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Identification and characterization of proteins from *Bacillus thuringiensis* with high toxic activity against the sheep blowfly, *Lucilia cuprina*

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Abstract

Current control of the sheep blowfly (*Lucilia cuprina*) relies on chemical insecticides, however, with the development of resistance and increasing concerns about human health and environmental residues, alternative strategies to control this economically important pest are required. In this study, we have identified several isolates of *Bacillus thuringiensis* (*Bt*), collected from various Australian soil samples, that produce crystals containing 130 and 28 kDa proteins. These isolates were highly toxic to feeding larvae in both in vitro bioassays and in vivo on sheep. By N-terminal amino acid sequencing, we identified the smaller crystal band (28 kDa) as a cytological (Cyt) protein. Upon solubilization and proteolytic processing by trypsin, the 130 kDa crystal protein yielded among others, a truncated 55–60 kDa toxin moiety which exhibited larvicidal activity against sheep blowfly. The amino-terminal sequence of the trypsin-resistant protein band revealed that this *Bt* endotoxin was encoded by a new cry gene. The novel cry protein was present in all the strains that were highly toxic in the larval assay. We have also identified from one of the isolates, a novel secretory toxin with larvicidal activity.

Keywords: *Bacillus thuringiensis*; Crystal endotoxin; Secretory toxin; Sheep blowfly; *Lucilia cuprina*; Bioassay; Biocontrol; Novel toxins; Larvicidal; Sheep assay

1. Introduction

Control of the sheep blowfly, *Lucilia cuprina* (Weidemann), relies heavily on chemical insecticides but their continued use over several decades has led to the emergence of resistance (Levot, 1995). Added to this are the problems of insecticide residues in meat and wool, and concerns about environmental issues. Because of this there is growing interest in using alternative pest management strategies. We have been exploring the development of a biopesticide for *L. cuprina*, based on the bacterium *Bacillus thuringiensis* (*Bt*).

The best known of the *Bt* insecticides are the crystal toxins (Cry and Cyt proteins; also called δ-endotoxins). During sporulation, the bacterium synthesises and packages into parasporal crystals, one or more proteins, which have specific toxicity to a range of insect species (Schnepf et al., 1998). When susceptible insect larvae ingest *Bt* spore–crystals, the crystal δ-endotoxins are solubilized in the alkaline environment of the midgut and then these protoxins are proteolytically cleaved by midgut proteases into active toxic peptides. The active toxin binds to receptors on the surface of midgut cells and is inserted into the membrane to form pores that destroy

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transmembrane potential, resulting in osmotic lysis of the cells lining the midgut (Schnepf et al., 1998).

Currently, preparations of *Bt* are widely used as a safe and effective pesticide in horticulture and forestry (Kellar and Langenfruch, 1993; Navon, 1993; Rajakulendran, 1993; Teakle, 1994) and to control mosquitoes and blackflies (Becker, 1997; Becker and Margalit, 1993; Mulla, 1990; Ritchie, 1993). Most studies have concentrated on the effect of *Bt* on insects that affect crops or are vectors of human diseases; few have investigated the toxic effects of *Bt* on insect pests of livestock. Pinnock (1994) reported several strains with activity against the sheep louse and sheep blowfly but these have not been characterized. Heath et al. (2004) demonstrated that *Bt* isolates producing Cry1Ba were toxic to *L. cuprina* larvae and provided protection against flystrike for up to 6 weeks. Isolates of the bacterium with toxicity to sheep blowfly larvae have been demonstrated in vitro (Gough et al., 2002). In this paper, new isolates are identified and characterized in more detail.

In addition to the crystal—associated toxic proteins linked to sporulation, some *Bt* isolates synthesise other unrelated proteins during vegetative growth. These proteins, termed vegetative insecticidal proteins (or Vips), have demonstrated insecticidal activity against a wide spectrum of lepidopteran insects (Estruch et al., 1996; Schnepf et al., 1998). Another secretory product, non-proteinaceous β-exotoxin, known to be particularly active against dipteran species (Pinnock, 1994), is not insect specific. The β-exotoxin is an adenine nucleotide analogue and because it interferes with protein synthesis it is considered harmful to mammals and is therefore unlikely to be approved for registration (Glare and O'Callaghan, 2000).

In this study, we evaluated 310 *Bt* isolates for crystal protein insecticidal activity against *L. cuprina* larvae and identified a strain of *Bt* that produces both a novel crystal protein and a secreted protein with potent larvicidal activity.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and spore–crystal preparation

Previously, uncharacterised isolates were selected from a *Bt* collection held by CSIRO Entomology (Australia). Three hundred and 10 isolates were grown to sporulation, at 28 °C, on Nutrient Agar (Oxoid) plates containing sporulation salts (Stewart et al., 1981). Spores and crystals were harvested into sterile water and centrifuged (10,000*g*, 10 min at 4 °C). Pellets were washed four times in water to remove cell debris and secretory products, including exotoxin(s) produced during cellular growth. Final pellets were resuspended in water and the volume of each adjusted to give an $OD_{600}$of 0.5 at a 1:100 dilution. These stocks were stored at −80 °C until required for insect screening. Stock *Bt* spore–crystal mixtures used in a sheep trial were freeze-dried and stored at −80 °C.

2.2. Purification of *B. thuringiensis* crystals

Crystals were purified from spore–crystal mixtures using differential ultracentrifugation based on the method of Luthe (1983). Bacteria were grown to sporulation and spore–crystal mixtures harvested and washed as described above. Following sonication to release crystals from the spores, spore–crystal mixtures were layered onto a linear sucrose gradient (60–90% w/v) and centrifuged (77,000*g*, 17 h at 4 °C) in a Beckman Coulter Avanti J-E centrifuge using a SW32 rotor. Fractions were collected with a pipette and purity checked by phase-contrast microscopy. The majority of crystals were recovered from the interface between the 60 and 70% sucrose layers, while spores formed a pellet at the bottom of the tube. The crystal band was removed and washed (three times in ice-cold sterile water) by centrifugation at 15,000*g* for 10 min at 4 °C. The final pellet was resuspended in 1-ml water, freeze-dried, and stored at −80 °C. Material from the pellet of the CAA 890 isolate was examined by scanning electron microscopy.

2.3. In vitro screening assay for Bt toxicity

Pupae of *L. cuprina* were supplied from a colony of flies reared by the Queensland Department of Primary Industries and Fisheries, Animal Research Institute, Brisbane. Eggs were obtained from female flies ovipositioning on homogenized liver and then stored overnight at 18 °C. Larvae hatched the next day following a 2–3 h incubation at 28 °C.

Larval diet medium consisted of a milk powder–yeast–agar base supplemented with sheep serum. One part 1% molten agar (Difco Noble) was added to three parts warm water containing 10.8% milk powder, 5% brewers yeast, and 10% sheep serum. One milliliter aliquots of molten diet medium were dispensed into wells of a six-well tissue culture plate (internal diameter of 35 mm) and left to solidify. Aliquots (100 μl) of *Bt* spore–crystal stock were pipetted evenly over the surface of each 1-ml diet medium (Gough et al., 2002). As a control, water replaced the spore–crystal mixture.

Ten neonate *L. cuprina* larvae were placed onto 4 mm-diameter filter pads (Millipore, Bedford, MA) and the pads were placed directly onto the diet medium surface. The plates were covered with gauze lids and incubated at 28 °C, 100% RH. Larval mortality was quantified by counting live larvae remaining after 45–48 h feeding. Larvae in control wells developed to third instar with the assay diet and incubation conditions.

In the initial screening assay, each *Bt* isolate was tested in duplicate; for reassessment of the efficacious isolates, each test was replicated four times.

2.4. Assessment of toxic activity

To assess toxicity caused by the crystal δ-endotoxin component of each *Bt* isolate, antibiotic was added to aliquots of spore–crystal stock and purified crystals (125 U/ml penicillin and 125 μg/ml streptomycin) and the assay diet medium (100 U/ml penicillin and 100 μg/ml streptomycin) before screening. Addition of antibiotic at these concentrations prevented germination of *Bt* spores and subsequent vegetative growth (with possible production of secretory toxic proteins and/or β-exotoxin). To assess whether heat-stable exotoxin remaining from spore–crystal preparations contributed to toxicity, aliquots of spore–crystal stocks were autoclaved (121 °C, 20 min) before re-screening in the assay.

In one isolate, CAA 890, freeze-dried crystals, purified from spore–crystal mixtures, were reconstituted in water then serially diluted from a protein concentration of 5.7 mg/ml and tested in the screening assay for toxicity to larvae.

To assess if the efficacious *Bt* isolates secreted toxic products during cell growth, CAA 890 and one non-toxic isolate (CAA 525, causing less than 10% larval mortality) were grown at 37 °C for 48 h in nutrient broth and their supernatants assessed in a feeding assay. In preparing the diet-medium, water was replaced with an equal volume of culture supernatant, either autoclaved or non-autoclaved.

2.5. N-terminal amino acid sequencing and SDS–PAGE analysis of trypsin digest

Purified crystals of *Bt* isolate CAA 890 were solubilized in 50 mM sodium carbonate buffer (pH 10.5), 20 mM dithiothreitol (DTT) at 37 °C for 1 h, dialysed against the same buffer without DTT and treated for 2 h with trypsin (Sigma). The ratio of enzyme to solubilized toxin was 1:25. Products of the trypsin digest were separated on 10% SDS–PAGE gel at 170 V for 1 h using NuPAGE Novex Bis-Tris gels and Xcell SureLock minicell (Invitrogen). Proteins were transferred to a polyvinylidene fluoride membrane (Applied Biosystems) and after staining (Coomassie brilliant blue G250), the ~55 kDa protein band was excised and subjected to N-terminal amino acid sequencing using an Applied Biosystems Procise (Model 490A) protein sequencer (Edman and Begg, 1967).

2.6. Protein estimation and concentration

Protein concentrations of spore–crystal mixtures and purified crystals were determined (Pierce, Rockford, USA) with albumin as a standard. The concentration of protein in each aliquot of spore–crystal mixture or purified crystal was calculated and the volume dispensed over the surface of the diet medium was expressed as mg protein/mm^2. The protein profiles of spore–crystal mixtures, purified crystal and culture supernatant (concentrated 100×) were determined using SDS–PAGE and Coomassie blue staining as described above.

2.7. Sheep trial

Three *Bt* isolates, (CAA 440, CAA 890, and CAA 938), that caused high mortality of larvae of *L. cuprina* in vitro, were tested in a sheep trial. The toxicity of the spore–crystal mixtures were tested in two ways; equal volumes of each of the three *Bt* spore–crystal stocks were pooled together and applied as a liquid and samples of the three isolate stocks were freeze-dried and 200 mg of each were pooled together as a *Bt* powder.

Six sheep were prepared for trial according to Eisemann et al. (1989). Six foam rings, with an internal diameter of 50 mm, were glued to closely shaven skin along each side of the spinal midline, from shoulder to flank, on the day preceding the trial.

Bt was applied to rings on five sheep as follows. A 150-μl aliquot of the pooled liquid spore–crystal stock was pipetted evenly over the skin within one ring. This volume was calculated to deliver the same concentration of protein per millimetre square and was equivalent to the volume used in vitro, taking the larger ring area in vivo into account. Thus the actual concentration of protein per millimetre square for both in vitro and in vivo was the same. In a second ring, *Bt* powder (10 mg) moistened with 50 μl sterile water was spread with a brush over the exposed skin. In a third ring, 1 mg of *Bt* powder was moistened with 50 μl water and applied as above. This concentration of spore–crystal powder was not equivalent to protein concentration used in vitro. Two control rings had water (50 and 150 μl) only. In the sixth ring, neither *Bt* powder nor water was added.

The sixth sheep was used to demonstrate that toxicity was due to spore–crystal activity. To prevent vegetative growth of the spores, penicillin and streptomycin (100 U/ml, 100 μg/ml) in 100 μl water were added to 10 mg spore–crystal powder samples and applied to two rings. In another two rings, 100 μl water containing antibiotic was applied to the skin. The final two rings, with 100 μl water only applied, acted as controls.

Once the formulations were applied to the rings, 50 neonate larvae were placed onto moist 22 mm Millipore-filter discs which were inverted inside each foam ring. Larvae were contained inside each ring as described in Eisemann et al. (1989). Larval mortality in

each ring was assessed after 48 h and mean weights of surviving larvae were calculated.

3. Results

3.1. In vitro screening assay and assessment of Bt toxic activity

The initial screen, using spore–crystal mixtures standardized to give an OD_{600} of 0.5 at a 1:100 dilution, indicated that 46 (15%) of the 310 isolates had high toxic activity (greater than 90% mortality) against feeding *L. cuprina* larvae in at least one of the duplicate wells. Larval mortality in control well replicates was generally less than 20% and in many assays, less than 10%. The 46 isolates were re-screened twice over two different days to confirm efficacy.

To determine the contribution of crystal δ-endotoxin to *Bt* isolate toxicity, penicillin/streptomycin was added to spore–crystal mixtures and larval diet-medium to prevent spore germination and possible production of secreted toxins during subsequent bacterial cell growth. At the completion of reassessment, 16 *Bt* isolates were identified with at least 50% of total larval mortality attributed to spore–crystal toxicity (Table 1). Penicillin/streptomycin in the diet-medium did not affect larval survival (less than 10% mortality), demonstrating that the concentration of antibiotic used in the screen was not toxic to feeding larvae.

To verify toxicity from the δ-endotoxin component, aliquots of spore–crystal mixtures from the efficacious *Bt* spore–crystal mixtures were autoclaved to abolish protein activity, and then re-assessed in the larval screen. Autoclaving caused a large reduction in larval mortality in the majority of the *Bt* isolates, suggesting that Cry protein was responsible. Given that larval mortalities in control wells were less than 20%, mortality above this in test wells where antibiotics was added was possibly a result of some heat-stable exotoxins remaining from spore–crystal preparation (Table 1).

Because the SDS–PAGE protein profiles and subsequent gene sequence analysis of the 16 efficacious isolates (see below) were similar, only one of the isolates, CAA 890 (Table 1), was selected for further analysis. Purified crystals from this isolate were bipyramidal in shape and of a uniform size (Fig. 1). The crystals were freeze-dried, reconstituted in water and tested in the larval feeding screen at a starting protein concentration corresponding to that of the toxic spore–crystal mixture (5.7 mg/ml; with surface application per plate well of 0.593 μg/mm^2). Crystals fed to larvae were still toxic (mortality greater than 80% after 48 h) at a protein

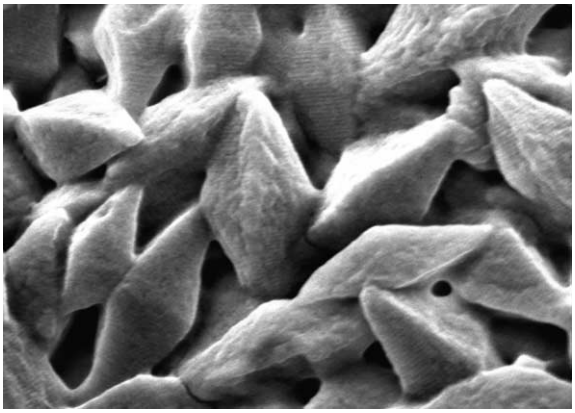

Fig. 1. Scanning electron micrograph of bipyramidal crystals from *Bacillus thuringiensis* isolate, CAA 890. 35,000×.

Table 1
Isolates of *Bacillus thuringiensis* with high toxic activity against larvae of the sheep blowfly, *Lucilia cuprina*

Bt isolate number CAA	Larval mortality (%) following 48 h feeding on spore–crystal mixtures of isolates			Sample type/locality/state collected
	No antibiotics	Plus antibiotics	Autoclaved/plus antibiotics	
437	100	54	46	Soil/Woodland/WA
438	100	91	9	Soil/Woodland/WA
440	100	92	28	Soil/Woodland/WA
441	100	77	23	Soil/Woodland/WA
442	100	77	23	Soil/Woodland/WA
853	100	86	18	Soil/Crop/Qld
854	100	59	41	Soil/Crop/Qld
857	100	81	18	Leaf litter/Eucalypt/Qld
890	100	89	11	Leaf litter/Eucalypt/Qld
894	100	71	29	Leaf litter/Eucalypt/Qld
898	92	78	14	Leaf litter/Eucalypt/Qld
917	100	72	28	Soil litter/Scrub/Qld
938	100	80	20	Sand litter/Beach/Qld
939	89	64	25	Soil/ Eucalypt/Qld
940	100	84	22	Soil /Eucalypt/Qld
1008	100	83	17	Soil/Creek/NSW

State collected: WA, Western Australia; Qld, Queensland; NSW, New South Wales.

concentration of 500 μg/ml (applied to diet surface at 0.05 μg/mm^2).

Supernatant from the *Bt* CAA 890 culture was toxic to larvae (93% mortality) after 48 h feeding. However, the toxicity was destroyed (0% larval mortality) when aliquots of supernatant were pre-treated with autoclaving, suggesting that a secreted protein and not β-exotoxin was responsible. Supernatant from *Bt* CAA 525 culture was not toxic after 48 h when fed to larvae, neither was the spore–crystal preparation from this isolate.

3.2. Protein profile and crystal protein analysis

Although the 16 efficacious isolates in the study were collected from various locations in Australia, they were shown to have very similar protein profiles by SDS–PAGE (Fig. 2). Each isolate produced a prominent band around 110–130 kDa and a minor band around 28 kDa. Amino-terminal sequence of the 28 kDa minor protein identified Cyt (cytolytic) protein toxin, Cyt 1BA1. We were unable to sequence the 130-kDa protein as the amino-terminal end of this protein appeared to be blocked. To obtain sequence information from the latter protein, we performed a limited tryptic digestion on the solubilized crystal proteins. Trypsin digestion resulted in the formation of three or more smaller fragments with apparent molecular masses of the major bands at 95, 80, and 55–60 kDa (Fig. 3). The 55–60 kDa protein band was excised for amino-terminal sequencing and the results revealed a 29 amino acid sequence (DIN DVKIRFISLDSLFTQSMPSFRIEGFQ). A BLASTP search of the Cry proteins from the GenBank database showed some similarity with Cry3 and Cry9 proteins, indicating possibly a new crystal protein.

3.3. Biological activity of the 55–60 kDa product of limited tryptic digestion of CAA 890 crystal proteins

The product of limited tryptic digestion of the crystal protein was purified by fast protein liquid chromatography (FPLC) using a Mono-Q column (Amersham). After protein elution, two peaks were obtained (Fig. 4). The minor peak (peak 1) contained the 55–60 kDa fragment while the major peak (peak 2) contained an 80–95 kDa fragment. The figure shows that the major peak was contaminated by the smaller component. When fed to larvae in the screening assay, the partially purified 55–60 kDa protein (protein concentration at 511 μg/ml) caused 80% mortality. The protein concentration required to give 100% larval mortality was calculated for the original spore–crystal mixture, purified crystal and the 55–60 kDa product (Table 2).

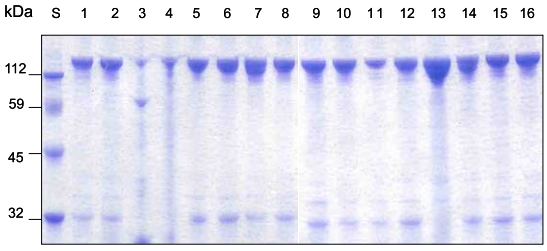

Fig. 2. Coomassie-stained SDS–PAGE (10%) of spore–crystal proteins from 16 *Bacillus thuringiensis* isolates with high toxicity (>90% mortality) against larvae of the sheep blowfly, *Lucilia cuprina*. Lane S, molecular-weight standards (Bio-Rad kaleidoscope); lane 1, CAA 440; lane 2, CAA 441; lane 3, CAA 442; lane 4, CAA 437; lane 5, CAA 438; lane 6, CAA 853; lane 7, CAA 854; lane 8, CAA 857; lane 9, CAA 890; lane 10, CAA 894; lane 11, CAA 898; lane 12, CAA 917; lane 13, CAA 938; lane 14, CAA 939; lane 15, CAA 940; and lane 16, CAA 1088.

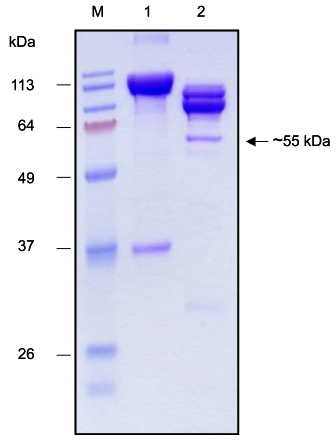

Fig. 3. Coomassie-stained SDS–PAGE (10%) showing proteolytic processing (trypsin digest) of crystal proteins from *Bacillus thuringiensis*, CAA 890. Lane M, molecular-weight standards (Fermentas prestained); lane 1, solubilized crystal proteins before trypsin digest; and lane 2, solubilized crystal proteins after trypsin digest. Arrow indicates trypsin-cleavage ~55 kDa product that was subjected to N-terminal sequencing.

3.4. Secretory toxins

The proteins from concentrated (100×) culture supernatant from *Bt* isolates CAA 890 (larvicidal) and CAA 525 (non-toxic) were visualised by SDS–PAGE. Comparison of secreted protein profiles of the two isolates revealed identical bands with the exception of a major band at approximately 90 kDa which was produced by CAA 890 and not by CAA 525 (Fig. 5). Amino-terminal sequencing of the 90 kDa band resulted in 12 residues (data not shown). No match was found in a BLAST search from the GenBank database.

3.5. Sheep trial

Larval mortality in each foam ring was assessed after 48 h (Table 3). In all sheep, *Bt* powder (10 mg) applied to a skin surface area of 1.96 cm^2 caused 100% larval mortality, while mortality in control rings was 18%

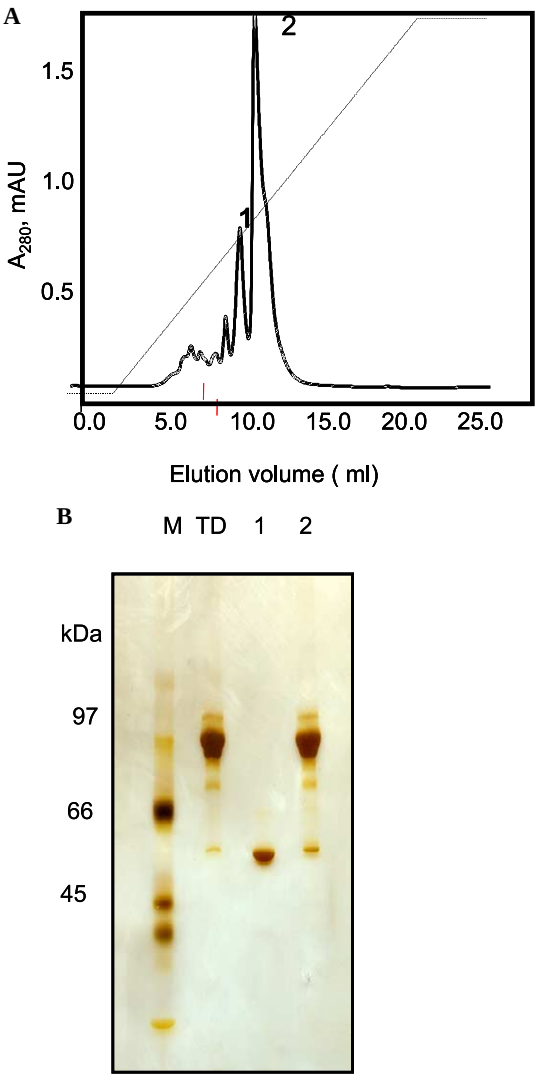

Fig. 4. Ion-exchange chromatography of trypsin-cleavage products from crystal proteins of *Bacillus thuringiensis*, CAA 890. The trypsin hydrolysate was applied onto a Mono Q column equilibrated with 50 mM Tris–HCl buffer, pH 8.5, containing 1 mM DTT. Proteins were eluted by a linear gradient of NaCl (0–1.0 M) in the same buffer. (A) Chromatographic tracing of digested CAA 890 crystals. Numbers 1 and 2 indicate the main chromatographic peaks. (B) SDS–PAGE (6–18%) analysis of pooled fractions, containing the 55-kDa fragment (peak 1) and 80–90 kDa proteins (peak 2). Lane M, molecular weight standards (Pharmacia, unstained); lane TD, trypsin hydrolysate before ion-exchange; lane 1, peak 1 proteins; and lane 2, peak 2 proteins.

Table 2
Comparative in vitro toxicity of *Bacillus thuringiensis* CAA890 preparations against larvae of the sheep blowfly, *Lucilia cuprina*

Preparation	Protein concentration (μg/ml) to give 100% larval mortality
Original spore–crystal mixture	5700
Purified crystal	1400
55–60 kDa protein fragment	639

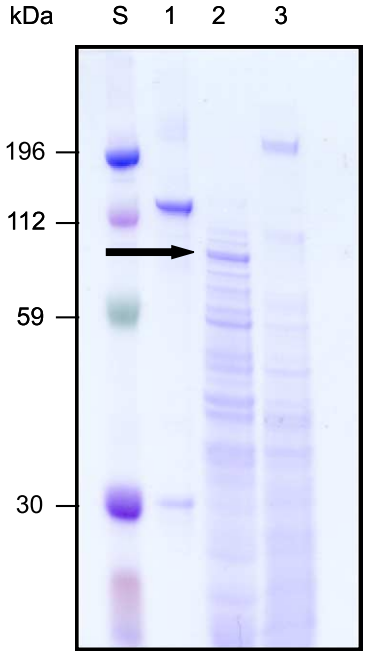

Fig. 5. Coomassie-stained (10%) SDS–PAGE of concentrated (100×) culture supernatant from *Bacillus thuringiensis*. Lane S, molecular-weight standards (Bio-Rad kaleidoscope); lane 1, crystal proteins from CAA 890; lane 2, supernatant from CAA 890 (larvicidal); and lane 3, supernatant from CAA 525 (non-larvicidal). Arrow indicates protein (90 kDa) present in larvicidal CAA 890.

($n = 5$). In rings where aliquots of only 1 mg *Bt* powder were applied, mean mortality was reduced to 58%; control rings returning a mean mortality of 18% ($n = 5$). However, weights of surviving larvae were the same as those of larvae in control rings (mean weights 5.0 and 4.7 mg, respectively). Aliquots (150 μl) of spore–crystal stock caused 72% larval mortality with mean weight of larvae 1.2 mg compared with control mortality of 16% and mean larval weight of 6.3 mg.

On the sixth sheep, high larval mortality (mean 92%) in rings with antibiotic added to *Bt* powder, demonstrated that toxicity was due to spore–crystal protein(s) (Table 4). In control rings with antibiotic but no *Bt*, mortality of larvae was 17%. However, weights of larvae recovered from rings with antibiotic were low (mean 1.91 mg) compared with larvae from control rings with water only (mean weight 7.47 mg), (Table 3).

4. Discussion

We have developed a feeding bioassay that is simple to conduct, reproducible and uses inexpensive dietary material to screen bioactives against the sheep blowfly. Using the in vitro larval feeding assay, we have identified 16 isolates of *Bt*, from various locations in Australia (Table 1), that express crystal protein with high toxic activity against larvae of the sheep blowfly, *L. cuprina*.

Table 3
In vivo assessment of toxicity of *Bacillus thuringiensis* preparations against larvae of the sheep blowfly, *Lucilia cuprina*. Five sheep, one ring per treatment

Sheep	1	2	3	4	5	Mean of 5 rings
Larval mortality (%) at 48 h						
Bt powder 10 mg	100	100	100	100	100	100
Bt powder 1 mg	40	92	38	80	42	58
Control	40	0	8	26	18	18
Bt spore–crystal 150 μl	100	64	34	64	98	72
Control, water 150 μl	28	24	0	14	14	16
Mean larval weight (mg)						
Bt powder 10 mg	—	—	—	—	—	—
Bt powder 1 mg	4.7	7.1	3.2	5.4	4.7	5.0
Control	3.4	6.0	2.4	8.3	0.5	4.7
Bt spore–crystal 150 μl	—	0.7	1.7	3.1	0.5	1.5
Control, water 150 μl	5.4	6.0	10.6	5.9	3.8	6.3

Table 4
In vivo assessment of toxicity of *Bacillus thuringiensis* preparations against larvae of the sheep blowfly *Lucilia cuprina*

	Ring 1	Ring 2	Mean
Larval mortality (%) at 48 h			
Bt powder 10 mg	100	84	92
Control 1	6	28	17
Control 2, water 150 μl	6	2	4
Mean larval weight (mg)			
Bt powder 10 mg	—	0.4	0.4
Control 1	1.5	2.3	1.9
Control 2, water 150 μl	9.1	5.9	7.5

Vegetative growth of spores inhibited with inclusion of penicillin (100 U/ml) and streptomycin (100 μg/ml). Control 1 with antibiotics, control 2, no antibiotics. One sheep, two rings per treatment.

Analysis by SDS–PAGE of sporulated culture from each of the bacterial isolates revealed two major banding patterns with several Cry protein bands around 110–30 kDa and a single band at 28 kDa. The latter band was identified, via amino terminal sequencing, as a cytological (Cyt) protein. Cyt toxins, produced by some isolates of *Bt*, are known to have activity against larvae of Diptera (Aronson and Shai, 2001). Because of the similar SDS–PAGE patterns for each of the 16 efficacious isolates and sequence data that verifies all 16 isolates are identical (data not shown), one isolate, CAA 890, was chosen for further characterization.

Crystals separated from the spores were as toxic to feeding larvae as the combined spore–crystal mixture, suggesting that the spores may not be essential to the overall virulence of CAA 890. Spores of some *Bt* strains are known to contribute to toxicity, often synergising the activity of the crystal δ-endotoxins when tested against some insect species (Porcar and Juarez-Perez, 2003).

Purified crystals from CAA 890 contained a prominent toxin band at 130 kDa. In vitro trypsin digestion of solubilized CAA 890 crystals produced several protease-resistant fragments with molecular masses ranging from 95 to 55–60 kDa. Following purification by FPLC, two elution peaks were obtained and these were subsequently assayed for toxicity against *L. cuprina* larvae. Bioassay results of the eluted fraction containing the 55–60 kDa fragment demonstrated high larvicidal activity (Table 2). These toxicity assays demonstrated that this activated fragment plays a major role in overall toxicity of CAA 890.

The toxicity of three of the efficacious isolates was confirmed on sheep infestations using freeze dried powder and *Bt* suspensions. The in vivo conditions were not ideal because in treatment rings it was observed that some surviving larvae were able to escape the effect of *Bt* by moving under the edge of the ring. The weights of larvae recovered from rings with antibiotic (Table 4) were low compared with larvae from control rings. The concentration of penicillin, streptomycin added to each ring was the same as the concentration used in vitro. We have demonstrated high dosage of penicillin, streptomycin is toxic to sheep blowfly larvae (data not shown). It is possible that the dose used was more toxic on the skin of the sheep than when applied to agar diet medium in vitro.

We used spent culture medium directly in the larval diet and demonstrated that a proteinaceous toxin secreted from CAA 890 contributes to the toxicity against sheep blowfly larvae. It is known that non-proteinaceous β-exotoxin, an extracellular compound synthesised by some *Bt* strains, contributes to virulence against Diptera (Pinnock, 1994). However, in our screening assay, toxic activity was abolished following autoclaving, confirming activity was due to protein(s). SDS–PAGE analysis of the spent culture medium revealed a number of bands including a prominent protein band of 90 kDa in CAA 890, that was not visible in protein analysis of culture from a non-toxic (CAA 525) *Bt* isolate (Fig. 5). Reports of secreted proteins from Bacillus isolates include phos-

pholipases (Ikezawa et al., 1989; Songer, 1997), hemolysins (Budarina et al., 1994; Granum, 1994), and chitinases (Sampson and Gooday, 1998). Novel vegetative insect proteins (Vips) secreted in culture during logarithmic growth of certain *Bt* strains may offer a novel alternative source of larvicidal toxins (Estruch et al., 1996; Yu et al., 1997). Donovan et al. (2001) found that synthesis of Vip3A protein after larval ingestion of spores was an important component of insecticidal activity against *Spodoptera exigua*. Secreted protein toxins could be useful additional candidates along with *Bt* crystal protein for managing sheep blowfly.

The possibility of identifying and using *Bt* strains active against blowflies has been considered before by Cooper et al. (1991), but to date, no commercial preparation to control insect pests of livestock has been reported.

Further studies on the contribution of the novel isolates identified in this study to sheep blowfly larvicidal toxicity are continuing with the aim of developing a biopesticide that is more attractive than current synthetic chemical control agents.

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