coleopteran activity. In addition to Cry3A and Cry1Ba, proteolysis within putative domain I regions with retention of toxic activity has been reported for both Cry4B (Angsuthanasombat *et al.*, 1993) and Cry2Aa (Nicholls *et al.*, 1989). Interestingly when the arginine at the Cry4B α5-α6 interhelical site was replaced by alanine (Angsuthanasombat *et al.*, 1993) the mutant toxin lost toxicity to *Aedes* cell lines *in vitro* but was at least twice as active as the wild type toxin *in vivo* against *A. aegypti* larvae. Although this result might suggest that prevention of interhelical proteolysis enhances toxicity, comparison with the Cry3A atomic structure (Li *et al.*, 1991) suggests that the interhelical loop in which the Cry4B cleavage occurs is exposed to the solvent and it is possible that in the gut environment, cleavage may occur at several additional positions in this loop which may only be rendered accessible through a conformational change resulting from the combined effect of the gut environment and the toxin binding to its receptor. If only one or a subset of these *in vivo* cleavages is necessary and sufficient to trigger the required conformational change, these various cleavage sites may possibly constitute alternative parallel routes for initiating membrane penetration.

The Cyt1A and Cyt2A δ-endotoxins both require proteolytic activation to express their toxicities (Drobniewski and Ellar, 1989; Knowles *et al.*, 1992; Koni and Ellar, 1994, Chilcott and Ellar, 1988). For both toxins proteolytic processing occurs mainly at the N-terminus (Koni and Ellar, 1994).

Although toxicity is dependent on activation, with one exception, the specificity of the activated toxin is not dependent on the source of the protease. When the dual specificity Bt subsp. *aizawai* IC1 130 kDa toxin was activated with lepidopteran gut proteases, it was cleaved to a 55 kDa lepidopteran-specific toxin, which on further processing with dipteran gut proteases resulted in a 53 kDa dipteran-specific toxin (Haider and Ellar, 1987).

δ-ENDOTOXIN RECEPTORS

Following solubilisation and activation the activated toxin has to pass through the peritrophic membrane in the insect midgut to gain access to the site of toxin action on the brush border membrane of midgut epithelial cells. The peritrophic membrane is a coarse fibrous mesh of chitin, protein and carbohydrate permeable to macromolecules of 60 kDa or less (Brandt *et al.*, 1978; Adang and Spence 1982). It remains to be seen whether this membrane has any role to play -negative or positive - in modulating the potency of these toxins. To account for the high degree of insecticidal specificity observed, Knowles and Ellar (1987) proposed that in an initial step prior to pore-formation, the δ-endotoxin binds to a specific receptor present on the midgut cells of susceptible insects. Studies using brush border membrane vesicles (BBMV) prepared from insect larval guts (Hofmann *et al.*, 1988a,b; Van Rie *et al.*, 1989, 1990a) and *in vitro* studies using insect cell lines (Knowles and Ellar, 1986; Haider and Ellar, 1987) provided convincing evidence for the existence of such receptors. Cry1Ac and Cry1Ab toxin binding proteins have now been purified, identified and cloned from *M. sexta* , (Knight *et al.*, 1994 and 1995; Vadlamudi *et al.*, 1995) as has the Cry1Ac receptor from *H. virescens,* (Gill *et al.*, 1995). The Cry1Ac receptors from both *M. sexta* and *H. virescens* are Aminopeptidase-N, a 120kDa major transmembrane glycoprotein in the brush border epithelial cell membrane. In the case of *M. sexta* the evidence indicates that the receptor belongs to the group of membrane proteins that are attached to the membrane surface via a glycosyl phosphatidyl inositol (GPI) anchor. (Garczynski & Adang, 1995; Knight *et al.*, 1995, Lu & Adang, 1996). Sangadala *et al.*, (1994) have purified the 120 kDa receptor from *M. sexta* using isoelectric focusing and immunoaffinity chromatography. When the purified protein was reconstituted into phospholipid vesicles it increased toxin binding by 35% and lead to a 1000 fold increase in the release of $^{86}Rb^{+}$ from the vesicles. A partially purified 100 kDa CryIA(c) binding protein from solubilised *L. dispar* BBMV was also found to be an aminopeptidase-N, (Valaitis *et al.*, 1994 and 1995; Lee *et al.*, 1996). The 210 kDa CryIA(b) receptor from *M. sexta* has been identified as a member of the cadherin family of proteins, (Vadlamudi *et al.*, 1993 and 1995), which have been associated with cell aggregation and sorting. The cDNA for this gene has now been subcloned into a mammalian expression vector and transfected into COS-7 cells, (Vadlamudi *et al.*, 1995).

The Cyt toxins appear to differ from the Cry toxins in that *in vitro* they display broad spectrum cytolytic activity, lysing most eukaryotic cells tested (Thomas and Ellar, 1983a). This has been attributed to their high hydrophobicity and ability to bind to unsaturated phospholipids (Thomas and Ellar, 1983a; Drobniewski and Ellar, 1989; Knowles *et al.*, 1992). Why therefore are Cyt toxins specific to dipteran larvae *in vivo*. Immunohistological studies involving sectioning of mosquitoes after feeding of Bt ssp *israelensis* inclusions, revealed that the Cry4 and Cyt1A components were localised to the gastric caeca and posterior stomach of the insect midgut (Ravaohangimalala *et al.*, 1993). However, separate incubation of Cry4 or Cyt1A with these sections of the mosquito midgut resulted in Cry4 still being localised to these specific regions whilst CytA was detected in nearly all the midgut cells (Ravaohangimalala and Charles, 1995). Therefore, although CytA appears to bind to the midgut in a non-specific manner, in the presence of the Bt ssp *israelensis* Cry toxins it is targeted to the same specific regions. The rate limiting step in pore formation by Cry toxins are the reversible binding to the receptor and membrane insertion, which is irreversible. However the rate limiting step for Cyt toxins *in vitro* is oligomerisation after binding to the membrane (Maddrell *et al.*, 1988.) If the presence of Cry toxins results in the Cyt toxin binding preferentially to the regions bearing specific Cry toxin receptors compared to none selective and more widely separated binding, then the local concentration of Cyt toxin in these regions might be increased to the point where self-association into pores is favoured. This is one possible explanation for the synergism between Cry and Cyt toxins observed with dipteran insects. There is considerable evidence that phospholipids with an unsaturated chain at the *syn*-2 position act as the ubiquitous receptor for Cyt δ-endotoxins (Thomas and Ellar, 1983b; Drobniewski and Ellar, 1989). Phosphatidylethanolamine is represented in dipteran insect membranes in greater quantities than other orders of insects (Fast, 1966; Luukkonen *et al.*, 1973; Jenkin *et al.*, 1976) and this is

associated with a higher proportion of unsaturated fatty acids in the phospholipids. Therefore if unsaturated phospholipids are the receptor for Cyt toxins then the higher abundance in dipteran membranes could account for the preferential dipteran toxicity of the Cyt toxins *in vitro* and *in vivo* (Li *et al.*, 1996).

PORE FORMATION AND CELL LYSIS

After binding to a specific receptor on the brush border membrane of insect midgut epithelial cells, activated toxins insert irreversibly into the plasma membrane of gut cells. Bt toxin:receptor interactions are biphasic, involving an initial reversible binding step which rapidly becomes irreversible (Van Rie *et al.*, 1989). The irreversible phase of the interaction is thought to reflect insertion of the toxin into the membrane which leads to the formation of a pore or lesion in the midgut epithelial membrane. This in turn results in the creation of leakage channels in the membrane which leads to cell lysis and eventually the death of the larvae (Knowles and Ellar, 1987). Both Cry and Cyt toxins have been shown to form pores in planar lipid bilayers and liposomes (Slatin *et al.*, 1990; English *et al.*, 1991; Schwartz *et al.*, 1993; Smedley *et al.*, 1997, Knowles *et al.*, 1989, 1992). Experiments done *in vitro* with cultured insect cell lines showed that both Cyt and Cry toxins elicited a graded efflux of molecules from cells and that cytotoxicity could be delayed or inhibited by osmotic protectants such as raffinose that are too large to penetrate the toxin-induced pores (Knowles and Ellar, 1987; Drobniewski *et al*, 1987). Using this approach a pore diameter of 1-2 nm was calculated for several Bt δ-endotoxins. Based on these results Knowles and Ellar (1987) proposed that Bt toxins kill cells by colloid osmotic lysis. Following receptor binding, toxins insert into or interact with the membrane to form a non-specific pore. Trans-epithelial ion gradients are collapsed by the leak, and there is an osmotically driven influx of water, resulting in cell swelling and eventual lysis.

The use of light scattering and carboxyfluorescein self-quenching to measure intravesicular volume changes in *Manduca sexta* midgut-brush-border-membrane vesicles (BBMV) treated with toxin (Carroll and Ellar, 1993,1997) has provided further evidence for the colloid osmotic lysis model, by showing that Cry1Ac induces or form pores freely permeant for raffinose (1.14 nm diameter.) Using non-electrolytes of increasing size the pores were estimated to have a limiting diameter of approximately 2.4-2.6 nm under alkaline pH conditions. Recent evidence suggests that Cry1Ac can form a 2 nm diameter pore in planar lipid bilayers containing fused *M. sexta* midgut BBMV (Martin & Wolfersberger, 1995). Cry toxin channel formation in lipid bilayers in the absence of receptors has been reported (Slatin et al., 1990; Schwartz et al., 1993; Grochulski *et al.*, 1995). This activity in lipid bilayers may be the result of a particular toxin activation regime (Smedley et al., 1997) or because the relatively high toxin concentration used (15-500 nM) in the experiment favoured channel formation.

THREE DIMENSIONAL STRUCTURES

Two of the most important advances in Bt δ-endotoxin research in recent years has been the elucidation of the three dimensional crystal structure of two Cry toxins (Cry3A; Li *et al.*, 1991 and Cry1Aa; Grochulski *et al.*, 1995) and a Cyt toxin (Cyt2A; Li *et al.*, 1996). The Cry3A toxin structures is composed of three clearly distinct domains (Figure 1). (The Cry1a structure solved by Grochulski *et al.* (1995) showed a very similar overall structure to Cry3A with an eight helix bundle in domain I and equivalent folds in domains II and III.) It is immediately apparent that domain I and domain II are likely to be pore-forming and receptor binding regions respectively (Li *et al.*, 1991). Reports that mutagenesis of loop regions in domain II affects toxin receptor binding, whereas domain I mutants have different pore-forming abilities (Smith and Ellar, 1994; Smedley and Ellar, 1996; Wu and Aronson, 1992; Chen *et al.*, 1995) support this view. In Cry3A, Domain I, from the N-terminus of the molecule to residue 290, is a seven helix bundle in which a central helix (α5) is surrounded by six outer helices. Five of these helices (α3-α7) are long enough to span a 30Å thick membrane bilayer, suggesting that these are candidates for forming the lytic pore in the insect membrane. However, the conformation

of domain I would have to be reversed during pore-formation to provide a hydrophobic outer layer contacting the lipid bilayer and a hydrophilic inner surface forming an aqueous channel. It was suggested that Cry3A domain I inter-helical proteolytic nicking may introduce the flexibility into the Cry toxin structure needed to allow unfolding and penetration of the structure

Figure 1 The three dimensional structure of CryIIIA (Li *et al.*, 1991 The three domains are: I, a seven helix bundle (upper left): II, a three-sheet assembly (bottom) and: III, a β sandwich (upper right).

into target insect membranes (Li *et al.*, 1991). Confirmation that this nicking takes place between helix 3 and 4 in Cry3A has recently been obtained (Carroll *et al.*, 1997). Our laboratory has also found that Cry1B is proteolysed between putative helices 3 and 4 when treated with gut extract from *P. brassicae*, a susceptible insect species (Carroll, unpublished). In addition to Cry3A and Cry1B, proteolysis within putative domain I regions with retention of toxic activity and no fragment separation has been reported for both Cry4Ba (Angsuthanasombat *et al.*, 1993) and Cry2Aa (Nicholls *et al.*, 1989). Domain II of the Cry toxins comprises folded β-strands surrounding a groove-like cavity terminating in the three exposed loops (β-prism). These three loops located at the apex of Domain II are strikingly reminiscent of the antigen recognition site of an immunoglobulin and were therefore suggested as a possible receptor recognition region (Li *et al* 1991). Previous work (Knowles and Ellar, 1986; Knowles *et al.*, 1991) has indicated that the specificity of the Cry toxins is determined by the oligosaccharides attached to the receptor. Indirect evidence that this β-prism structure is a carbohydrate-targeting binding site came initially from a comparison of Cry toxin structure with the crystal structure of a protein (VM0-I) found in the vitelline membrane of hens eggs which is composed *entirely* of this β-prism structure (Shimizu *et al.*, 1991) and which is thought to function as an oligosaccharide binding protein. More compelling indirect evidence for role of Domain II in receptor recognition comes from the recent description of the X-ray structure of the plant lectin, jacalin (Sankaranarayanan *et al.*, 1996). Each subunit of this tetrameric protein contains the β-prism fold which can be superimposed on the corresponding Cry toxin domain. Jacalin binds specifically to a tumour associated T-cell disaccharide and the X-ray structure of the form with bound methyl-α-D-galactose shows that the carbohydrate binding site of jacalin is composed of the exposed loops that connect the folded β-strands.

Domain III, from residues 501 to 644 at the C-terminus, is a compact sandwich of antiparallel

β-sheets originally proposed to protect the active 60-70 kDa N-terminal of the Cry toxins from further proteolysis, (Li *et al.*, 1991). More recently it has been demonstrated that domain III is also involved in determining toxin specificity. Domain III exchange experiments between Cry1Aa and Cry1Ac hybrids demonstrated that residues 451-623 from Cry1Ac directed binding to a 120 kDa aminopeptidase-N receptor from *Lymantria dispar*. The same residues from Cry1Aa resulted in a hybrid toxin that did not bind the 120 kDa receptor but did bind to a 210 kDa protein, (Lee *et al.*, 1995).

Further domain II/III swapping experiments between Cry1C and Cry1Ab, (de Maagd *et al.*, 1996a), and between Cry1Ab and Cry1Ac, (de Maagd *et al.*, 1996b), have shown the importance of domain III in toxicity and ligand binding specificities of Cry toxins. The initial Cry1C / Cry1Ab domain swapping experiments took domains I and II from Cry1Ab and domain III from Cry1C. This hybrid toxin was found to be highly toxic toward *S. exigua* compared to Cry1Ab and significantly more toxic than Cry1C. In semi-quantitative binding assays the hybrid toxin was reported to bind BBMV from *S. exigua* larvae but in direct ligand binding assays binding to a 205 kDa CryIA(b) binding protein had been abolished. The reciprocal hybrid formed from domains I and II of Cry1C and domain III of Cry1Ab did retain binding specificity toward the 205 kDa Cry1Ab receptor. The second set of domain swapping experiments using Cry1Ab and Cry1Ac gave some unexpected results with domain III of Cry1Ac appearing to direct hybrid toxin binding to a 120 kDa major binding protein in both *M. sexta* and *S. exigua* BBMV, whilst Cry1Ac domains I/II appeared to direct binding to a 210 kDa protein from *M. sexta* BBMV. In contrast domain II from Cry1Ab directed toxin binding to a 210 kDa protein from *M. sexta* BBMV, which is presumably the same cadherin protein identified as the Cry1Ab receptor in *M. sexta*, (Vadlamudi *et al.*, 1993 and 1995). However, domain III from Cry1Ab bound to a 250 kDa protein. There has only been one previous report that native Cry1Ab binds a *M. sexta* BBMV protein of approximately this size, (Feldmann *et al.*, 1995). The results of these domain swapping experiments would appear to suggest that there is a complex relationship between receptor binding and domains II and III of the toxin.

The atomic structure of Cyt2A from Bt ssp *kyushuensis*. solved by Li *et al.* (1996) revealed it to have a single domain of α/β architecture but a novel connectivity comprising two outer layers of α-helix hairpins wrapped around a mixed β-sheet (Figure 2).

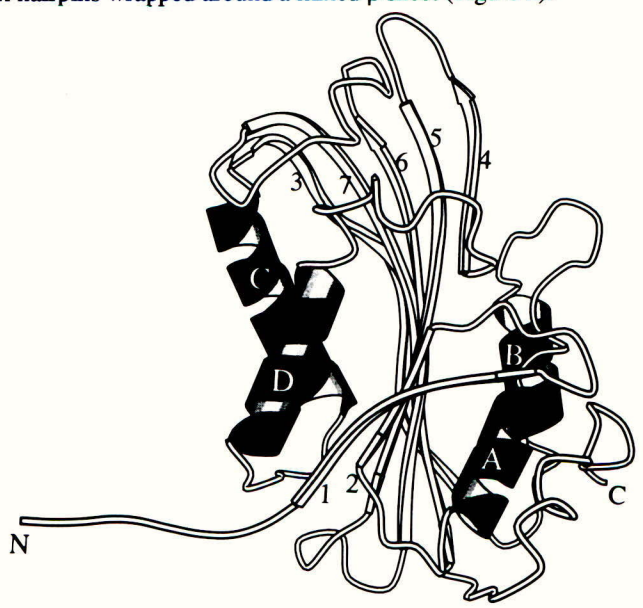

Figure 2 Schematic ribbon diagram of the Cyt2A monomer. The edge view shows the three layered α/β architecture (Li *et al.*, 1996).

plasmid encoded proteins in Bt, but the presence of other factors involved in virulence on these plasmids cannot be ruled out. In this study (Dunn & Ellar, submitted) we identified a novel genetic locus on the 130 MDa plasmid of Bt ssp *fukuokaensis* (*Btvir*) adjacent to the gene encoding a 24 kDa crystal component (Orf1). One of the genes in this locus, *btcap1* was found to have a Gram positive homologue in *S. aureus* implicated in the synthesis of the polysaccharide capsule of this organism and a Gram negative homologue in *E. coli* associated with the polysaccharide chain-length determinant of LPS. Most strains of *S. aureus* produce capsular polysaccharides which confer on the organism virulence and resistance to phagocytosis . The capsule has been shown to mask surface-bound antigen and thus prevent interaction with receptors on phagocytic cells. Capsular polysaccharide may prevent vegetative Bt which has invaded the haemocoel of the infected insect from being attacked by the host insect defence mechanisms.

A second gene in this locus, *btpk1* was found by database searches to show similarity only to eukaryotic protein kinases. The similarity has been concluded to be specific for two reasons. Firstly the range of organisms to which similarity was noted (from yeast and spinach to human protein kinases). Secondly the extent of similarity is not restricted to one or two subdomains of the protein kinases but all eleven subdomains are represented in the Btpk1 sequence. Ser/Thr protein kinases play essential roles in signal transduction in organisms ranging from yeast to mammals, where they regulate a variety of cellular activities. Although they have long been considered to be confined to eukaryotes, recently genes encoding eukaryotic-type protein kinases have been found in several bacterial species (Zhang, 1996).

While Ser/Thr protein kinases are known to be involved in eukaryotic cellular signal transduction it has been found that such a role can be directly associated with the virulence of a pathogenic bacterium. *Yersinia pseudotuberculosis* possesses a virulence plasmid (much in the same way as the 130 MDa plasmid of Bt ssp *fukuokaensis* can be regarded) which encodes a number of secreted proteins (Yops) (Cornelis, 1992). Amongst these Yops is the secreted protein kinase YpkA (Galyov *et al.*, 1993) which displays extensive homology to eukaryotic Ser/Thr protein kinases. Specific mutations in *ypkA* resulted in avirulent strains while the wild-type protein was found to mediate morphological changes in infected HeLa cells (Håkansson *et al.*, 1996). YpkA was shown to be transported into the HeLa cells and targeted to the inner surface of the plasma membrane where it would be able to interfere with cell-signalling apparatus (Håkansson *et al.*, 1996). It was therefore proposed that this protein kinase is an essential virulence factor in *Y. pseudotuberculosis*.

The phosphorylation of eukaryotic proteins involved in signal transduction would be of great importance in the virulence strategy of a bacterium. Virulence may however be mediated in other ways using protein kinases. Enteropathogenic *E. coli* (EPEC) have been found to stimulate the phosphorylation of host tyrosine residues (Rosenshine *et al.*, 1996). This phospohorylation was found to be essential for the adherence of the *E. coli* to the host cells and thus permit colonisation of the organism in the initial stages of pathogenesis. We are currently examining the role of Btpk1 in the virulence and specificity mechanisms of Bt ssp *fukuokaensis* and of other such protein kinases in different Bt subspecies.

Release of nutrients from the toxin-damaged gut epithelial cells has been shown to result in the lowering of the midgut pH and thereby provide a suitable environment for the spore to germinate (Dadd, 1975; Van-Nguyen, 1995). After germinating spores invade the haemocoel the expression of additional virulence factors could play a major role in pathogenicity/potency. For example the immune inhibitor A produced by vegetative Bt specifically degrades the antibacterial proteins (attacins and cecropins) produced by the insect. Other Bacilli which are not entomopathogenic have not been found to express this protein (Lövgren *et al.*, 1990). A new class of insecticidal proteins recently identified in Bt; the Vip proteins (Estruch *et al.*, 1996) are vegetatively expressed and share no similarity to the δ-endotoxin proteins produced by Bt. On the basis of protein similarity, we have proposed (Dunn & Ellar, submitted) that Btcap1 and Btpk1 may play a role as virulence factors. Expressed in vegetative cells, the Btcap1 protein may have a function in the production of the bacterial capsule. The capsule

In the protoxin form, Cyt2A exists as a dimer linked by the intertwined β-strands in a continuous 12-stranded β-sheet. Protease processing cleaves the intertwined N-terminal arm, including the β1 strand responsible for dimerisation, releasing the active toxin as a monomer (Koni and Ellar, 1994; Li *et al.*, 1996). Proteolytic activation therefore removes structural barriers to pore formation. It was proposed that membrane-bound Cyt toxin molecules oligomerise to form the trans-membrane pore (Maddrell *et al.*, 1988). Although both the Cry and Cyt toxins use the same cytolytic mechanism of pore formation, the 6 helices in the Cyt2A molecule are too short to span the 30Å width of the hydrophobic region in a biological membrane. Because β–strands β5, β6 and β7 are sufficiently long to span the bilayer and over these strands the sheet shows an amphiphilic or hydrophobic character, Li *et al.* (1996) proposed that the Cyt pore could be based on a β-barrel in which the strands oligomerise to form the β-barrel structure.

ADDITIONAL BACILLUS THURINGIENSIS POTENCY DETERMINANTS

Taxonomic studies have indicated that Bt is practically indistinguishable from *B. cereus* except for its capacity to produce crystalline δ-endotoxins (Drobniewski, 1993). Bt may thus be considered an opportunistic insect pathogen. Disruption of the cellular barrier between the gut lumen and the haemolymph by the δ-endotoxins leads to death or weakening of the susceptible insect thereby creating favourable conditions for spore germination in the gut environment and subsequent septicaemia.

The high potency of many of these δ-endotoxin proteins has tended to obscure the effects of secondary factors which are potentially involved in the enhancement of the pathogenic mechanisms of this bacterium. For Bt subspecies like *israelensis* whose δ-endotoxin crystals display an LC_{50} of between 9-12 ng/ml, the effect of secondary pathogenic mechanisms is not immediately noticeable. However, for some Bt subspecies (for example *kurstaki* HD1) the effect on the LC_{50} can be enhanced 150 fold in the presence of spores (Miyasono *et al.* (1994)). In this way it has been shown that for certain Bt subspecies the spore can play an important role in enhancing the effect of the δ-endotoxin crystal. Enhancement has been thought to result from germinating and vegetative Bt and not merely due to the physical presence of the spore (Van-Nguyen, 1995). The use of other bacillus spores has little effect on the potency of the bacterium and homogenates of spores or UV irradiated spores are also ineffective potentiators of potency. The ability to identify vegetative Bt in the haemolymph of larvae fed spores plus one fifth of the lethal dose of pure δ-endotoxin (Miyasono *et al.*, 1994) strongly suggests that bacterial infection resulting from the invasion of germinating Bt into the haemocoel from the gut is a major factor in reducing the LC_{50}. The specific ability of Bt spores to enhance toxicity could be due to the activity of other species specific factors such as spore resistance to gut conditions, speed of germination, spore adhesion to cell surfaces, "invasiveness" of the outgrowing spore and the possession of additional 'virulence' factors.

The previous identification of virulence factors in Bt such as β-exotoxins (Levinsson *et al.*, 1990; Moar and Trumble, 1987), phospholipase (Taguchi *et al.*, 1980), the immune inhibitor A protein which specifically degrades the antibacterial proteins produced by the insect (Dalhammer and Steiner, 1984), flagella (Lövgren *et al.*, 1993) and chitinase (Lüthy, 1980) could in some way explain the enhancement of potency resulting from spore germination. For example, chitinase which has been identified in several Bt strains would have a role in virulence by degrading the chitin component of the insect peritrophic membrane (Lüthy, 1980). Recent reports have shown that the pathogenic mechanisms of Bt are more complex than had been previously thought. Zhang *et al.* (1993) identified a pleiotropic mutant of Bt which displayed reduced levels of flagellin, β-lactamase and phospholipases suggesting that the expression of these factors is co-regulated. The recently discovered gene which regulates the expression of phosphatidyl choline-specific phospholipase-C in Bt (Lereclus *et al.*, 1996) could be one such global regulator of virulence.

We undertook an investigation to determine the genetic context of a gene whose product forms a component of the crystalline inclusion of ssp *fukuokaensis*. Little is known about the other

would also help Bt to evade the antibacterial mechanisms of the susceptible insect and thus allow the organism to proliferate in the haemolymph, and express the other secreted factors necessary for virulence. Btpk1 would be predicted therefore to have a more direct role in virulence in that if secreted, it could interfere with the cell-signalling apparatus of the insect larva and thus contribute to the pathogenic mechanisms.

For many years Bt has been known to possess other factors which could potentiate the effect of the δ-endotoxin for example the α and β exotoxins. It is however becoming clear that as an opportunistic insect pathogen Bt can rely on many more resources to fulfil its potential as an effective entomopathogenic bacterium. Improvement of the target range and potency of Bt will inevitably require a comprehensive understanding of these additional virulence factors.

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