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Report on Overseas Travel

R.J. Akhurst, CSIRO Division of Entomology, Canberra

Aim of Travel: To participate in the VIth International Colloquium on Invertebrate Pathology and Microbial Control. The Colloquium also incorporated the Second Iuternational Conference on *Bacillus thuringiensis*.

Summary:

- The Bt conference was held over the five days of the conference and covered issues such as resistance, novel Bt toxins, synergism between toxins and other bacterial components, mode of action of Bt toxins, genetics, and transgenic plants.

- To date field populations of only two species are known to have developed resistance to Bt. However, there was a new report of resistance to *Bacillus sphaericus* in mosquitoes in Brazil. Resistance to Bt endotoxins has now been detected in 11 species of insects through laboratory selection, including resistance to the CryIC toxin by *Spodoptera exigua* and *S. littoralis*; in most of these, the resistance is broad-spectrum but the mechanisms are not yet identified. Various selection procedures (crystals + spores, crystals alone, activated toxin) have been used successfully. Resistance in *S. exigua* was apparently linked to an inducible detoxification mechanism.

- Selection with multiple toxins showed that pyramiding toxins decreased the rate at which resistance occurred but did not prevent resistance developing in *Culex quinquefasciatus* selected against CryIV and Cyt toxins.

- There are now 49 different Bt endotoxins identified. A new system of classification of the toxins is being developed by an international committee and will be pnblished shortly.

- Only one paper on transgenic plants was presented at the meeting. Ciba-Geigy reported on its transgenic maize that utilises CryIA(b) under the control of tissue-specific promoters (green tissue, pollen-specific, pith). Good control but some leakiness with tissue-specific promoters was reported. When the CaMV 35S promoter was used Bt production declined as the plants matured; there was no corresponding decline with the tissue-specific promoters.

- *H. armigera* was reported to be most sensitive to CryIA(c), *ca* 15-fold less to CryIA(b) further 6-fold less to CryIA(a); there was no measurable susceptibility to Cry IB, IC, or ID.

- Clones of *cryIA(c)* were reported to produce toxins that differed significantly, with LC_{50}s ranging between 5.6 and 23.9.

VIth International Colloquium on Invertebrate Pathology and Microbial Control/Second International Conference on *Bacillus thuringiensis*

The Colloquium was attended by 600 participants from 55 countries who presented 470 papers and 161 posters and was held over five days (August 29 - September 2) in Montpellier, France. On each day the Colloquium commenced with a plenary symposium before going to concurrent sessions that covered all aspects of invertebrate pathology (bacteria, viruses, protozoa, fungi, nematodes).

Bt Issues

1. New Toxins/Host Range

There are currently 49 different Cry toxins in the databank and some new cyt toxins. A new classification system being developed by an international committee is expected to be finalised by December. This system will be based only on amino acid sequence homology, not host range.

As well as activity for various Lepidoptera, Coleoptera (including Scarabaeidae), Diptera, and Nematoda, Bt isolates with activity for Orthoptera were reported. The latter were some Spanish Bts with some, not terribly high, activity for locusts; a *kurstaki* strain with 140 and 130kDa toxins only, not exotoxin; there were also two isolates of *konkukian* and three of *aizawai* that showed some activity.

Two other Gram positive spore-forming bacteria, *B. popilliae* and *C. bifermentans,* have been reported as having toxins with sequence homology to Bt endotoxins. *Bacillus sphaericus*, although superficially similar to Bt, has binary (51 + 42kDa) and MTX (100kDa) toxins that have no sequence homology to the Bt toxins. Only DNA homology group IIA *B. sphaericus* produce these toxins; it is likely that *B. sphaericus* will be subdivided into several species

2. Structure

Previously the only Bt toxin for which the crystal structure had been elucidated was CryIIIA. Since the mode of action of this coleopteran toxin differs from that of the CryI toxins in Lepidoptera the general applicability of the CryIIIA model had been questioned. At the Colloquium Grochulski *et al.* presented a paper on the crystallography of CryIA(a) that showed that it has same 3 domains as CryIII with differences from CryIIA in the structure of the putative binding region but not in the putative pore forming region. Yamamoto & Powell (Sandoz) also presented a molecular modelling analysis of CryIA(b) that was in general agreement with Grochulski.

Although domain III is considered a hinge domain and II is thought to be the binding domain, Bosch *et al.* showed by interchanging domains of CryIC and CryIE that changes in domain III affected specificity. Donald Dean reporting his mutagenesis studies offered the opinion that domain III was involved in determining specificity. In commenting on domain I function he noted that changes in this domain reduced toxicity for one species but had no impact on toxicity for another. He also found that

changes in domain II of CryIIIA that reduced binding for *Tenebrio molitor* actually increased toxicity for this species, presumably because it enhanced the ability of the toxin to insert into the membrane and form a pore.

3. Mode of Action

An aminopeptidase N has been identified as the receptor for CryIA(c) in *Manduca sexta* by Ellar's group at Cambridge and Adang's group at the University of Georgia. Ellar's group has cloned most of the gene; their cDNA clone is truncated at 5' end. They have shown that it has high homology to mammalian aminopeptidases but lacks the hydrophobic N-terminal; Adang reported that the *M. sexta* aminopeptidase is bound to the membrane by a glycosyl phosphatidylinositol anchor which is apparently critical in establishing the correct steric relationship to allow the toxin to create a pore in the membrane. In mammals aminopeptidase N has also been implicated as a receptor (e.g. for coronaviruses in pigs).

CryIA(c) recognises carbohydrate determinants on the aminopeptidase N receptor. It is not yet clear whether some amino acids may be also be involved. The non-specific binding that has been detected with CryIA(c) in various insects may result from the carbohydrate determinant occurring on other proteius. However, binding will not lead to toxicity unless these other proteins are in correct steric relationship to membrane.

Ferré *et al.* reported that CryIA(a) and CryIA(c) recognise the same receptor protein in *M. sexta* and in *L. dispar* bnt not in *H. virescens* and suggested that the receptor binding story may not be complete. However, it may be that CryIA(a) and CryIA(c) recognise different carbohydrate determinants that happen to occur together on a protein in some species but on differeut proteins in others.

Ligand binding studies show only one binding protein in the *M. sexta* BBMV but multiple bands (ca 6) with *H. zea*, *H. virescens* and *Trichoplusia ni* BBMV proteins. The toxin also binds to bands with aminopeptidase activity in *H. virescens* though in neither *M. sexta* nor *H. virescens* do binding and aminopeptidase activities correlate. After *H. virescens* BBMV proteins were column purified three positive binding fractions were identified. Cowles *et al.* have cloned the highest aminopeptidase activity gene but have not determined if it binds CryIA(c).

Feldman reported some binding of CryIVD to *M. sexta* BBMV (but no binding of CryIA(b) to *Tipula* or *Anopheles* BBMVs). Ligand blotting revealed 3 bands binding CryIVD for *Tipula* and *Anopheles* but only 1 major band for each (78 and 148kDA, respectively).

5. Resistance

Georghiou noted that there is now resistance to Bt in field populations of two species and in laboratory populations of 11 species. He also reported that resistance to *B. sphaericus* toxins has been detected in *Culex quinquefasciatus* in Brazil.

Ferré *et al.* obtained 31-fold resistance in 7 generations of selection with *Trichoplusia ni*. Although CryIA(b) and IA(c) competed in in vitro binding assays the resistance

was only to CryIA(b). Does the very high non-specific binding by CryIA(c) confer toxicity, are the IA(b) and IA(c) receptors different but on the same protein, or is resistance due to other factors? Unfortnnately the culture was lost to viral infection and these questions could not be answered.

Two reports of resistance to CryIC in *Spodoptera* spp. were reported. Moar, using purified crystal, selected *S. exigua* for resistance to CryIC. After resistance was detected (9-12 generations) he selected with trypsin-activated toxin and with protoxin and found that resistance increased more rapidly in those insect challenged with the activated toxin (1000-fold after 32 generations). He found no resistance in *S. exigua* from selection against spore/crystal preparation of HD-1 (no CryIC) and no resistance to formulated products. The resistant S. exigua were also cross resistance to CryIH and CryIIA but not to XenTari (IA(a), IA(b), IC, ID, IIB). Differences in binding affinity did not account for level of resistance detected. There is apparently an inducible detoxification mechanism - if grown to 3-4th instar before exposnre to toxin, the resistant line was susceptible. Moar suggested that rapid regeneration of stem cells (as proposed by Whalon for CPB) may be the mechanism. Resistance in *S. exigua* was stable over 10 generations without selection. There was synergism between CryIC and spore for susceptible and resistant strains, but synergism with exotoxin was limited to the resistant strain.

The results reported for *S. littoralis* by Müller-Cohn differed in several respects from those of Moar. Selection of *S. littoralis* against *cryIC* in a *cry*$^-$ strain produced 10-50-fold resistance in a strain that had been maintained as a laboratory population for 20 years. Resistance was detected in 5 generations and involved cross-resistance to CryID and CryIE but not CryIF; it was not as resistant to *aizawai* as expected. The resistance was a recessive trait and was not stable; it was lost in 3 generations of non-selection. No mechanism was postulated.

Bauer selected cottonwood leaf beetle (Chrysomelidae) for resistance to CryIII using the CellCap formulation M-Trak. Resistance was detectable at 7 generations and rose to >30,000-fold by 30 generations. No differences in gut pH or protease could be detected.

Heckel reported his progress on identifying markers for Bt resistance in *H. virescens*. He has a linkage map for all 31 chromosomes. In the Monsanto resistant line resistance loci map to 3 linkage groups (3, 11, 22) whereas in the highly resistant Gould line (YH-2) resistance mapping to linkage group 9 accounts for 80% of the 3000-fold resistance.

McGaughey & Johnson selected *Culex quinquefasciatus* against one or more CryIV toxins and CytA. The development of resistance was slowed but not prevented by the use of multiple toxins.

Field and laboratory populations of *C. quinquefasciatus* have developed resistance to the *B. sphaericus* binary toxin (10-fold and 10000-fold, respectively). Resistance in the laboratory selected line involved a change in receptor; field resistance did not.

Hughes presented a plenary symposium paper on insect resistance to viruses. Resistance is known more with granulosis viruses rather than with NPVs but this may be a distortion due to the relative numbers of studies condncted. Only one field resistance - *Phthorimaea operculella* - has been recorded. Hughes concluded that resistance to insect viruses was more likely to develop at the establishment phase (primary and secondary infection) and so there was unlikely to be a greater risk with engineered viruses.

6. Transgenic Plants

The only paper on transgenic plants presented at this major meeting on Bt was on the Ciba-Geigy maize with *cryIA(b)* for *Ostrinia nubilalis* control. The toxin gene is under tissne-specific expression - green tissue, pollen-specific, pith promoter - in these plants. Little in the way of data was presented bnt the authors acknowledged that there was some leakiness with tissne-specific promoters. They also reported that there was a drop in toxin production (ng/mg tissue) over 7 weeks with the CaMV35S promoter but no drop with tissue-specific promoters.

7. Genetics

Lereclus and Peferoen presented general reviews of the potential for improving Bt strains through classical and molecnlar genetic strategies. The issnes covered were expansion of the activity spectrum (conjugation, cloning, altering binding to access alternative receptors), enhancing toxicity (synergism by adding new genes, engineering to alter the efficiency of binding and of pore formation), enhanced toxin production (gene regulation and copy number), resistance management (introdnction of additional genes with different specificity domains), and improving persistence (through genetic engineering). Ellar reported that mutation has been used to enhance toxicity by 4.5 to 10-fold.

Baum reported on the use of transposon mutagenesis with Tnp1 from *Bt morrisoni* for Bt. Peferoen is linking phage display libraries to mutagenesis to enhance binding efficiency.

Agaisse *et al.* presented an analysis of *cry* promoters. The promoters vary between gene classes: *cryI* has σ^{35} and σ^{28}, *cryII* has σ^{35} or σ^{28}, *cryIVA* has σ^{35} and σ^{E}, and *cyt* has σ^{35} (and σ^{E}?) or σ^{28} (and σ^{E}?). Unlike the other *cry* genes, CryIIIA is not dependent on sporulation expression. The promoter may be σ^{A} and a repressor or inactivator are probably also involved.

8. Methods

Some novel and some adapted methods for investigating the binding efficiency, specificity and pore forming ability of Cry toxins were described. Vachon *et al.* showed that the use of fluorescent dyes to monitor specific ionic permeability extends the analytical value of using cell cultures to monitor pore formation. BBMVs still have significant advantages over cell cultures because BBMVs require specific binding ; Eschriche *et al.* described the preparation of BBMVs from whole Lepidoptera that overcomes the problems of dealing with small insects. Carroll

described a light scattering assay for determining vesicle permeability; probably best used in mutation studies to characterise pore efficiency but may be useful for comparative studies of different toxins. McCarthy claimed that the use of midgut epithelial cell suspensions to measure cell lysis (detect release of LDH by TTC reduction) was as good as or better than using cell cultures.

MacIntosh showed how high resolution video imaging can be used for measuring dynamic cellular events. This technology can be used to measure ion concentrations, membrane potential, enzyme activities, conformational changes of proteins, etc. It provides good temporal resolution (confocal laser microscopy gives good spatial resolution). She used it to show that CryIA and IC caused Ca^{2+} influx in SF9 cells but IIA did not. Novo Nordisk has created a new subsidiary, BioImage S, around this technology.

Various labelling strategies are being used in ligand binding studies and for mapping binding sites. Fiuza *et al.* used laser scanning densitometry to map distribution of binding sites in gut. They used a streptavidin-enzyme conjugate to localise ICPs and showed that CryIA(b) and IA(c) receptors were distributed throughout the length of the *Chilo suppressalis* midgut whereas CryIB receptor distribution was very patchy. De Maagd *et al.* described a ligand binding assay using chemiluminescence; biotin labelled toxin and anti-biotin antibodies.

A major problem for Bt producers has been real-time quality control. Two approaches that seem to have resolved this problem were presented. Chen *et al.* described an MTT spectrophotometric assay that assesses cell viability. It is quantitative, faster than trypan blue and can be used to assay fermenter production of Bt toxins. It gives good correlation with bioassay data. However, this method does not discriminate between the various types of Cry toxin that are produced by commercial strains. Pustzai-Carey and Devidas described the use of HPLC with various columns (MonoQ, Waters EDTA and 5PW for monitoring Bt toxins. This method provides a quantitative measure of each Cry toxin in the fermentation liquor within 30 minutes and so is highly suitable for in-line monitoring of the fermentation process. The data provided from this method correlate well with bioassay data. The technology has been patented by Abbott and will be made available on licence to research institutions.

Jarrett reported that *H. armigera* was most sensitive to CryIA(c), ca 15-fold less to CryIA(b) further 6-fold less to CryIA(a); there was no measurable susceptibility to Cry IB, IC, or ID. Feitelson reported that clones of *cryIA(c)* produced toxins that differed significantly, with LC_{50}s ranging between 5.6 and 23.9.