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Abstract

Novel strains of the bacterium *Bacillus thuringiensis* were isolated which had larvicidal activity against some tropical species of blowfly. It was shown that this specific toxicity could be attributed to the 8-endotoxin proteins produced during sporulation. The biochemistry, bioactivity and genetics of the production of these 8-endotoxins was characterised.

The parameters for the industrialised production of these insecticidal bacteria were deduced. A suitable pilot-scale producer was identified in Africa. An insecticidal preparation was formulated which could be applied to fish prior to sun-drying.

Toxicity tests with rats indicated no hazard resulting from exposure to very high levels of *B. thuringiensis* insecticide. Separate tests gave severe cause for concern with respect to two toxins, potentially presenting a hazard to humans, (3-exotoxin and *Bacillus cereus-type e*ntertoxotin. Attempts to remove the former toxin from our strains of *B. thuringiercsis* ultimately proved to be unsuccessful.

Due to the unacceptable risks considered to be presented by the unwanted toxins the objective of the project was altered to the use of our bio-insecticide as a bait in fish processing and drying sites. The insecticide was reformulated and tested under field conditions in Sri Lanka. The results showed that the insecticide might prove to be a useful component of an overall regime designed to reduce losses by insects to postharvest fish. The use of low level technology to produce B. *thuringiensis* for insecticidal baits was reviewed briefly.

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Chapter 1

Introduction to the Work

The importance of dried fish in the nutrition of people in developing countries is well documented (see Borgstrom, 1962, FAO Fish.Tech.Pap., 1981). It is a rich source of protein complete in the essential amino acids, and with a net protein utilisation rating close to that of eggs, the protein bio-availability is excellent (MAFF, 1978). As protein deficiency is commonplace in the developing world, increasing the quantity of dried fish could help to improve the nutritional status of large numbers of people (Borgstrom, 1972). Improving utilisation through reducing losses is an important means of increasing the production of traditionally processed fish in developing countries.

Traditionally processed fish is highly susceptible to attack by insect pests, principally blowflies (Diptera: Calliphoridae) and *Dermestes s*pp. beetles (Coleoptera: Dermestidae). Damage resulting from infestation is a major cause of post-harvest losses in the traditional fish processing industry. Whilst extensive quantitative studies of insect related losses have yet to be carried out, it is generally accepted that losses of 25% are not uncommon, and, in extreme cases, losses in excess of 90% occur (Meynall, 1978, Golob et aL1987, Young and Esser, 1992).

Each insect group attacks the fish at a different stage in the processing. Primarily, blowflies are attracted to the fish during the salting and sun-drying stages, whilst beetles infest the dried, stored product. There are exceptions to this with larvae of the dipteran species *Piophila* casei and Lucilia cuprina cuprina being reported to infest cured fish (FAO Fisheries Technical Paper 303 , Esser, 1994). There may also be seasonal variations in the predominance of pest species, with blow-flies being of most importance during the wet seasons and beetles during the warm, dry seasons (Walker, 1981) Damage through blowfly infestation results from larval feeding packs forming within the flesh of the fish. Deep burrows are created which subsequently cause the dried product to fragment, increasing losses and making transportation difficult (Meynall, 1978). During drying, the fish muscle surrounding the feeding larvae becomes putrid probably due to the combined erects of enzyme secretion onto the food initiating tissue breakdown, and pH change due to nitrogenous waste excretion in the form of ammonia. Larvae usually leave the fish to pupate, often in the earth beneath the drying racks. Thus, adults emerge within close proximity of the fish and the cycle repeats. There are two main routes of infestation: (1) larvae hatch from egg batches laid (or in viviparous species hatched larvae are deposited) on or adjacent to the fish during the early stages of processing, and; (2) larvae move onto the fish from other oviposition sites and food sources (Johnson and Esser, 1996). To be successful, control measures need to target both routes of infestation.

The beetles most commonly found infesting dried fish are *Dermestes maculatus* and *Dermestes frischii,* although *Necrobia rufipes* is also a frequent pest (Osuji, 1975a). Both adults and larvae feed on the fish causing extensive damage through tissue loss and fragmentation. Factors such as temperature, relative humidity and moisture content of the fish influence the level of infestation present. Osuji (1975b) found that daytime temperatures of around 50oC cause the beetles to migrate into the centre of fish stacks where average

temperatures are 35oC. Temperatures above 40oC prevent development in the larvae of these species (Amos, 1968), whilst temperatures of 50oC for more than 15 minutes can be lethal (FAO Fish.Tech.Pap., 1981). Relative humidities of 50% or above are generally necessary for survival in all species (Howe, 1965). These factors can be manipulated for insect control purposes. However, for optimum development and minimal mortality the moisture content of the food source should be between 10.5 and 15.9% (Scoggins and Tauber, 1951). This range coincides with that normally found in unsalted, dried fish making it an ideal food source.

Frequent and extensive losses from insect infestation cannot be sustained by processors or traders. Control measures recommended by extension workers combine the use of chemical and non-chemical methods. Of primary importance is the following of good hygiene practices. The removal of any fish waste from the vicinity of the fish processing operation will help to reduce the number of flies present at the site, as well as limiting the possibility of larval crossinfestation from other food sources. The provision of adequate physical protection to the fish during the early stages of processing can have a profound erect on reducing infestation by blowflies. Infestation during salting was prevented by simply fitting an insect proof lid to brining tanks in Indonesia (Esser, 1991). Similarly, erecting mesh screens or netting over drying racks can prevent gravid females gaining access to the fish during drying, thereby limiting infestation without markedly reducing drying rates (Esser, 1992). Salting the fish prior to sun-drying may also have a role to play in preventing blowfly infestation (Walker, 1986, Johnson and Esser, 1996).

Chemical control, through the application of insecticides to the fish during drying and storage, is a highly effective way of combating infestation by blowflies and beetles. Over 30 active ingredients have been considered for use on fish. A full review of this work can be found in Walker (1987). At present only two insecticidal compounds have recommended Maximum Residue Levels (MRL) for use on fish. These are the synergised pyrethrins and prim1phosmethyl. In the former, the pyrethrins are synergised with piperonyl butoxide (ratio 1:10). Several laboratory and field studies were conducted in the UK, Canada and Norway during the 1960's. All showed that good control against blowflies could be achieved by dipping or spraying fish in emulsions of the synergised pyrethrins at concentrations of 0.3% pyrethrin or below (Olley, 1961, Somme and Gjessing, 1963, Morris and Andrews, 1968). The MRL was set at 0. lmg/kg pyrethrin and l.Omg/kg piperonyl butoxide by the FAO/WHO Joint Meeting on Pesticide Residues (FAO/WHO, 1970), and later increased to 3mg/kg and 20mg/kg respectively (WHO, 1973). In tropical countries, early work carried out by McLellan (1963) in Uganda gave good protection to Tilapia spp. from the blowfly Chrysomya marginalis (Weid.). Data for residue levels, however, are not stated. More recent work conducted in Africa reveals that in order to gain adequate protection against insect pests, residues of synergised pyrethrins, in particular of the piperonyl butoxide component, are well in excess of the MRLs (Diouf, 1980, Walker, 1981, Walker and Donegan, 1984). Indeed Golob et aL(1986) found residue levels of 5.7mg/kg for pyrethrins and 225mg/kg for piperonyl butoxide six months after application to stored fish in Turkana, Kenya. Such residue levels are wholly unacceptable, and yet, despite these findings, synergised pyrethrins are still used to combat insect infestation by fish processors in developing countries (Azeza, 1986, Walker, 1987).

Pirimiphos-methyl underwent extensive trials in Malawi where it was found to provide excellent protection against blowflies and fleshflies (Walker and Donegan, 1984). Trials

conducted in The Gambia by Walker and Evans (1984) showed that control against D. *maculatus* could be achieved, although the protection declined after one month's storage. Esser (1986) found that marine catfish, *Atrius* spp., could be protected against *Chrysomya megacephala* (Fab.) during drying, and *Dermestes* spp. for ten weeks during storage, following a single dipping in a 0.03 % emulsion of pirimiphos-methyl. A MRL of 10mg/kg has been recommended for pirimiphos-methyl use on fish. Mean residues determined on experimental fish after drying have been between 4.6mg/kg and 9.7mg/kg (Esser, 1986, Walker and Evans, 1984). Residues at this level gave good control of insect pests for prolonged periods. However, liberal application can result in residues in excess of the MRL. Commercial fish sampled in Zambia had pirimiphos-methyl residues of 26mg/kg (Walker, 1987). Walker suggests that problems such as this can be overcome with good extension practices.

Whilst pirimiphos-methyl is by far the safest insecticidal preparation available, its use appears to be limited, possibly because of its restricted availability or comparative cost in some countries (Walker, 1987). Lack of training or accessible information means that many fish processors resort to the use of inappropriate, often dangerous pesticides (Walker, 1988). Products such as DDT and lindane, which are organochlorines and thus accumulate in the body, and the acutely toxic dichlorvos and phosalone, are in widespread use (Walker, 1987). Many of these products are available as household insecticides. Of additional concern is the apparent tendency to incorporate kerosene as a formulation component (Walker, 1986). Esser *et al.* (1986) reported a total absence of insects on fish treated with non-approved insecticides, suggesting that the concentration of insecticide applied to the fish was in excess of that needed to obtain effcacious blowfly control.

On consideration of the available data it is apparent that the control of insect infestation in traditionally processed fish is important for social and economic reasons. Furthermore, the willingness of processors to increase their expenditure by purchasing insecticides suggests that significant financial gains must result from controlling infestation. The lack of available foodsafe insecticides has obvious grave implications to human health. Whilst a complete move towards the use of pirimiphos-methyl is to be desired for safety reasons, the likelihood of rapid pest resistance developing is great. It is therefore essential that alternative insecticides be developed for use on fish.

This project was funded with the intention of developing a microbial insecticide for use on fish. Such pesticides have several advantages over their synthetic counterparts. They have been shown to be far safer with respect to both human health and environmental considerations (Drobniewski, 1994). In addition, they are cheap and easy to produce using many locally available substrates and can therefore often be produced within the country of intended use, thus benefiting the local economy and avoiding unnecessary foreign exchange (Jones, 1988).

Several micro-organisms are currently used in pest control, the most predominant of these being *Bacillus thuringiensis. B. thuringiensis* is a gram positive, spore-forming bacterium which produces insecticidal crystal proteins (ICPs) at sporulation (Hdfte and Whiteley, 1989). The spectrum of activity exhibited by each strain is dependant upon the class of protein or proteins produced (Hofte and Whiteley, 1989). It is an ubiquitous soil-dwelling organism which has been isolated world-wide (Martin and Travers, 1989). It has been widely used in pest control over many years (Perferoen, 1991, Feitelson *et al.* 1992) currently accounting for

92% of the biological control market (Powell, 1993). Apart from use as an agricultural pest control agent, its major success has been in vector control. B. *thuringiensis* van. *israelensis* (Bti) has been effectively used in malarial and onchocerciasis control programmes across the developing world (Walsh, 1986, Becker, 1990). Its use for these purposes is actively promoted by the World Health Organisation.

The safety record of *B. thuringiensis* is excellent (Drobniewski, 1994, Hadley *et al.* 1987, Lamanna and Jones, 1963, Siegel and Shadduck, 1990 and Siegel *et al.,* 1987). In contrast, the World Health Organisation estimates that chemical pesticides are responsible for poisoning over 1 million people each year, and 2% of these will prove fatal (Ambridge and Haines, 1987).

B. thuringiensis has commercial drawbacks being of comparatively low toxicity with high host specificity and low persistence following application (Dent, 1993). This is in marked contrast to chemical insecticides. However, these limitations offer environmental benefits which enable its use within integrated pest management programmes as well as alleviating public concerns over environmental issues. With the increase in insect resistance to chemical insecticides, *B. thuringiensis* has gained importance in the control of several important pest species. Amongst these are the diamondback moth, *Plutella xylostella,* and larvae of the blackfly, Similium *damnosum* (Jones, *et al.,* 1993). Estimates of the numbers of chemical resistant insect species were 392 in 1980 compared with 25 in 1955 (Youdeowei and Service, 1983). Assuming this apparent trend continues, *B. thuringiensis* is likely to become increasingly important in the control of major pest species. However, insect resistance is not wholly restricted to chemical insecticides and some resistance to B. *thuringiensis* has been reported (McGaughey, 1985, McGaughey and Beeman, 1988, Stone *et* al. 1989). Identification of the genes which code for the 8-endotoxin (Hofte and Whiteley, 1989) has enabled the toxin to be incorporated into hosts such as plants and other bacteria thus extending the insecticidal capability of the organism (Vaeck *et al.,* 1987). This will lead to an inevitable increase in the exposure of target insects to the toxin and so further resistance may develop.

B. thuringiensis produces a number of metabolites with insecticidal properties. Work has concentrated on two of these, the (3-exotoxin and 8-endotoxin or insecticidal crystal protein (ICP). The former is structurally similar to ATP and acts by inhibiting protein synthesis through competing for binding sites thereby interfering with DNA-dependant RNA polymerase during RNA synthesis (Sebasta *et al. , 1981*). In immature insects, exposure to O-exotoxin manifests at critical development stages such as moulting or pupating. This results in prolonged development or mortality (Bond *et al.,* 1971). Adults are often infertile or have reduced fecundity (Sebasta *et al.,* 1981). Whilst the j3-exotoxin is effective at killing insects its action is not specific to invertebrates (Faust, 1.975). Vertebrates, including man, are susceptible to the toxin, with high levels of exposure resulting in death. Levinson *et al.* (1990) suggest that a single gene codes for the (3-exotoxin, and as such genetic manipulation may allow for this toxin to be removed from the strain when its presence is undesirable.

The mode of action of the 8-endotoxin is complex (Li *et al.,* 1991, Knowles, 1994). A full review of the mechanism of action is given by Knowles and Dow (1993). It is thought to require solubilization of the protein in the midgut of the insect. The protoxin is then proteolytically cleaved to yield an active toxic fragment of 60-65 kDa. This then penetrates the peritrophic membrane and binds to specific receptors situated on the midgut epithelial wall

where it induces the formation of pores in the cell membranes (Mathavan, *et al., 1989).* The result of this action is a net influx of ions and accompanying water (Knowles and Ellar, *1987),* eventually causing the cells to swell and lyse leading to massive epithelial disruption (Endo and Nishiitsutsuji-Uwo, *1980,* Reisner *et al., 1989).* The action of the crystal alone is often suffcient to cause death. However, many authors have reported an important role for spores in enhancing the action of the toxin (Burges *et al., 1976, Li et al., 1987,* Miyasono *et al., 1993).* Ellar *et al. (1990)* suggest that this role is one of exacerbating the onset of septicaemia. The insect pests of drying or dried fish often feed by burrowing into the flesh. As the mode of action is through ingestion of the active ingredient rather that by contact, extra care must be taken in the formulation and application to ensure that the concentration and distribution of the toxin on the target surface is sufficient to ensure adequate exposure whilst the insect is browsing on the surface.

The remit for this project was to investigate the potential role of B. *thuringiensis* in controlling the insect pests of drying and dried fish. If strains were identified with activity against these pests their application to protecting fish was to be investigated. This would entail: the safety of the strains to be assessed; the determination of fermentation parameters using substrates which are readily and cheaply available in developing countries; the development of a formulation for use on fish which would not prohibitively increase the production costs; the identification and development of a quality control method for use during production; the identification of a potential producer within the African continent. The remainder of this report details the work conducted to achieve these goals.

Chapter 2

Strain Isolation and Screening

At the onset of this project, it was not certain whether strains of B. *thuringiensis* could be identified which exhibited activity against Calliphoridae and Dermestidae. Several coleopteran active strains had been reported but few dipteran active strains, apart from the mosquitocidal strains. Hodgman *et al.* (1993) had demonstrated novel activity against larvae of the housefly in the isolate YBT-226, but, unfortunately, this strain was the property of E.I. Dupont de Nemours and was not intended for commercial release. A second report by Indrasith *et al.* (1992) showed the strain *B. thuringiensis* subsp. *kurstaki* HD-1 to be active against adult houseflies but not larvae. There had been no published reports of blowfly or fleshfly active strains. A screening programme was therefore undertaken to screen many known strains of *B. thuringiensis* for activity against species of tropical blowflies and *D. maculatus.* A toxin type was identified which exhibited activity against tropical blowfly larvae, but, despite extensive screening of known coleopteran active strains, no toxin type was found which showed activity against *D. maculatus.* Having identified the blowfly active toxin type, novel strains were isolated from soil samples collected from areas where high numbers of blowflies are found. These isolates were screened for activity against both blowfly larvae and D. *maculalus.* Several strains were identified with Calliphoridae activity, but none with Dermestidae activity. In view of the time and expenditure involved in carrying out the screening programme for Dermestidae activity, it was agreed that this aspect of the project should be curtailed. Screening of any newly available toxins continued but the isolation programme was halted.

This chapter describes the isolation and screening procedures undertaken. The LD50 values obtained for the active strains and their insecticidal crystal proteins (ICPs)are listed. Studies into aspects of the pathogenicity of the active strains relevant to larval infestation behaviour are described.

2.1 Materials and Method

2.1.1 Bacterial strains and growth conditions.

The following commercial strains were used in the screening: *Bacillus megaterium* N.C.I.B 7581 (obtained from Dr. P.J. White). *B. thuringiensis* strain 4412 (obtained frpm Dr. Peter Luthy) *B. thuringiensis* subspecies *tenebrionis* (from DSM, Germany) *B. thuringiensis* subspecies *israelensis* (donated by Dr. David Ellar) *B. thuringiensis* strains HD 1, HD2, and HD73 (donated by Dr. David Ellar) *B. thuringiensis* strain Buibui MT44 (obtained from Mycogen)

All bacterial strains were grown on Nutrient Broth or Nutrient Agar (Oxoid) supplemented at l ml 1-1 with CCY salts (Stewart *et al.* 1981) to aid sporulation. This medium was termed `BGM' medium. Liquid cultures were incubated in an orbital shaker at 30oC and 200 r.p.m. for 48 hours or until sporulation was complete. Spores and crystals were harvested by centrifugation for 60 minutes at 4,000 r.p.m. in an MSE

Mistral 3000E centrifuge. The pellet was washed twice with deionised water then resuspended in deionised water to give a final volume which represented a fifty-fold concentration with respect to the initial culture. These preparations were referred to as "fifty-fold concentrates" and were stored in small aliquots at -20oC.

2.1.2 Isolation of Pure Crystals.

Pure crystals were isolated by the method of Thomas & Ellar (1983).

2.1.3 Maintenance of Insect Colonies.

Fly colonies

Breeding populations of tropical blowfly species were established from imported larvae (MAFF licence number AHZ/980E/94/46). These comprised *Chrysomya megacephala* (Fab.) from Thailand, *Lucilia cuprina* (Weid.) from Senegal and *Chrysomya alhiceps* (Weid.) from Uganda. *Musca domestica* (Linn.) (WHO strain) was obtained in the UK. Colonies were maintained at 28 oC, 68 % RH with a 12:12 L:D photoperiod. Adults were giyen a maintenance diet of *ad-lib* sugar, milk powder and water, and were offered tinned cat-food as an oviposition medium on a twice weekly basis from the third week post-eclosion. Larvae for replacement colonies were reared on a yeast and agar based diet comprising 50g active dried yeast (DCL), 8g agar (Oxoid Bacteriological No 1), 250ml distilled water and 750ml UHT full cream milk, prepared by microwaving to boiling point.

Beetle colonies

Adults and larvae were reared in demi jars covered with filter paper and sealed with parafin wax. Whole, dried *Tilapia* spp. were placed in the jars as a food source for both adults and larvae. The insectory was maintained at 70% RH and 30°C, with a series of light:dark photoperiods.

2.1.4 Bioassay Procedure.

Fly Assay

The larval rearing diet defined above was used in the bioassays. *M. domestica,* acted as an indicator species for initial screening of toxins. Those protoxins exhibiting activity against *Mdomestica* were then screened against the tropical species.

The insecticidal suspension was dispensed into plastic pots (No 2 size, Ashwood Timber & Plastics Ltd.). Suspensions of pure crystals were prepared giving final concentrations of crystal protein in an exponential range from 2 to 128 ~Ig ml-l. The required volume of the spore-crystal fifty-fold concentrate was rediluted in distilled water, to give a final volume of lml. Molten diet, to give a total volume of 25mls, was added to the suspension of crystals, thoroughly mixed by agitation, then allowed to

set. The surface of the diet was scored to provide feeding crevices. 20 neonate larvae were transferred onto the diet. Pots were covered using filter paper held in place with elastic bands and placed in an incubator maintained at 27oC and 70% RH. Mortality was recorded after 48 hours. Moribund larvae were counted as living. The following controls were used for all bioassays unless otherwise specified: *B. thuringiensis* subsp. *tenebrionis* was used as a negative control at a dose equal to the highest dose used in the test range; the strain 4412 was used as a positive control at a dose of 201igml-1. All doses were replicated 5 times, and assays were repeated at least twice.

Beetle Assay

Toxins were screened individually. Assays were conducted in triplicate using 5 first instar larvae for each pot. 1 ml of the fifty-fold spore crystal concentrate was mixed into 5g of fish meal placed in 20ml plastic pots. Filter paper was secured over the pots to allow ventilation but prevent moisture build-up which would cause the fish meal to become too moist for the beetles to survive on. Pots were placed in an incubator maintained at 30oC and 70% RH. Mortality was recorded after 48 hours. A negative control was provided by using known non-toxic strains. No positive control was available.

2.1.5 Isolation procedures

About 0.25 g **(dry** weight) of environmental sample were placed in test-tubes containing 2 ml of a Nutrient Broth (Oxoid). The tubes were heat-shocked at 70oC for l0min in a water-bath. The contents of each tube were poured into 250M1 flasks containing 50 ml of Nutrient Broth (Oxoid) supplemented with CCY salts (Stewart, 1981), lm 1-1 to aid sporulation, and 20 International Units ml-I of penicillin G (Sigma Chemicals).The flasks were incubated at 30°C at 200 r.p.m. until sporulation was complete. The particulate matter was recovered by centrifugation for 60min at 3,600 rpm in an MSE Mistral 3000E. The pellets were resuspended in 2ml aliquots of liquid growth medium and put through a second cycle of the procedure above. The pelleted matter from the second centrifugation step was resuspended in 2ml of broth. Serial dilutions of these suspensions were plated out onto Nutrient Agar (Oxoid) containing CCY salts and supplemented with 20 International Units ml- 1 of penicillin G. After incubation at 30°C until sporulation was complete individual beige colonies with a matt texture were examined microscopically for the characteristic spores and crystals of B. *thuringiensis* (Bulla *et al.* 1980).

2.1.6 Identification of Isolates with Activity Against Blowfly Larvae.

Individual isolates were grown and harvested as previously described. Pooled samples comprising 300 pd of the fifty-fold concentrate from each of three isolates were initially bioassayed. Where larvicidal activity was exhibited, each isolate was individually screened to identify the toxic strain(s).

2.1.7 Electrophoresis of Crystal Proteins.

SDS-PAGE was carried out by the method of Laemmli (1970).

2.1.8 Protein Determination.

Protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin (Sigma Chemicals) as a standard.

2.1.9 Investigation into the Pathogenicity of **the Novel Isolate 13B.**

2.1.9.1 *Effect of Replacing Spores with Vegetative Cells.*

Vegetative cells, grown overnight in liquid media, were harvested and washed twice in distilled water, then kept on ice until the population density of the viable cells could be assessed by plate counts. Cells, at a level of colony forming units equivalent to the number of spores normally associated with an LD50 dose of crystals, were added to pure crystal preparations constituting an LD50 dose, then dispersed into the larval diet.

2.1.9.2 *Investigation into the Potential for Survival Following Limited Exposure to a Lethal Dose of B. thuringiensis.*

Large numbers of neonate larvae were placed onto diet containing a lethal dose of spores and crystals. Twenty larvae were transferred in triplicate to untreated diet at the following time intervals: 1 hour, 2 hours, 4 hours, 12 hours and 24 hours after the initial exposure. Pots containing recovered larvae were placed in an incubator at 27°C and 67% R.H. and monitored until all animals had either died or completed their life cycles. The experiment was repeated four times.

2.1.9.3 *Lethality of the Isolate 13B to Adult Flies.*

Toxicity of the ICP was assessed against adults of both M. *domestica* and *L. cuprina.* Four mesh cages covered with netting were set up to house the adult flies. Water and carbohydrate were provided respectively in the form of damp cotton wool pads and 250mg finely ground sucrose contained in an open dish. Ten, two day old flies were placed in each cage. Assays were set up as follows: Test cage 1: SOmg of 13B pure crystals were thoroughly mixed in with the sugar; Test cage 2: SOmg of 13B pure crystals were thoroughly mixed in with the sugar and 100mg of crystals were evenly spread onto the water pad; Control cage 1: SOmg of pure crystals of *B. thuringiensis* subsp. *israelensis* were thoroughly mixed in with the sugar; Control cage 2: an additional SOmg of sucrose was added to the sugar. Mortality rates were recorded daily for each cage. Water and sugar / sugar crystal mixes were replaced as necessary. The trial was considered complete when 100% mortality was attained in the test cages. To ensure that the lethality observed was a result of exposure to the B. *thuringiensis* and not a factor associated with the rearing conditions, controls remaining alive at the end of the experiment were maintained under the same conditions for a further 2 weeks.

2.2 Results

2.1 Strain assessments.

Of the known strains of *B. thuringiensis* that were bioassayed against fly larvae, only strains HD-2 and 4412 were found to be active. Isolation of novel strains from soil samples revealed six isolates exhibiting larvicidal activity out of 37 assayed. Three of these were classed as high activity **(1313, 113 and 313)** and three as moderate activity (l OB, 3A and AB). LD50 values were calculated using probit analysis of the bioassay data. Values are detailed in Table 2.1. The lethal dose range for the fifty-fold concentrate of spores and crystals of each strain assessed, against the larvae of a given species of fly, is given in Table 2.2. Values obtained from the protein analysis showed the protein production to be comparable in all strains.

Pure crystals of five of these isolates were analysed by SDS-PAGE (Fig 2.1). Four of the isolates produce very similar profiles indicating the presence of two protoxins in the 130-140 kDa range and one in the 60-70 kDa range. One of the isolates however, strain 1B, had only one protoxin type in both size categories. In spite of the similar electrophoretic pattern of the protoxins in the four strains, their bioactivity against fly larvae was markedly different, indicating that they are not the same isolate.

None of the strains screened for activity against *D. maculatzrs* were found to be toxic.

Table 2.1 LC50 values, calculated by logit transformation, for the two commercial strains, 4412 and HD-2, and the novel isolate 13B, against neonate larvae of *M. domestica* and two tropical blowfly species, *L. cuprina* and *C. albiceps.*

Species	Strain	LC5o (jig ml-1)	Standard **Error**	**95% Confidence Interval**
M. domestica	13B	77.6	1.0	(72.5, 83.0)
	4412	79.4	1.0	(76.7, 82.3)
	HD-2	81.3	1.0	(78 5, 84 21)
L. cuprina	13B	150	21.1	(107, 193)
	4412	303	75.1	(151, 454)
	HD-2	200	25.8	(148, 252)
C. albiceps	1313	607	141	(300, 914)
	4412	807	1320	(118, 1495)
	HD-2	1 390	316	(0, 4270)a

a Concentration required to effect an LC50 response is in excess of the maximum feasible concentration used thus resulting in a very poor logit fit for these data.

Table 2.2.

The minimum concentration of the fifty-fold concentrated spore/ crystal preparation required to effect an LID 100 response.

Strain	**Fly Species**		
	Alfn.sca domestica	*Lucilia cirprirra*	*Chrysomya megacephala*
4412	10	50	200
	40	300	450
HD-1	NA	NA	NA
HD- *t2*	NA	NA	NA
B. thuringiensis ssp. israelensis	NA	NA	NA
B. thuringien.sis ssp. tenebrionis	NA	NA	NA
Isolate 13B	25	100	200
Isolate 1B	30	150	250
Isolate 3B	30	100	300
Isolate AB	150	170	500
Isolate 1013	100	500	1000
Isolate 3.A	200	200	1000

NA not active

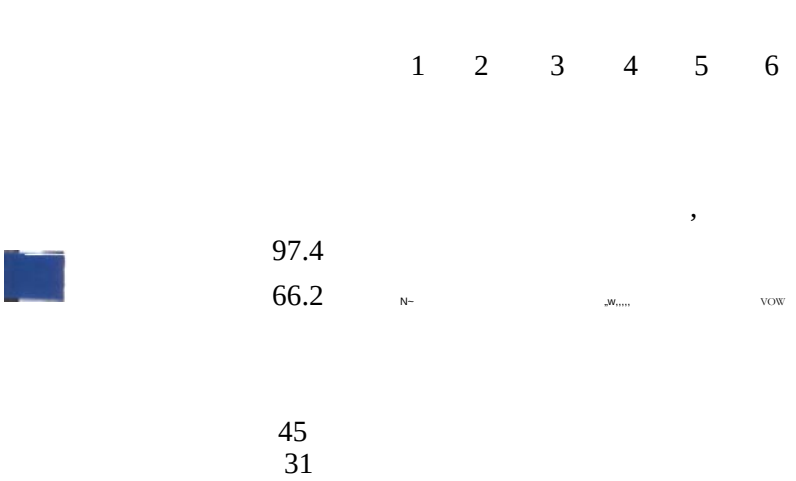
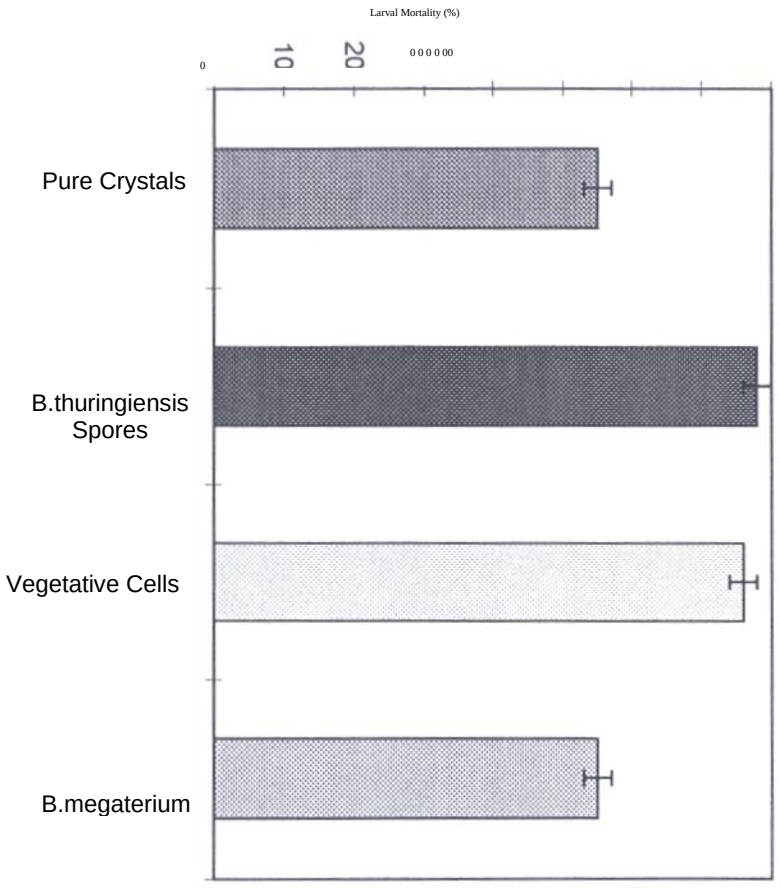


Fig. 2.1. Polypeptide profile of 8-endotoxins for the B. *thuringiensis* novel isolates on an SDS/10% polacrylamide gel (Coomassie Blue-stained). Lane 1, ~*Lr* marker; lane 2, 1.3B; lane 3, 1B; lane 4, 10B; lane 5, AB; lane 6, 3A



ot spores did, however, increase the rate of mortality (data not shown)

with the survival rate following each period of exposure being sporadic. Surviving larvae exhibited severe lethargy, and a reluctance or inability to move, but continued to feed within a confined area as measured by their weight gain and instar changes. This perhaps suggests that the observed lethargy is not attributable to paralysis, unless it is partial in effect. The variability in survival may be a result of natural variations in the population.

2.3 Discussion

These results extend the findings of Hodgman *et al. (1993)* in showing that blowflies as well as the house fly are susceptible to ICPs of B. *thuringiensis.* The latter report is, in fact, erroneous in that strain YBT was claimed not to be a producer of (3-exotoxin Hodgman *et al. (1993).* This strain has subsequently proven to be a strong producer of this toxin (Yu Ziniu, pers. comm.). The bioassay data cited are therefore wrong. Further work has shown that the ICPs of strain YBT are toxic to house fly larvae, in addition to the toxic effect of the P-exotoxin. Our work shows that it is exclusively the CryIB protoxin that is responsible for this toxicity. This has not been shown before. No other protoxin type had this activity. This makes the work of Indrasith *et al. (1992)* even more suspect in that they claimed that a CryIA toxin resulted in the death of adult and larval flies. We could not find any toxic effect of our Cry IB crystals on adults.

The variation in LC50 required for different strains of B. *thuringiensis* is typical of the species variation seen with this organism when applied to, say, lepidopteran larva. There is also an interesting variability between strains in that the most effective one against one larval species is not necessarily the most potent against another. Unfortunately the blowfly larvae are the most resistant to the ICPs,

Chapter 3

Strain Development and Safety

The expected end use of the insecticidal product is for application to fish intended for human consumption. The safety status of the strain to be used is therefore of primary importance. Hazardous metabolites potentially produced by B. *thuringiensis* include P-exotoxin and *B.cereus-type enterotoxin* and emetic toxin. A strain suitable for the purpose of this project must be unable to produce any of these products. Autoclaving (3-exotoxin at pH 3 destroys its toxic activity; it is stable in neutral or alkaline solutions (Levinson *et al.,* 1993).

3.1. O-Exotoxin Assessment

3.1.1. Materials and Methods

a) Bacterial strains and growth conditions
Bacterial strains and growth conditions were as detailed in section 2.1.1. with the following variations: following centrifugation the supernatant liquid, which will contain the water soluble, heat-stable (3-exotoxin if present, was retained and autoclaved for 15 minutes at 121°C to destroy any non-heat stable insecticidal metabolites.A second assessment was made using triplicate samples with their pH adjusted to 3, 7 and 12 respectively, prior to autoclaving. The sterilised culture fluids were then adjusted to pH 7.

b) Maintenance of insect colonies
Insect colonies were maintained as in section 2.1.3.

c) **Bioassay Procedure** Bioassays were carried out as described in section 2.1.4., with the following variations. In place of the spore/ crystal concentrate, 3mls of the autoclaved supernatant was dispersed in 22 mls of molten diet. Positive and negative controls were provided by using the autoclaved culture fluids from *B. thuringiensis* strain HD-2 and *B. thuringiensis ssp. tenebrionis* respectively. For assays involving the supernatant liquids whose pH values had been adjusted further controls were provided which incorporated a volume of acid or alkali, equivalent to that which was required to adjust the pH value, into the molten diet. Larvae were neared on the assay diets until they either died or completed their life-cycles. Death at any instar change, or failure to successfully pupate, strongly indicated the presence of (3-exotoxin. Successful pupation and adult emergence confirmed the negative O-exotoxin status of the strain.

3.1.2. Results

Bioassay results showed that all six of the novel isolates were positive for P-exotoxin as was *B. thuringiensis* strain 4412. Adjusting the pH prior to autoclaving further confirmed these results. Growth and mortality rates in larvae exposed to the

supernatant at pH 7 and pH 12 were comparable to one another and equivalent to the positive control, whilst those exposed to the supernatant at pH 3 were equivalent to the negative control (see table 3.1.1). No attempt was made to quantify the level of (3exotoxin production as this was not considered to be relevant to the project purpose (any strain producing P-exotoxin will be unacceptable).

Table 3.1.1: **O-Exotoxin assessment of strains using autoclaved supernatant fluids**

Strain	Exotoxin assessment
13B	positive
113	positive
3B	positive
3 A	positive
AB	positive
1 OB	positive
4412	positive
B. thuringiensis subsp. *israelensis*	negative
Controls'	negative

1 Culture fluid of strain 13B autoclaved at pH ..3 and then neutralised.

3.1.3. Discussion

These results show that all blowfly toxic strains in our possession are P-exotoxin producers. In view of the non-specific action of this type of toxin, the strains as they are cannot be used for their intended purpose of application to a food product. Several commercially available strains are P-exotoxin producers, but, more recently, agrochemical companies will only market P-exotoxin minus strains.

Levinson *et al. (1990)* found that P-exotoxin production was carried on a single plasmid. It may therefore be possible to remove this plasmid either by conjugation or through plasmid curing (growing the organism under conditions which will make it lose some or all of its extrachromasomal DNA). The success of this, however, may be dependent upon the plasmid encoding for the P-exotoxin not also encoding for the 8endotoxin. In their investigation, Levinson *et al. (1990)* found that in the five strains of *B. thuringiensis* producing P-exotoxin they assessed, the (3-exotoxin plasmid also encoded one or more insecticidal crystal protein.

3.2 Strain Development to produce O-exotoxin minus status

3.2.1 Introduction

The finding that all of the fly-toxic strains in our possession produce P-exotoxin lead to the decision to try to remove this trait. Theoretically the simplest way to achieve this would have been to clone the gene encoding the active 6-endotoxin and express it in an

acceptable vector such as *B. thuringiensis* subspecies *israelensis*. The problem with this approach is that it is currently highly unlikely to obtain permission in any country to liberate any micro-organism which has been genetically manipulated. This term covers techniques which alter the genetic material of an organism in a way that could not occur naturally. Fortunately bacteria are adept at transferring DNA between themselves and also seem to lose DNA, particularly extrachromosomal DNA quite easily. Two strategies, involving naturally-occurring processess, designed to arrive at a strain of *B. thuringiensis* incapable of producing (3-exotoxin were adopted. These were plasmid curing and conjugation.

3.2.1(i) Plasmid curing.

The development of a strain lacking the ability to synthesise exotoxin by the conjugational approaches described below is a time-consuming task and the frequency of transfer of plasmids carrying the ciy genes may be low. In addition, there is no guarantee that these genes will be transcribed and then translated into crystal proteins which retain the same high activity as they exhibited in the donor strain.

Alternatively, plasmid curing could be used to achieve the same end result as exotoxin is thought to be plasmid encoded. This would provide a rapid, simple means of obtaining a strain unable to synthesise exotoxin, but synthesising a highly active 8endotoxin as the `donor' strain could be rid of exotoxin directly. Such an approach was validated by the fact that a gene for a 32kDa protein (not a toxin) was found to be lost preferentially during heat curing of plasmids (Aileen Van Nguyen, PhD thesis). Plasmid cured colonies were first examined by phase-contrast microscopy to make sure crystal producing ability was retained and then tested for exotoxin production by bioassay.

3.2.1(ii) Conjugation.

This approach was used to transfer DNA from a donor strain of B. *thuringiensis* synthesising a highly active toxin to an exotoxin negative, recipient strain of B. *thuringiensis* in such a way as would occur in Nature. Any resultant strain would therefore stand a good chance of being approved for release into theenvironment by the appropriate regulatory bodies. The problem with such an approach proved to be a quick, reliable way in which to distinguish donor from recipient and transcipient colonies following conjugation without the use of plasmid antibiotic markersTwo possible means by which this could be achieved were devised and tested:

(a) *Antibiotic Selection on the basis of minimal inhibitory concentrations (MICs).* Many different bacterial species are inherently resistant to certain inhibitory compounds.It is well known that some species will synthesise compounds toxic to susceptible, or less resistant species. This puts such species at a competitive advantage in terms of survival, especially in situations where essential nutrients are limiting i.e. in a contained, finite system such as an agar plate or an insect larva. It would be highly desirable if the recipient strain was resistant to an antibiotic and the donor strain was

not. Following conjugation, the cells could be plated out onto agar plates containing the antibiotic and only recipient and transcipients would be able to grow. As the donor and recipient strains are of the same species and as such, highly homologous, this would be unlikely to occur naturally. All strains of B. *thuringiensis* are penicillin resistant but it would be feasible that the degree of resistance could differ between strains. This would be reflected by differences in the minimal inhibitory concentrations of penicillin between strains. Another possibility was that these strains could have differing degrees of resistance to other antibiotics such as chloramphenicol and streptomycin. Strains 4412 and 13B were selected as potential `donors' as had been previously shown to synthesise a highly active crystal protein. Strain 1013 was selected as the `recipient' as it was shown to be exotoxin negative and originally thought also to be enterotoxin negative. In order to determine whether or not it was possible to transfer crystal-producing ability from a donor to recipient strain, an acrystalliferous recipient strain was required. This was achieved by `plasmid curing'.

(b) *Selection against donor strain achieved by growth in nonpermissive growth medium.* It was discovered that the novel isolates varied in their ability to sporulate on a particular medium (BFB). All, however, could produce good vegetative growth. This couad be taken advantage of if the donor strain was unable to sporulate and the recipient strain (a cry- mutant) sporulated well in a particular medium. It would mean that if conjugation was carried out in such a medium and the resultant cells allowed to sporulate, only recipient and transcipient cells should have the ability to produce heat resistant spores. In this way, after heat shock, there would be no donor cells present and transcipients (of Cry' phenotype) could be subsequently identified by phase contrast microscopy. These would be recipient cells which had gained the ability to produce ICP from the donor i.e. they would have gained the blowfly-toxic gene.

3.2.2 Materials and Method

a) (3-exotoxin Assessment

The protocol followed was as described in section 3.1. Individual colonies from 44 plates grown at 42°C were bioassayed for exotoxin activity.

b) Conjugation

(Based on method of Jarrett *et al., 1990).* `BFB' medium is composed of:
Blood, fish and bone-meal (B&Q) 18g/ 1
Chickpea flour (Holland and Barrett) 18g/ 1
Molasses (Holland and Barrett) 5ml/ 1
To make solid media Technical Agar (Oxoid) was added at 1.5%(w/v).

Donor and recipient colonies were grown separately in boiling tubes containing BGM or BFB growth media (5ml), respectively. These were incubated overnight for approximately 18h at 30°C. Aliquots of donor and recipient cultures 100 pd) were

added to fresh medium (5ml). Aliquots of each (100 pd) were added separately to fresh medium as a control. These were incubated at 30°C, 40 r.p.m for 24hrs, then at 200 r.p.m until sporulation was complete. Samples of culture (lml) were transferred to Eppendorfs tubes (1.5ml) and heat-shocked at 60°C for 10 minutes. Serial dilutions were made and these plated out onto BGM or BFB agar plates then incubated at 30°C overnight.

c) **Minimal Inhibitory Concentration (MIC) determination -liquid medium.**

B. thuringiensis strains AB, 4412 and 1313- were grown on BGM + CCY plates at 30°C overnight. A single colony was then resuspended in sterile BGM medium and an OD_{600} measured, then adjusted to a value of 0.6. An inoculum (101) was added to BGM + CCY test tubes (5m1) containing different volumes of a filter sterilised solution of penicillin G (l0mg/ml). Penicillin concentrations tested ranged from 0 - 500 International units/ml. The tubes were then incubated at 30°C overnight and growth examined the next day.

-antibiotic plates.

Lawns of novel strains l OB and AB (medium activity isolates - potential recipient strains), 4412 and 13B (high activity isolates - potential donor strains) were spread over BGM agar plates containing either a 1/50 concentration or a 1/100 concentration of a penicillin/streptomycin (5,000 I.U./ ml and 5mg/ ml, respectively) mixture (Sigma) or chloramphenicol (CAP) at 511,g/ ml. Control lawns were also spread onto BGM plates with no antibiotic present.

(d) `Total' plasmid curing to remove the ability to produce ICPs

Strain AB, which was to be the recipient strain, was streaked out to single colonies on BGM + CCY agar plates, incubated at 42°C until sporulation was complete (as determined by phase contrast microscopy) and then restreaked and incubated at 30°C, again until sporulation. Colonies were then microscopically examined for the absence of crystalline inclusions in the spore mother cell (mutants with the spo+ crygenotype). Sometimes, several rounds of curing were required to result in the disappearance of crystals. A better method involved incubating at 42°C for 24h and then transferring the plates to 30°C directly. In this way it was possible to determine the phenotype immediately.

(e) **8-Endotoxin assessment of cured strains**

Bioassays were carried out as detailed in Chapter 2.

(f) **Partial plasmid curing to lose the ability to produce (3-exotoxin**

Strains were streaked out onto nutrient agar plates supplemented with CCY salts (Stewart *et al. 1981).* Plates were incubated at 42°C until sporulation was complete.

Individual colonies were then examined microscopically for the presence of crystal inclusions. Those colonies retaining crystal production were restreaked onto Nutrient Agar and grown at 30°C, then re-examined for the presence of crystal inclusions.

(g) **Conjugation using antibiotic selection**

I.Three boiling tubes containing 5ml of BGM + CCY were inoculated with either donor strain 4412 pBC 16 (TetR) , 4412 pC 194 (CapR) or recipient strain l OB cry-. These were incubated overnight at 30°C. 2. The OD_{600} of each tube was measured and, where necessary, made equivalent. 3. A sample (100p1) of donor and recipient was added to fresh BGM + CCY medium (5ml) and incubated at 30°C with very gentle shaking (40 r.p.m) for 24h. A sample (100p,1) of donor and recipient cultures were also added to fresh BGM + CCY (5ml) and incubated separately as controls. 4. A dilution series was performed for each tube (recipient strain lOB cry-, donor strains 4412 pBC 16 and 4412 pC 194 alone ; strain l OB cry+ strain 4412 pBC 16 ; strain l OB cry- + strain 4412 pC 194) and appropriate dilution(s) plated out onto BGM

CCY and/ or BGM + CCY + CAP/TET plates. These were incubated at 30°C overnight and examined the next day.

(h) Electroporation

From Bone & Ellar (1989), modified by S. Gash (pers. comm.). 1. A recipient lawn of isolate 4412 was grown on BGM, overnight at room temperature. 2. Sterile water (2m1) was added to the lawn and the bacteria scraped off into an eppendorf tube. 3. The tube was centrifuged for 10 secs. and the resulting pellet resuspended in electroporation buffer (272mM sucrose; 8mM HEPES pH 7.4 ; 15% glycerol). 4. Centrifugation was repeated followed by resuspension in electroporation buffer (120p,1 per intended electroporation). The electroporator (Bio-Rad, with Pulse controller) was set at 400W ; 25mF ; 1.8 kV) 5. Plasmid DNA solution(1ml) was added to 120p1 of 4412 recipient cell suspension in 0.4 cm gap electroporation cuvettes (Bio-Rad). 6. Electroporation was carried out then cuvette contents transferred to a boiling tube containing l ml of LB and incubated at 30°C, 200 rpm for 40 mins. 7. Chloramphenicol (CAP) was added to the tube at a concentration of 5pg/ml, and the incubation continued for a further 20 min. 8. Dilutions were plated out onto LB + CAP (5mg/ml) or LB + TET (25pg/ml) plates and these incubated overnight at 30°C. NB. Recipient lawns incubated overnight were left for no longer than 17 h before electroporation was carried out.

3.3 RESULTS

3.3.1 (a) Conjugation: selection against donor by growth on a medium inconducive to sporulation

Initial observations of the variablity of sporulation and ICP formation in BFB medium are shown in the tables, below. All strains were able to produce excellent vegetative growth in this medium. Cultures exposed to a heat shock (after an incubation period adequate for sporulation to be completed) will only give rise to colonies if spores have, indeed, been formed. Thus strains with a decreased capacity for sporulation in this medium will give rise to fewer colonies than those able to sporulate successfully.

Table 3.2.1 Microscopic analysis of strains of *B. thuringiensis* grown in BFB medium with and without CCY salts.

Strain	Liquid medium	
	BFB medium minus CCY salts	BFB medium plus CCY salts
Isolate 13B	A few spores and crystals	Many spores and crystals
Strain 4412	Crystals, no spores	Crystals, v. few spores
Isolate l OB cry-	A few spores	Many spores and crystals

Strain	Solid medium	
Isolate 13B	Spores and crystals	Many spores and crystals
Strain 4412	v.few spores and crystals	Few spores and crystals
Isolate lOB cry-	man sores, few c stals	Spores and c stals

Table 3.3.2 Plate counts (10-6 dilution) of strains of *B. thuringiensis* grown on BFB medium with and without CCY salts.

Strain	BFB medium - CCY salts	BFB medium+ CCY salts
Isolate 13B	156	170
Isolate 4412	38	52
Isolate lOB	276	>300

The results obtained from the preliminary experiments strongly suggest that when incubated in BFB medium strain 4412 sporulates very poorly. Isolates 13B and lOB are able to sporulate more efficiently, with the latter yielding the most spores. This is true in either the presence or absence of CCY salts, although all three strains fare better when CCY salts are included in the growth medium.

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3.3.1 (b) Conjugation and selection of *B. thuringiensis* transcipients grown in BFB medium.

Experiments involving the conjugational transfer of plasmid DNA were subsequently carried out using isolate 4412 as donor and the plasmid-cured acrystalliferous mutant strain, l OB *cry-*, as recipient. The conjugation method described in 3.2.2 (b) was used. As a direct consequence of the results obtained from the preliminary experiments, a method was devised to separate the donor from recipient strain following conjugation. It was so envisaged that only recipient and transcipient colonies would ultimately survive and these could be distinguished by phase-contrast microscopy. 4412 was chosen as donor in preference to 13B as it contains only one type of *cry* gene, cry IB(a), whereas 13B contains additional *cry* genes (inferred from SDS PAGE analysis, see previous chapter). In addition, strain 4412 produces characteristic bipyramidal crystals. It was hoped that in transcipient cells the crystals would retain this conformation, facilitatin g the screening of large numbers of colonies.

It was discovered that donor colonies still had the ability to grow on the final plates following the heat- shocking treatment i.e. strain 4412 was sporulating in the `nonpermissive' growth medium. In an attempt to eliminate the donor strain, a more intense heat-shock was applied (70°C for 30 min.) but this made no significant difference. The experiment was then again repeated, but higher dilutions plated out as it was assumed that even though the donor strain was sporulating, the level at which it was doing so was likely to be much less than that of the recipient/transcipient strain. This dilution idea, however, also proved unsuccessful and it was concluded that the donor strain could not be successfully `weeded out' using this approach.

There was one useful observation resulting from these experiments in that 4412 donor and isolate l OB *cry-* recipient colonies were of differing morphology when grown on either BFB or BGM plates: the latter strain produced colonies with ragged edges, those of the former strain were very crisply defined. This clear difference in colony morphology was thought to be a potential method for distinguishing donor from recipient colonies (as opposed to the traditional method of exclusively preventing the growth of the donor colonies). In such a way, it would be possible to then identify colonies with isolate lOB type morphology and screen these for the presence of crystals i.e. identify the transcipients.

A possibility creating cause for concern was the fear that there was no DNA transfer (or a very small, undetectable amount) occurring at all. An initial approach was consequently devised to (i) demonstrate DNA transfer was in fact taking place, (ii)separate out donor colonies on the basis of differing morphology and (iii) show that crystal producing ability was specifically being transferred to the recipient strain. Were the results of this approach encouraging the antibiotic selection could be omitted so that the desired transcipient could be sought using methods not requiring genetic manipulation. In order to demonstrate DNA transfer was occurring, plasmids pBCl6 (TetR) and pC 194 (CapR) were first isolated from P. Jarrett's strain of *B. thuringiensis* subsp. aizawai and *B. subtilis* 168, respectively, using the alkaline lysis method (section 4.2.2). These were then separately electroporated into donor strain 4412.

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Colonies to which either antibiotic resistant plasmid had been transferred (those growing on the antibiotic plates) were subsequently conjugated with the acrystalliferous recipient strain 1013 using using the antibiotic selection method as described in 3.2.2 (g). Selection for tetracycline resistance was made at 25~tgml-".

It was discovered that when grown separately (i.e. in the control tubes), an equivalent number of donor 4412 and recipient l OB cry- colonies resulted. The strains could be seen to differ in morphology as strain 4412 produced small, well-defined round colonies whereas strain lOB colonies were larger with ill-defined, ragged edges. When grown together in order to allow conjugation to take place, however, no colonies of isolate lOB type morphology resulted on BGM + CCY + TET/CAP plates. This could have meant that the antibiotic resistant plasmids were not being transferred from donor to recipient ie. no DNA transfer had taken place. Colonies of isolate l OB type morphology did, however, grow on the BGM + CCY plates, but in a number significantly less than that of 4412 colonies.

From this, it was concluded that perhaps strain 4412 had some kind of competitive advantage over isolate I OB since when cultures of identical optical density were incubated together, 4412 colonies greatly outnumbered 1013 colonies. It would be reasonable to suggest that this could possibly be due to the fact that strain 4412 is exotoxin positive while strain lOB exotoxin negative. It has been previously shown that (3-exotoxin inhibits the polymerase activities of strains of both *Escherichia coli* and B. *thuringiensis* (Johnson, 1978). It could well be imagined that this toxin could effect the replication of a non-producer, such as strain lOB, thus giving the producing organism, strain 4412, a competitive advantage.

Following this assumption, the conjugation method previously described was modified slightly. The 4412 donor cultures were diluted 5 x and 25 x, before 1001 of each was added to 100~tl of recipient strain lOB *cry-* and conjugation allowed to take place. Strains were again distinguished on the basis of differing colony morphology, the results obtained were as shown below.

Table 3.3.3. Controls of electroporation experiment

Ratio	**4412 pBC16 (dil. 10-4)**		**4412 pC194T ~~ lOB cry-(dil. (dil. 104)**		**10-4)**
	BGM+CCY	**BGM/CCY/ TET**	**BGM+CCY**	**BGM/CCY/ CAP**	**BGM+CCY**
1:1	>300	273	>300	>300	>300
5:1	>300	218	>300	>300	>300
25:1	>300	191	>300	>300	>300

It was observed that a greater number of colonies were present when the antibiotic resistant strains of 4412 were plated out onto BGM + CCY as opposed to BGM + CCY + TET/CAP. This was put down to the fact that as the strains were incubated in

would be no selective pressure or advantage in exhibiting antibiotic resistance. When conjugation cultures of strain 4412 pBC 16 + l OB *cry-* were plated onto BGM + CCY + TET no growth of either strain on any of the BGM + CCY + TET plates was observed.

Table 3.3.4. Conjugation culture plated onto BGM + CCY

Ratio-.- 4412 pBC16 + lOB cfy_- ..... **4412 pC194 + lOB cry_**	**(dil. 10-'')**		**(dil. 10-')**	
....	*IOB_ moj~holo~*	`I`ll *2_ mor~holo~y*	*IOB. morpholo~y :~41:~. mor~holoa*	....
1:1	22	188	56	200
5:1	>350	0	292	65
25:1	>350	0	360	10

'	**4412 pC194 + IOB cry- (dil. 10~) colonies**				
	1:1 ratio		**5:1 ratio**	**25:1 ratio**	
Strain IOB Strain 4412 Strain JOB Strain =1412 Strain JOB Strain 4412					
__ morrhofty_ .... _ morpholo,gy., ... mo2holo~y.__._, morphofty._ _, morphofty ..... _ morpholo~y__					
0	218	2	78	ND	ND
0	238	2	71		
0	280	0	65		
0	230	4	75		
0	292	1	83		
0	280	1	145		
0	222	1	116		
0	224	10	70		
0	300	0	68		
0	380	3	87		
0	218	1	87		
0	252	0	104		
1	70	16	50		
1	44	4	52		
0	200	4	70		
2	103	1	67		
1	300	0	83		

Determination of Transformation Efficiency
Total number of strain l OB cry- (BGM+CCYplates) = 292 colonies/ plate
(This representslOB cry- CA PR + lOB cry- CAPS)

Number of strain l OB cry- (BGM + CCY + CAP plates) = 50 colonies/ 18 plates
= 2.78 / plate

Therefore the percentage 1013 cry- CAPR = 2.78/ 292 x 100 = 0.95%

The results show that plasmid pC 194 (Cap) has been transferred from the 4412 donor to isolate l OB cry- recipient as colonies of lOB - type morphology were present on the BGM + CCY + CAP plates. DNA transfer has taken place. Subsequently, these colonies were examined by phase contrast microscopy for the presence of crystals in order to ascertain whether or not the plasmid(s) for crystal-producing ability had been transferred along with that (those) for antibiotic resistance. None of the isolate l OB cry- colonies appeared to contain convincing parasporal bodies. In view of the very large size of the plasmids of *B. thuringiensis* (Gonzalez *et al.* 1981) one would expect that transformation by conjugation of ICP-encoding plasmids would be a very rare event. There can be little doubt that it is possible, nevertheless, as the profusion of strains of *B. thuringiensis* carrying similar protoxin genes would seem to testify.

3.4 Plasmid curing to lose the ability to produce (3-exotoxin

The development of a strain lacking the ability to synthesise exotoxin by the conjugational approaches previously described is a time-consuming task and the frequency of transfer of plasmids carrying the *cry* genes may be low. In addition, there is no guarantee that these genes will be transcribed and then translated into crystal proteins which retain the same high activity as they exhibited in the donor strain. Alternatively, plasmid curing could be used to achieve the same end result as Pexotoxin is thought to be plasmid encoded (Levinson *et al.*, 1990). This would provide a rapid and simple means of obtaining a strain unable to synthesise exotoxin, but synthesising a highly active 8-endotoxin. Such an approach was validated by the fact that a gene for a 32kDa protein (not a toxin) was found to be lost preferentially during heat curing of plasmids over plasmids encoding cry genes (Aileen Van Nguyen, PhD thesis). Plasmid cured colonies were first examined by phase-contrast microscopy to make sure crystal producing ability was retained and then tested for exotoxin production by bioassay.

Two separate attempts were made to `cure' strains of the ability to produce (3exotoxin: the inital attempt gave rise to several mutants which were shown conclusively by bioassay to have lost the ability to produce (3-exotoxin. These mutants retained the ability to produce ICPs and were, in fact, used in the studies on fermentation media (Chapter 5). During a programme of sub-culturing, designed to check the stability of these mutants and which took place over a period of about 9 months these strains slowly regained the ability to produce (3-exotoxin. An attempt to explain this very unexpected occurrence is made later in this chapter.

During the re-emergence of the production of P-exotoxin in the mutants obtained in the first round of curing a second attempt was made. The mutants derived from this attempt were studied more closely, the results are tabulated in Table 3.4.1. Of the 44

colonies assayed 9 appeared to have lost the ability to produce (3-exotoxin, as determined by larval survival through to adult emergence. Colonies which were deemed (3-exotoxin minus were restreaked to assess strain stability. These were then re-assessed by bioassay. Only three of the cured strains remained j3-exotoxin minus (A1, S3 and S9). Larvae exposed to Al and S9, however, grew more slowly and pupated at significantly lower body weights than those exposed to S3 or the control. This perhaps suggests that a reduced level of (3-exotoxin production had resumed in these two cured strains.

The 8-endotoxin activity of these cured strains was then assessed (see Table 3.2.2). 8-Endotoxin activity equal to that of the parent strain was present in strains Al and S9. 8-Endotoxin activity was absent in S3.

Repeated P-exotoxin assessments using bioassays for the plasmid cured strains indicated that (3-exotoxin production increased with each subsequent generation in strains Al and S9, but remained absent in strain S3 (see Table 3.4.1). The 8-endotoxin activity in strains Al and S9 was also found to increase with the increase in (3exotoxin production (see Table 3.4.2). As mentioned above (3-exotoxin production was also found to resume in colonies from plates which represented earlier generations originally found to be (3-exotoxin minus (data not shown).

Table 3.4.1: (3-Exotoxin Assessment of Plasmid Cured Strains

Cured Strain	**Plate 1** **Exo. 'Larva Status 1 Weigh** t m		**Plate 2 (restreak)** **Exo. Larva Status I Weigh** t m		**Plate 3** (rest ea Exo. **'Larva Status** 1 Weigh t m		**Plate 4 (rest eak)** Exo. **'Larva Status I** Weigh t m	
Al	-	4.1	-	3.7	+	3.0	++	2.5
D 1	-		+++	1.9	+++	1.9	+++	1. 8
D3	-		+++	2.1	+++	2.0	+++	2.1
D5	-		+++	1.6	+++	1.6	+++	1.7
D6	-		+++	2.0	+++	1.9	+++	2.0
E2	-		+++	2.1	+++	2.1	+++	2.1
E3	-		+++	1.6	+++	1, 6	+++	1. 6
S3	-	4.4	-	4.1 .	-	4.4	-	4.3
S9	-	4. 5	-	3 . 8	++	2.6	+++	1.9
Controls								
Btt	-	4.4	-	4.1	-	4.3	-	4.4
4412	+++	1. 8	+++	1. 8	+++	1.9	+++	1. 8

Larval weight represents the mean larval weight calculated from the gross weight of 20 larvae recorded at 48 hours.

\- (3-Exotoxin production absent : determined by normal larval growth and development through to adulthood.

+ Some (3-exotoxin production likely : determined by reduced larval growth and failure to pupate.

++ Increased P-exotoxin production : determined by further reduction in larval growth and failure to pupate.

+++ Confirmed (3-exotoxin producer : determined by severe effect on larval growth and death at instar change.

Table 3.4.2: b-Endotoxin Activity of Plasmid Cured Strains A1, S3 and S9.

Cured Strain	Plate 2 (restreak)		Plate 3 (restreak)		Plate 4 (rest (rest	
	Endotoxin m	Larval Status Weight	Endotoxin m	Larval Status Weight	Endotoxi a n	Larval Status Weight
A 1	+	3 .3	++	2.6	++	2.0
S3	-	4.3	-	4.4	-	4.3
S9	+	3.3	++	2.5	+++	All dead
Btt	-	4.5	-	4.4	-	4.4
4412	+++	All dead	+++	All dead	+++	All dead

Dose rate used was that which would result in 100% mortality with the parent strain.

Larval weight represents the mean larval weight calculated from the gross weight of 20 larvae recorded at 48 hours.

+ Low activity : some reduction in growth in comparison to the negative control. Larvae pupated but adults failed to emerge.

++ Medium activity: Further reduction in growth. Some larvae pupated, many died prior to pupation.

+++ High activity: lethality equivalent to that of the parent strain at the same dose rate.

3.4.3. Discussion

The plasmid which encodes for (3-exotoxin production can be removed by plasmid curing. From our results, however, elimination of the capability to produce (3-exotoxin, in some cases, also detrimentally affected the 8-endotoxin activity. This is in partial agreement with the findings of Levinson et al. (1990). These authors, however, found that in the cases they studed all (3-exotoxin minus mutants were also devoid of ICPs (`cry-'genotype). Prolonged exposure to elevated temperatures will, as this report, for example, shows, cause all plasmids to be lost. In this instance we used limited exposure to elevated temperature in the hope of obtaining partial plasmid loss. In the case of S3, crystal proteins were still produced at sporulation but activity towards our target insects was no longer present. It may be that only the CryIB toxin is absent and that other toxins remain complete. If this is so, then it may be possible to remove the (3exotoxin plasmid and retain the CryIB activity using plasmid curing. Some of the initial

(3-exotoxin minus mutants that we isolated retained comparable activity in their ICPs with the parent strains. These, along with the other such mutants that we isolated regained the ability to produce (3-exotoxin with successive sub-cultures. This observation is hard to explain. One explanation could be that the initial heat-cured population was not entirely homogenous: a small proportion of parental-type, (3exotoxin producers may have remained. If the ability to produce (3-exotoxin confers a selective growth advantage over non-producers, as is hypothesized above, 3.3.L(b), the former type will, upon sub-culture, become more prevalent in the population. This would explain the apparent re-appearance, over time, of the ability to produce (3exotoxin in a population which was thought to have lost this ability. Alternatively, the observed initial absence of (3-exotoxin production in the cured strains Al and S9 is difficult to explain. It is possible that the (3-exotoxin production is switched off when the organism is under extreme stress as occurs during growth at elevated temperatures.

Attempts to transfer the plasmid carrying the 8-endotoxin gene of strain 4412 to a strain incapable of producing (3-exotoxin and enterotoxin were unsuccessful. The initial stumbling block in the conjugation process was the effective removal of the donor cells from the population after any genetic transfer had taken place. The approaches that we devised to circumvent the inability to use any sort of antibiotic selection produced by

genetic manipulation were unsuccessful. Control experiments showed that the conditions that we used were suitable to facilitate conjugation. It must be borne in mind, however, that the antibiotic resistance plasmids that were transfered are small. In contrast the plasmids of *B. thuringiensis* that carry the 8-endotoxin genes of *B. thuringiensis* are very large (Gonzalez *et al., 1981;* Carlton and Gonzalez *1985).* Since ease of transfer is inversely related to plasmid size one might have to be rather optimistic (or have a very good selection method) to be able to isolate transconjugants of *B. thuringiensis* plasmids with ease.

3.5. Mammalian toxicity assessment

The mammalian toxicity assessment was carried out under Home Office Licence number PPL 30/1130. Whilst the toxicological safety of *B. thuringinesis* has been well studied (see Drobniewski, 1994), its previous applications have been for non-food use'. In view of this it was considered necessary, ethically, to establish the toxicity status of our strain.

3.5.1. Materials and Methods

a) Toxicological Study

A total of fifty female rats were used for the study. Exposure was via the oral or subcutaneous routes. The oral dose comprised 10'2 spores administered daily for 21 days. The sub-cutaneous dose involved a single injection of 106 spores, administered on Day 1 of the treatments. Controls were provided by equivalent doses of autoclaved preparations. Ten rats were used in each treatment group. A further ten untreated rats

1 Plants intended for human consumption are not classified as food until after harvesting.

were used as a control against the handling involved in administering the treatments. Rats were kept for seven days prior to commencing the treatments to ensure all animals were healthy.

Treatments were carried out by trained personnel in accordance with Home Office legislation. Rats exposed via the oral route were orally intubated and the contents of one mini-centrifuge tube administered by injection into the stomach via the tube. Rats exposed via the sub-cutaneous route were injected into the fat pad situated dorsally at the base of the neck.

All rats were maintained for 23 days from the commencement of the treatments.

b) Preparation of inoculum

Strain 13B was grown up and harvested as detailed in section 2.1.1. The concentrated spore/crystal mix was held on ice at 4°C until the population density of viable cells could be assessed by plate counts. The spore/crystal concentrate was then resuspended in sterile distilled water to give a final concentration of 10'2 spores ml-1. Half of this stock was autoclaved to provide a control. A total of 210 aliquots (l ml) of each stock, representing a single oral dose, were then dispensed into labelled, sterile minicentrifuge tubes (Eppendorf). Tubes were batched in tens (a single day's treatment per batch of rats) placed in plastic bags and immediately placed in a freezer at -20°C until needed. Both stocks were continuously stirred on magnetic stirrers whilst the aliquots were being dispensed.

The remaining stocks were further diluted with sterile distilled water to give a final concentration of 106 spores ml-1. A further 10 aliquots(1 ml), representing a single subcutaneous injection dose, of each stock were dispensed into labelled, sterile minicentrifuge tubes (Eppendorf). These were again batched and placed in a freezer at -20°C until needed.

c) Post-treatment analysis

On completion of the treatments rats were weighed on a top pan balance in groups of five, each group having undergone the same treatment. They were then sacrificed by cervical dislocation.

Full post-mortem examinations were conducted by a veterinary surgeon experienced in pathology. Blood samples were collected from the heart using sterile needles with heparinised vacutainer tubes. The following organs were removed for culturing for the presence of B. *thuringiensis:* liver, heart, spleen, kidneys, lungs, section of small intestine, and in those animals exposed via the sub-cutaneous route the fat pad was also removed. All organs were incised after removal and examined for pathological abnormalities. Organs were pooled according to exposure and mean weights were recorded using a top-pan balance. Organs were held on ice at 4°C until results from cultures were obtained.

With the exception of the viscera, organs were homogenised in sterile Ringers solution (Oxoid) and plate counts from the homogenate carried out using nutrient agar plates. Blood samples were mixed by inverting several times then 1 ml samples spread on nutrient agar plates. Viscera were incised using a sterile blade and the contents emptied into 10 ml aliquots of sterile distilled water. Plate counts from serial dilutions were carried out. These dilutions were then heat shocked at 80°C for 10 minutes to eliminate any vegetative cells as well as the non-spore forming bacteria naturally present in the gut. Comparative plate counts were carried out on the heat shocked dilutions.

3.5.2. Results

Table 3.5.1 details the mean weights recorded for total body weights and organs pooled by treatment. Total body weight for control rats was lower than that recorded for the other groups. In contrast, all weights recorded for organ types were heavier than for any of the other groups. Whilst this may suggest that organ development was retarded by the treatments carried out this does not explain the lower total body weight recorded. It is possible that organs were not removed with the same degree of presision between treatment groups - a reduction in tissue recovered resulting in a reduction in weight recorded. Alternatively, the difference may be explained by biological variations between animals. If the weight difference was directly related to the exposure to *B. thuringiensis,* one would expect to see enlargement of the spleen and thymus with a concurrent weight increase. As this was not the case, it appears unlikely that the weight differences observed are a factor of the treatments carried out.

All animals were in excellent body condition. This, coupled with total body weights above those of the controls, indicates that exposure to the organism did not (a) affect feeding or the general health and well-being of the animals, or (b) result in large energy expenditure in clearing the organism from the animal's system. The increased weights recorded for treated animals over the untreated control animals suggests that no adverse effects resulted from handling the animals or administering the treatments.

Post-mortem examination did not reveal any gross abnormalities. There were no visible tissue changes, oedema or localised inflammation in any of the organs from any of the rats. The following points are of particular relevance with relation to the route of exposure: (i) in those animals exposed via the oral route, the gut contents were normal and there was no evidence of gut inflammation or luminal fluid accumulation; this indicates that the organism did not cause infection or a local reaction during its passage through the intestinal tract, nor did it appear to affect the gut transit time of faecal matter, and (ii) in animals exposed by sub-cutaneous injection there was no localised reaction around the injection site nor any swelling in the lymph nodes responsible for draining the area. This indicates that the organism did not appear to cause either a local or more general infection and was rapidly cleared from the site of exposure.

B. thuringiensis was not isolated from any of the tissue samples taken, with the exception of the visceral contents of orally treated animals (see Table 3.5.2). This confirms that the organism was rapidly cleared from the body of those animals exposed by sub-cutaneous injection, and there was no evidence to suggest that the organism crossed the intestinal wall following oral treatment.

The number of colony forming units isolated from the visceral contents following heat shocking was lower than was found prior to heat shocking, but there was no significant difference between those rats given oral doses of active *B. thuringiensis* and the controls. This suggests that the colonies destroyed by heat shocking were non-spore forming gut flora and not vegetative cells of B. *thuringiensis.*

Table 3.5.1: Table of mean weights of total body and of organs for rats following each of the procedures.

Dose Type	Route	Mean Weight (g)						
		Total body	Heart	Lungs	**Liver**	Kidney	Spleen	Thymus
Active	Oral	181.5	1.28	2.30	8.67	2.31	1.07	0.89
Autoclaved	Oral	182.7	1.52	2.22	7.91	2.07	1.35	1.13
Active	Sub-cut.	179.3	1.33	1.82	8.13	2.35	1.19	1.12
Autoclaved	Sub-cut.	181.6	1.13	2.31	8.56	2.08	1.20	1.23
None	None	175.4	1.82	2.78	8.84	2.84	1.92	1.86

Weights are means of ten pooled samples.

Table 3.5.2: Isolation of B. *thuringiensis* **from tissue samples**

	Dose Type				
Route	**Active Oral**	**Autoclaved** Active **Oral**	**Sub-cut**	**Autoclaved Sub-cut**	**None** None
Heart	x	x	x	x	x
Lungs	x	x	x	x	x
Liver	x	x	x	x	x
Kidney	x	x	x	x	x
Spleen	x	x	x	x	x
Thymus	x	x	x	x	x
Blood	x	x	x	x	x
Fat Pad	ND	ND	x	x	x
Visceral Contents	+	x	x	x	x

x = No *B. t.* isolated + = *B. t.* isolated ND = Not Done

3.5.3. Discussion

Our findings are in keeping with the other studies have been published concerning the toxicity to mammals of entomopathogenic bacilli administered by various routes. Not

surprisingly toxicity can be demonstrated by intraperitoneal (Lamanna and Jones 1963; Siegel and Shadduck 1990) and intracranial injection (Siegel *et al.* 1987). These are obviously not realistic routes of entry from normal exposure of bioinsectides. Clearance of bacteria from such challenging sites of infection seems to be slower if both vegetative and spore forms are present (Lamanna and Jones 1963; Siegel and Shadduck 1990). Clear evidence for multiplication of bacteria is debatable, however.

No reports exist of fatality following oral nor subcutaneous administration of *B. thuringiensis* although the latter route of exposure was lethal when spores of the closely related *B. anthracis* were injected (Lamanna and Jones 1963). Oral dosage (4 x 10' spores) of *B. thuringiensis* subsp. *israelenesis* to mice gave rise to no illness in the subsequent seven days (Siegel *et al.* 1987). The feeding of a commercial preparation of *B. thuringiensis* (DiPel) to sheep over five months gave rise to insignificant physiological changes in comparison to controls: no change was seen with respect to body weight nor food consumption (Hadley *et al.* 1987). Considering the differences in digestive system the relevance of this finding to humans should, perhaps, not be overemphasised. The fact that no ill effects were observed from sub-cutaneous injection of rats would seem to be an observation which could be extrapolated to human safety. Exposure of humans to spores of B. *thuringiensis* through cuts or abrasions in the skin appears to be without undue risk.

These results would indicate very strongly that there was no risk to humans from the ingestion of large numbers of spores of *B. thuringiensis.* The oral dose given dailyto the rats (1012 spores) represents a very significant challenge. Furthermore, in terms of dosage/ kg body weight this would be massively above any dosage which would conceivably be consumed by a human. The strain used (1313) has been shown to be a producer of (3-exotoxin. It is also a producer of *B. cereus-type enterotoxin,* see below. The complete absence of deleterious effects to rats seems hard to reconcile with the production of these toxins. The only plausible explanation is that spores of *B. thuringiensis* do not germinate in the gut of the rat and are thus precluded from producing these toxins. The use of rats as models for the safety assessment of oral dosage of B. *thuringiensisto* humans must, therefore, be seriously questioned.

Few reports exist of human trials although Fisher and Rosner (1959) exposed humans to doses of a commercial preparation of *B. thuringiensis* strain HD-1 orally and by inhalation over five days. There were no adverse clinical indications in the subjects nor in laboratory tests on them. This strain was indicated in both of the tests that we used to be an enterotoxin producer. Conceivably the level of toxin produced is too low to cause symptoms of food poisoning or else, other factors such as diet are important. In contrast most of the volunteers eating food contaminated with a different strain, *B. thuringiensis* subsp. *galleriae,* showed signs typical of food poisoning following an incubation time of eight hours (Ray 1991). The possibility that the symptoms were due to b-exotoxin were not ruled out. This toxin is not produced by strain HD-1. It is,
i however, produced by the strain used in the toxicity tests in this report. The fact that we found no evidence of germination of these spores in rats would negate any ability to produce toxins which only occur in the vegetative form.

3.6. Enterotoxin Assessments

3.6.1. Materials and Methods

a) **Bacterial Strains and Growth Conditions**

The following strains were cultured for enterotoxin assessment: six novel isolates (13B, 113, 3B, AB, 3A, 10B) two plasmid cured mutants (13 BA 1, 4412E) five commercial strains (4412, HD-1, HD-2, B. *thuringiensis* var. *tenebrionis, B. thuringiensis* var. *israelensis).* Strains were grown up in Brain Heart Infusion Broth (Oxoid) supplemented with lOgll of glucose, at 30°C for 12 hours at 200 rpm. Cultures were centrifuged at 3,400 r.p.m. for 10 minutes at 4°C in an MSE Mistral 3000E centrifuge. The supernatants were filtered through 0.22 pm Millex-GV filters (Millipore SA. France).

b) Assay

Enterotoxin status was initially assessed using a commercially available reversed passive latex agglutination (RPLA) assay (Unipath BCET-RPLA TD950). The protocol followed was that supplied by the manufacturers.

A selection of these strains were submitted to the Central Public Health Laboratory Service, Collingdale, for independant verification of results. These comprised: all commercial strains with the exception of HD-2; the novel isolates 13B and AB; two of the plasmid cured mutants 13BAI and 4412E. The assays used by the CPHLS are the RPLA kit (Unipath BCET-RPLA TD950) and the diarrhoea] enterotoxin visual immunoassay (Tecra). Analysis for emetic toxin was also carried out by the CPHLS.

3.6.2. Results

All strains assessed were found to be positive for enterotoxin production but negative for emetic toxin production. Results obtained in the laboratory mirrored those obtained by the CPHLS. There was good correlation between results obtained by the CPHLS using the two different test methods. All results are tabulated in Table 3.6.1. The commercial strains B. *thuringiensis* var. *israelensis* and B. *thuringiensis* var. *tenebrionis* seem to be poorer producers than do the other strains assessed. The mutant strains appear to have increased their enterotoxin production, although how this may be genetically or biochemically possible is uncertain.

Table 3.6.1: Assessment of enterotoxin status in six novel isolates and five commercially available strains.

Strain	Laboratory RPLA test	RPLA test titre	CPHLS Tecra kit
13B	positive	positive (128)	positive
113	ND	ND	ND
3 B	ND	ND	ND
3A	ND	ND	ND
AB	ND	positive (2048)	positive
1013	positive	ND	ND
13BAI	positive	positive (1024)	positive
4412E	positive	positive (512)	positive
4412	positive	positive (512)	positive
HD-1	positive	positive (512)	positive
HD-2	positive	ND	ND
B.t. var. *tenehrionis*	positive	positive (16)	positive
B.t. var. *israelensis*	positive	positive 32	positive

3.4.4. Discussion

Controversy exists about the reliablity of the two kits used in this study (Buchanan and Schultz 1994). These authors favoured the Tecra ELISA kit in comparison to the BCET-RPLA kit. The latter was recommended as a simple and reliable method for detecting enterotoxigenic strains of *B. cereus,* however, by Granum *et al.* (1993). False positive and negative results have been reported with the RPLA method (Day *et al.* 1994; Lund and Granum 1996). Variation between the two products was also noted by Rusul and Yaacob (1995). In this study we used both methods to determine the ability of strains of *B. thuringiensis* to produce diarrhoeal enterotoxin and found complete agreement in the limited number of strains tested. The use of an *in vitro* cell assay using transformed human colonic cells was unsuccessful (data not shown): lysis of these cells could not be correlated with the addition of preparations of enterotoxin. The only reliable method of assessing enterotoxin production is the use of ileal loops of live rabbits (Duncan and Strong, 1969), a practice now regarded as unethical.

Rusul and Yaacob (1995) assessed the prevalence of *B. cereus* in various foods. Most of the cooked and all of the dried foods that they tested contained *B. cereus, B. thuringiensis* or *B. mycoides.* Over 80% of the isolates tested were deemed to be enterotoxigenic. These authors suggested that more than 105 cfu g-1 food are required to cause food poisoning in humans. Granum (1993) suggested that levels of enterotoxigenic bacilli in foods above 103 to 104 cfu g'1 could pose a health risk. Interestingly he suggested that only strains capable of producing high levels of enterotoxin should be of concern and that their prevalence is normally quite low. The rats in this study were, however, given a dosage of about 5 x10'° spores/ d for three weeks. This represents a much higher dose of enterotoxigenic organism and, on a dose

per body weight basis, an even larger, relative challenge. Despite high doses of spores to rodents no reports exist of signs of food-poisoning. Our results support these findings in spite of the evidence that the strain of *B. thuringiensis* used was capable of producing the enterotoxin.

These results strongly indicate that all strains assessed are enterotoxin producers. This includes those strains which are commercially available and extensively used in agriculture and horticulture, as well as *B. thuringiensis* var. *israelensis* which is incorporated into drinking water in some temperate and many tropical countries to combat the vector borne diseases malaria and onchocerciasis. Strain HD-1 is also available for home use as a bio-pesticide for organic produce. It would seem probable that large quantities of these products are consumed on vegetable and grain produce, or in drinking water, on a regular basis without any apparent detrimental effects. Their safety must therefore be dependant upon the food source to which they are applied being unsuitable for the production of the enterotoxin *in situ.* Fish however, being rich in protein would readily support enterotoxin production. As such the possibility of utilising these strains as insecticidal preparations for application to fish can no longer be considered.

These results potentially have relevance to the traditional application of *B. thuringiensis.* With current trends for increasing popularity of organically grown foods and decreasing cooking times for vegetables a potential food-poisoning risk exists with the use of *B. thuringiensis* as a biopesticide. Some strains of *B. thuringiensis* can, obviously, produce *B. cereus-type enterotoxin;* extrapolation of safety tests using rodents to risk assessments for humans appears to be unreliable . A further problem in the use of this biopesticide on foods is that if an enterotoxigenic strain of *B. cereus* grew in, say, a dairy product to such a level that there was a risk of food poisoning the food would become unacceptable for consumption. Spraying precultured spores of *B. thuringiensis* on leaf crops will not produce a comparable decline in the organoleptic qualities of the food and so it would still be considered suitable for consumption. The residual spore load that can remain on leaves shows that a potentially significant number of spores can be ingested after normal food preparation practices. Should washed salad leaves, for example, be supplemented with carbohydrate- or protein-rich foods and kept unrefrigerated the potential for replication, from an already high inoculum, is obviously great. Harvesting leaves the day after spraying was intended to show as a high a residual spore load as possible; instructions concerning a period after spraying during which the crop may not be consumed are, however, not generally given with insecticides of this type.

As public health laboratories become more conversant with the identification of *B. thuringiensis,* as distinct from *B. cereus,* it is possible that the instances of food poisoning being attributed to the former organism will increase. To ensure that biopesticides containing members of the genus *Bacillus* continue to enjoy the excellent safety record that they have to date it would seem to be advisable to ensure that existing and newly registered products are reliably tested for their potential as food poisoning agents.

With respect to this project even if a pesticide is produced for use in a bait it will have to be enterotoxin-negative. This is obvious from a point of view of the people producing it and from the possible cross contamination risk to the drying fish (perhaps from adult flies). No attempts were made to develop an enterotoxin-negative strain from a positive one as was attempted with the (3-exotoxin. This was partly as a result of (a) the experience with the latter toxin, (b) the lack of a reliable assay and (c) the complex nature of the polypeptides required to produce toxicity (Lund and Granum, 1996).

Chapter 4

Genetic analysis

At the outset of the project the identity of the 6-endotoxin type found to be toxic to larvae of *M. domestica* (Hodgman *et al.,1993)* was unknown. From the established strains of B. *thuringiensis* found to be active against larvae of blowflies we deduced, by a process of elimination and using a knowledge of the 6-endotoxin types that these strains produced, that toxins of the CryIB family were involved. Since this toxin type (nor any other) had not previously been associated with this spectrum of toxicity we decided to characterise the relevant genes. A further incentive to this work was that the CryIB(a) protoxin produced by *B. thuringiensis* strain HD-12 was completely inactive, even against M. *domestica.* This was regardless of whether the native crystal or crystal which had previously solubilised or activated *in vitro* were used. The specificity of this activity was, therefore, very precise. As a corollary of this it would not be unexpected if the spectrum and potency of toxicity varied with the exact DNA sequence within the sub-family of CryIB found to be responsible. To try to ensure that we were working with the strains of *B. thuringiensis* that had the highest potency and most appropriate spectrum of activity we carried out a selective DNA sequencing programme.

As the project progressed it became known that a former colleague had devised pairs of polymerase chain reaction (PCR) primers which were specific for almost all known 6endotoxin gene sub-families (pers. comm. Dr S. Gash, University of Cambridge). Having been given access to this information and permission to use it our task of obtaining DNA sequences and of characterising our strains was greatly facilitated.

4.1 Materials & Methods

4.1.1 Chemicals used and their abbreviations (unless stated otherwise the supplier was Sigma Aldrich)

Agarose (Type IA Low EEO)
Agarose (NBL Gene Sciences Ltd.)
Ammonium acetate
Ammonium persulphate (AMPS)
5-bromo-4-chloro-3-indoyl-(3-D-galactopyranoside (X-gal)
Chloramphenicol (CAP)
Chloroform
Dithiothreitol (DTT)
Ethylenediamine tetraacetic acid (EDTA)
Ethanol (EtOH)
Ethidium bromide
Hydrochloric acid (HCl)
Isopropyl (3 - D - thiogalactopyranoside (IPTG)
Magnesium chloride (M9C12)
N,N,N,'N' tetramethylethylenediamine (TEMED)
Orthophosphoric acid

Penicillin G (pen G)
Phenylmethylsulphonylfluoride (PMSF)
Phenol (Fisons)
Potassium acetate
Sodium acetate
Sodium chloride
Sodium dodoecyl sulphate (SDS)
Sodium hydroxide
Tetracycline JET)
Trichloroacetic acid (TCA)
Tris (hydroxymethyl) - methylamine (`Tris buffer')

4.1.2 Buffers

SMM	0.5M sucrose
	0.02M maleic acid
	0.02M MgC12
	adjusted to pH 6.5 with conc. NaOH
TBE : 5x conc.	Tris buffer 54.0g/1
	Boric acid 27.5g/1
	EDTA (pH8.0) 20ml/1
TE (Tris.EDTA)	Tris.Cl (pH 8.0) lOmM
EDTA	(pH8.0) 1mM
SDS PAGE:	
Gel running buffer	Tris buffer 6.0g/1
	Glycine 28.8g/l
	SDS 0.1% w/v
Gel sample buffer	SDS 0.1% w/v
	EDTA.Na2 1mM (inhibits metallo-proteases)
	PMSF 2mM (inhibits serine-proteases)
	Glycerol 10% w/v
	Tris.Cl (pH7.5) 50mM
	Bromophenol blue 0.5% v/v of a 0.5% w/v soln.
	Fresh DTT added at 25mM (3.8mg/ml) just before use.

4.1.3 Solutions prepared

SIDS PAGE	30% acrylamide stock: acrylamide 300g/1
	bis-acrylamide 20g/l
	1.5M TriS.Cl (pH8.8)
	0.5M Tris.Cl (pH6.8)
	10°/o w/v ammonium persulphate

m

	10% w/v sodium dodoecyl sulphate TEMED
Gel stain	Coomassie blue R 0.1% w/v
	AR quality methanol 50%
	Acetic acid 10%
Gel destain	AR quality methanol 10%
	Acetic acid 10%

4.1.4 DNA sequencing regents

Annealing buffer (5x conc.)	200mM Tris.Cl (pH7.5)
	100mM MgCl2
	250mM NaCl
Dithiothreitol (DTT)	O.1M

Labelling nucleotide mixture (use with radiolabelled dATP):
1. 5 pM dGTP, 1. 5 ~tM dCTP and 1. 5 ~M dTTP

Termination nucleotide mixtures (one for each dideoxy nucleotide):

Each mixture contains 80p,M dGTP, 80p,M dATP, 80p,M dTTP, 80W dCTP and 50mM NaCl.

In addition, the `G' mixture contains 8p.M dideoxy-dGTP; the `A' mix, 8p.M ddATP and the same for the `T' and `C' mixes.

Stop solution:	95% formamide 20mM EDTA 0.05% bromophenol blue 0.05% xylene cyanol FF

Labelled dATP (Amersham):
[a,-35 S] dATP specific activity 1000-1500 Ci/mMol

DNA SEQUENCING GEL:

l Ox TBE:	Tris base 108.0g/l Boric acid 55.0g/1 EDTA.Na2 9.3g/1
40% acrylamide stock:	acrylamide 380.0g/1 bis-acrylamide 20.0g/1

0.5x TBE 6% gel mix:	40% acylamide stock 15m1/0.11 l Ox TBE 5mL/0.11 Ultrapure urea 46g/0.11

25% ammonium persulphate solution (AMPS)
3 5 % TEMED

4.1.5 Media

LB	lOg/1 Bacto-tryptone
	lOg/1 NaCl
	5g/l Bacto-yeast extract
BGM (Basic growth medium)	See section 2.1.1
BHI (Brain Heart Infusion)	Oxoid

4.1.6 Enzymes (Sigma)

Lysozyme
Mutanolysin
Proteinase K
RNAse A
Trypsin

4.1.7 Kits for DNA manipulation

DNA Sequencing - Sequenase° Version 2.0 (Promega)
pGEM Cloning - pGEM" - T Vector System (Promega)

4.1.8 Bacterial strains and plasmids

Bacillus cereus strain FM-1 (obtained from Prof P.E. Granum
Bacillus thuringiensis (novel isolates: 13B, 313, 1B, IOB, 3A, AB)
Bacillus thuringiensis subsp. israelensis (Bti.)
Bacillus thuringiensis subsp. tenebrionis (Btt)
Bacillus thuringiensis strains HD-1, HD-2, HD-12, YBT, 4412.
Escherichia coli inv cc F' } purchased from
Escherichia coli JM109 } Promega

Plasmids:
pBC16 TetR **}obtained from**
pC194 CapR } P. Jarrett
pSV5 (cloned cryIB(b) gene from HD12 in Bti cry- strain IPS78/1 1)
pSV4 (C terminally truncated version of SV5 full protein product)

4.2 Methods

4.2.1 Total DNA preparation

(a) Method from personal communication, Dr E. Bone.

1. LB medium was inoculated with a strain of B. *thuringiensis.*
2. A sample of culture (20ml) was removed, centrifuged at 6 000 r.p.m for approximately 20 minutes in an MSE HSI 8 centrifuge, the supernatant discarded and the pellet resuspended in lml of TE buffer (pH 8.0).
3. Addition of lysozyme (2mg), 24011 0.5M EDTA (pH 8.0), 2611 proteinase K (10mg/mL) and 1401 of 10% SDS, was followed by incubation at 37°C for 1-2 h.
4. The DNA was precipitated with 2 x vol. EtOH (95%), and left for 5 min at 37°C.
5. The DNA was spooled into 500~il TE using a sealed Pasteur pipette.
6. Steps 4 & 5 were repeated.
7. The DNA was dissolved into TE at 37°C for 1-2 h.
8. Extraction with 1:1 phenol: chloroform was carried out.
9. of The aqueous phase)about 40011) was removed, 133pl of 7.5 M ammonium acetate and l ml of EtOH (95%) added. The tube was inverted and left to stand at room temperature for 2mins.
10. The-tube was microcentrifuged at 13 000 r.p.m for 10 mins.
11. The EtOH was aspirated off and the pellet washed with EtOH (70%). This was microcentrifuged for 2 mins, the supernatant removed and the pellet aspirated then desiccated for 30-60 mins.
12. The pellet was dissolved in 200 ~tl TE (pH 8.0) and approximately 2 pl were run on a 0.6 or 0.8 % agarose gel at 50mA for approximately lh.

(b) Adams *et* al., (1989)

1. LB medium (2m1) was inoculated with a colony of *B. thuringiensis* and incubated at 37°C overnight no shaking).
2. This was transferred to a 21 flask containing 250m1 LB and grown at 37°C, 200 r.p.m, until an OD600 = 0.4 was reached.
3. The flask contents were harvested at 8,000 r.p.m for 45 min. using a Sorvall G.S.A rotor.
4. The cell pellet was resuspended in 20ml of TE (pH 8.0), lysozyme(20mg) added, the tube inverted to mix and then incubated at 37°C for 1-2 h.
5. The addition of lml of 0.5M EDTA (pH8.0) was followed by 0.5ml of 2M Tris.Cl (pH 7.9) and the tube was inverted to mix followed by incubation at 37°C for 15 min.
6. RNAse A (10mg/ml) solution (2001.11) was added and the tube incubated at 37°C for a further 15 min.
7. Addition of of 10% SDS (2.3ml) resulted in considerable clearing of the cell suspension due to cell lysis.
8. Incubation for 2 h at 50°C with 2mg of proteinase K (or trypsin) was carried out and the suspension then split into 2 tubes.
9. Two phenol extractions were followed by two with phenol : chloroform (1:1)

10. Total DNA was precipitated with 0.1 vol. 3M sodium acetate and 2.5 vol. EtOH (95%).
11. After mini-entrifugation at maximum speed for 5 min. the pellet was washed in EtOH (70%), re-centrifuged and then aspirated and desiccated.
12. The pellet was resuspended in 10 ml TE and dissolved by heating at 60°C.
13. Samples were run on a 0.6% agarose gel at 50mA for approximately l h.

4.2.2 Plasmid preparation

This method was developed and initially follows the procedure of Adams *et* al.(1989) followed by the alkaline lysis protocol (Maniatis 1989).

1. *B. thuringiensi.s* was streaked out onto nutrient agar + 54g/ml CAP and incubated overnight at 37°C.
2. A single colony was used to inoculate 2ml LB medium + 54g/ml CAP and incubated overnight at 37°C.
3. This was transferred to LB medium + 54g/ml, CAP (250ml) and grown at 37°C, 200 r.p.m until an OD_{600} = 0.4 - 0.6 was reached.
4. Flask contents were harvested for 45 min at 8000 r.p.m in a Sorvall G.S.A rotor.
5. The-pellet was resuspended in 20m1 TE and lysozyme (20mg), inverted to mix and incubated for 1 - 2 h at 37°C.
6. Addition of 0.5M EDTA(lml) + 0.5ml of 2.OM Tris.Cl (pH 8.0) was followed by mixing by inversion and incubation for 15 mins. at 37°C.
7. A sample (2.5H) of the suspension was removed (the remainder frozen) and 1.25ml of 0.8M NaOH/ 4% SDS added then mixed by inversion and put on ice for 5 min.
8. Ice cold potassium acetate (pH 4.8), 1.875ml was added, the tubes inverted to mix and put on ice for a further 5 min.
9. The suspension was separated into Eppendorf tubes and mini-centrifuged at 13000 r.p.m for 5 mins.
10. The supernatant layer was transferred to new tubes and an equal volume of phenol: chloroform (1:1) added, the tubes were inverted and minifuged at 13000 r.p.m (max. speed) for 2 min.
11. The supernatant layer was again transferred to clean tubes and ethanol precipitated with 2 vol 95% EtOH. The tubes were then mixed by inversion and left to stand at room temperature for 2 min.
12. The tubes were minifuged (max. speed), the supernatant layer removed by aspiration and the remaining pellet washed in 70% EtOH, minifuged then the pellet aspirated and desiccated.
13. The pellet was dissolved in approximately 20041 of TE (pH8.0), RNase treated (204g/ml) and incubated at 37°C for 30 mins.
14. Samples were run on a 0.6% agarose gel at 45mA for about 1.5 h

4.2.3 Polymerise chain reaction (PCR) amplification of the cryIB gene.

The method and primers were developed by Dr S. Gash, Univ. of Cambridge (pers. comm.).

1. Bacterial lawns of high activity (13B) and medium activity (3A, AB) isolates, together with strains YBT, HD2 and HD 12 (positive controls for cry IB(a) and cry IB(b) genes, respectively) were grown up from single colonies by being incubated at 30°C overnight.
2. Resulting growth was scraped off into O.SmI sdH20, whirlymixed briefly, frozen at -20°C for approximately 15 min, thawed, then boiled for 10 min and the process repeated.
3. The suspension with microcentrifuged for 20 - 30 s and the tube placed on ice until the supernatant was required as the template in the PCR reaction mixture.
4. All constituents were added to autoclaved Eppendorf tubes (0.75m1), the Tail polymerise last of all, and a `hot start' PCR method used. This involved incubating the reaction mixture at 85°C for 1 min, pausing whilst dNTPs, prewarmed to 60°C were added, then continuing to incubate at 85°C for a further minute.

PCR reaction mixtures:

Constituent	Volume (~tl)
NH4 reaction buffer	10.0
MgC12 (23mM)	39.3
sdHZO	18.5
specific primer, forward (0.25~M)	2.5
specific primer, reverse (0.25p,M)	2.5
`red hot' DNA polymerise (Adv. Biotech Ltd.)	0.2
dNTP s (2. S mM)	12. 0
template	15.0

cry IB(a) specific primer sequences:

1 Ba f3 :	ATGCAAGAACGAGAAGTGTTCTTTATACC	position 425 - 453 in the coding sequence
1 Ba rl :	CCTATTGGCCGAGATTGAATCGTGTGC	position 1079 - 1053 in the coding sequence

PCR thermal profile using a Techne 3A thermal cycler:

Programme 35

1.	85°C	2 min

Programme 36

1.	94°C	1 min
2.	54°C	1 min
3.	72°C	1 min

24 cycles

Programme 37

1.	94°C	1 min
2.	54°C	1 min
3.	72°C	7 mins
4.	4°C	24 h

4.2.4 Agar6se gel electrophoresis

Agarose gels (0.4/ 0.5/ 0.6 and 1.2%) were prepared by dissolving the appropriate amount of agarose in 0.5 x TBE buffer(50 ml). This was done by placing the mixture in a 650w microwave oven at medium heat for approximately 2 min. The agarose solution was allowed to cool, 0.51.1 of ethidium bromide dissolved in ethanol (10mg/ml) added and the gel mix poured into minigel apparatus (N-BL). The gels were run under the appropriate conditions (current and voltage settings) dependent upon the DNA being analysed.

4.2.5 pGEM cloning protcol

4.2.5.1 *Ligation of the p-GF.IINI®-T vector and PCR product*

1. The PCR product was purified from the reaction mix by RNAse A treatment, followed by ethanol precipitation, centrifugation, aspiration and desiccation. The resulting pellet was resuspended in 25p.1 TE (pH 8.0).
2. The DNA was quantified by measuring its *OD26o.*

DNA Quantification

Isolate	_ODZ6o dil. *x* 250	DNA ,gg/ml)
13B	0.008	100.0
3 A	0.021	262. 5

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Calculation:

(a) The DNA was diluted 1/ 250 then its OD260 measured using a Lamda 3 spectrophotometer.

(b) The amount of DNA, in p,g/ml = Absorbance (260nm) x 50 x 250 (since OD260 = 1 - 501.tg/ml DNA)
The amount of DNA was also quantified by visualising it on a gel when run against a DNA standard (a molecular weight marker, X174, Hae III digest, NBL) of known concentration. In this way, an approximate determination was made as to how much DNA was present in the 13B and 3A products by comparing the intensity of the band with those of the standard.

3. The pGEM'~'-T Vector and pGEM`p'- T Vector control tubes were centrifuged to collect contents at the bottom of the tube.

4. Ligation reactions were set up as follows:

Constituent	Sample Reaction	Control Reaction
T4 DNA Ligase 1 Ox Buffer	1 ~t1	1 -LI
pGEM°-T Vector (50ng/l.il)	l Eel	l ~11
PCR product (or pGEW'-T Vector control)	x~tl	1 ~t1
T4 DNA Ligase (1 Weiss unit/ml)	1 ~~l	1 Pl
dH20	xpl	511
final volume	lOl.il	101,1

For an optimal ligation, a 1:1 molar ratio of vector : insert (PCR product) was recommended.The following equation was used to calculate the appropriate amount of PCR product:

$$\frac{\text{na vector x kb size of insert}}{\text{vector}} \times \text{insert : vector molar ratio} = \text{ng of insert kb size of}$$

T- vector supplied at 50ng/~LL
T- vector z~ 3 kb
Insert (PCR product) -z~ 655bp

5. Ligation mixtures were incubated at 15°C for approximately 24 h. and a sample taken after overnight incubation at 15°C and kept at 4°C for 6 h.

6. A 2p1 aliquot of each sample was used for the transformation step.