POSTER PRESENTATIONS

Poster Session Organiser

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BACULOVIRUS INSECTICIDES: DETECTION OF LATENT BACULOVIRUSES IN NATURAL INSECT POPULATIONS

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ABSTRACT

Baculoviruses have long been used as natural biological control agents for insect pests. Attempts to shorten the time taken to kill the pest by using genetically modified viruses incorporating insect-specific toxins genes have proven successful in both laboratory and field trials. An important safety consideration concerns the possibility of genetically-modified viruses recombining and exchanging genetic information with indigenous baculovirus isolates, or activating latent or occult viruses. As very little is known about the distribution of latent or occult virus infections in natural insect populations, we have developed a simple PCR-based method that can detect the presence of virus DNA in all stages of the insect life cycle.

INTRODUCTION

Baculoviruses are insect-specific viruses that have been used for many years as natural agents for the control of insect pests. The success of baculoviruses as insecticides has largely been attributed to the ability of the virus to persist in the environment by protection of virus particles in proteinaceous occlusion bodies or polyhedra (Hunter *et al.*, 1984). Polyhedra are composed mainly of a single polypeptide, polyhedrin, encoded by the virus genome, which forms a quasi-crystalline structure into which the virions become embedded (Rohrmann, 1986). Natural virus epizootics have been recorded in many insect species, however, virus must persist for long periods when the insect hosts are not available. Survival may be achieved by persistence of polyhedra in soil or decaying leaf matter for example, or the virus may persist within the host population in an occult or latent state (reviewed by King *et al.*, 1994). Several reports have shown that insects infected with one baculovirus actually die from the activation of a supposedly latent virus, rather than the applied virus (Longworth & Cunningham, 1968, Jurkovicova, 1979). However, the mechanisms of baculovirus latency and the activation of occult virus are poorly understood. Indeed until recently (Hughes *et al.*, 1993), no one had ever demonstrated the presence of latent virus in an unchallenged insect population. In this report we demonstrated that a laboratory culture of *Mamestra brassicae* insects (MbLC) harboured a latent or occult baculovirus infection. The virus was activated by feeding the MbLC larvae with either the closely related *Panolis flammea* nucleopolyhedrovirus (PfMNPV) or the unrelated *Autographa californica* (Ac)MNPV. DNA profiles showed that the activated virus was closely related, if not identical, to MbMNPV.

We were unable to detect the presence of latent virus DNA using conventional Southern blotting and DNA hybridisation techniques (Hughes *et al.*, 1993), however, using the more sensitive technique of DNA amplification by polymerase chain reaction (PCR) we have been able to detect specific latent virus sequences. PCR has also been used to detect baculovirus DNA on the surface of gypsy moth eggs (Burand *et al.*, 1992). In particular, we have shown that sequences specific for the polyhedrin gene are present in all stages of the MbLC life cycle (Hughes *et al.*, 1993; 1994). As the polyhedrin gene sequences are very highly conserved between different baculovirus isolates, DNA amplification of this gene provides a convenient tool for the detection of latent baculoviruses in naturally occurring insect populations. In this paper we describe a method for the extraction of baculovirus DNA from individual insects (larvae, pupae, adults and eggs) collected from natural habitats and a method for the detection of polyhedrin-specific DNA sequences using PCR.

MATERIALS AND METHODS

Collection of insects

Insects were collected from four study sites using sweep netting and tree beating techniques together with light trapping and butterfly netting. Larvae were obtained either by sweeping low level foliage and grasses with a sturdy net or were dislodged from trees and shrubs using a beating stick above a white ground sheet. Adult moths were collected using an ultra-violet light moth trap and butterflies were caught on the wing using a net. Adults were bred where ever possible, to obtain second generation larvae; allowing various larval instars to be tested.

Extraction of virus and host genomic DNA from individual insects

Each individual larva, pupa or adult was homogenised in 600 µl ice-cold TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 0.1% sodium dodecyl sulphate (SDS) using a dounce homogeniser. To 300 µl homogenate was added 200 µl 0.1 M sodium bicarbonate. After 10 min at 37°C, 200 µl lysis buffer (50 mM Tris-HCl, pH8.0, 5% β-mercaptoethanol, 10 mM EDTA, 0.4% SDS) and 2.5 µl RNase A (10 mg/ml) were added and the incubation continued for 30 min. Following the addition of 15 µl proteinase K (10 mg/ml), the incubation was continued for a further 30 min. The mixture was then extracted once with phenol (equilibrated in 100 mM Tris-HCl, pH 8.0), twice with phenol:chloroform (1:1) and once with chloroform. The DNA was then precipitated with ethanol, washed in 75% ethanol and the air-dried pellets were dissolved in 100 µl d.water.

DNA amplification using PCR

The PCR primers were synthesised as 21-mers consisting of DNA complementary to the 5' (5'ACCCGTTACAGTTACAATCCG3', nt 5'-7 to 3'-28) and 3' (5'GGCGGGTCCGTTGTACAGAGG3', nt 3'-735 to 5'-714) regions of the 740 bp polyhedrin gene coding region of MbMNPV (ref). Control MbMNPV DNA (25 ng) or insect DNA samples (1 µg) were used in a PCR mix using a final concentration of 1.0 µM of each primer, 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM of each dNTP and 2 units *Taq* polymerase (Promega). Each reaction consisted of 30 cycles of

amplification using a step programme (94°C, 1 min; 45°C, 2 min; 74°C 2 min) followed by a 7 min final extension at 74°C.

Random amplification of polymorphic DNA

Random amplification of polymorphic DNA (RAPD) was carried out on the DNA samples extracted from insect larvae, pupae or adults essentially as described by the manufacturer (Pharmacia Biotech). Briefly, 5-50 ng DNA were added to a single RAPD bead (containing buffer, dNTPs, bovine serum albumen and AmpliTaq polymerase) and 25 pmol of RAPD primer 2 (5' [GTTTCGCTCC]3'). The volume was adjusted to 25 µl with d.water and overlaid with 50 µl mineral oil. Each reaction consisted of 1 cycle (95°C, 5 min) followed by 45 cycles (95°C, 1 min; 36°C, 1 min; 72°C, 2 min). The kit provided control DNA extracted from *Escherichia coli* (Cla) and *E. coli* (BL21).

RESULTS AND DISCUSSION

Collection of insects

To examine the extent of latent or occult baculovirus infections in natural insect populations, insects were collected from four habitats in Oxfordshire during the summer of 1996. Standard methods were used to collect samples of adults, pupae, larvae or eggs (Table 1). Where possible, collected adults were bred to produce second generation larvae for further testing. Larvae were maintained on natural or semi-synthetic diets. All individuals were identified to the species level and catalogued before freezing at -80°C. All insects collected were apparently healthy and had no obvious signs of disease.

Table 1. Summary of insect species collected from habitats in Oxfordshire

Habitat	Number of species	Number of individuals			
		Adults	Pupae	Larvae	Eggs
Deciduous wood-land & mesotrophic grassland	24	52	24	280	30
Fenland	17	191	17	80	-
Calcareous grass-land	9	78	4	35	5
Light trap	12	84	1	138	5
Other	4	27	18	35	-

Rapid amplification of polymorphic DNA

Individual insects (pupae, adults or larvae) were homogenised and DNA was extracted using standard techniques. The DNA extraction method used has been previously optimised to extract virus and host genomic DNA from individual insect species (Hughes *et al.*, 1993). Samples of DNA were analysed by electrophoresis through agarose gels to confirm the integrity of the high molecular weight DNA (data not shown). To confirm that the extracted DNA was suitable for amplification purposes and to aid confirmation of the identity of different insect species, we performed a RAPD analysis. In this technique, the DNA is subjected to amplification using a primer which should anneal to sequences randomly distributed throughout the insect genome. The amplified DNA products are analysed by agarose or acrylamide gel electrophoresis and should produce a banding pattern unique to each species tested. In Figure 1 we illustrate the results obtained from testing four different insects collected in our survey of Oxfordshire habitats. Each insect species tested to date has produced a unique banding pattern (lanes 3, 6-8, Figure 1). The banding pattern was consistent in all individuals of the same species where the identity of the species using conventional methods was certain (for example see lanes 3 and 4, Figure 1).

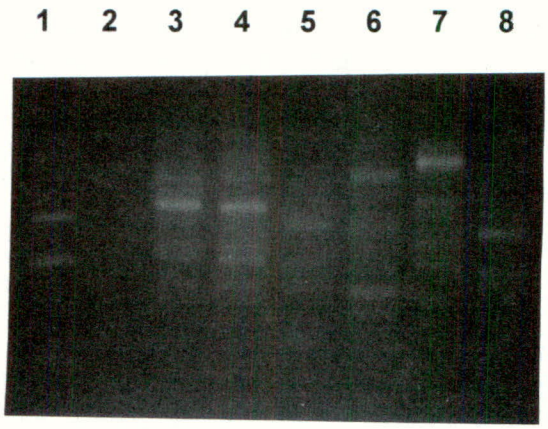

Figure 1. RAPD analysis of DNA samples extracted from six individual insects: *Noctua comes* larvae (lanes 3 & 4), putative *N. comes* larva (lane 5), *Tyria jacobaeae* larva (Cinnabar moth, lane 6), *Mimas tiliae* pupa (Lime Hawk moth, lane 7) and *Vanessa cardui* adult (Painted Lady butterfly). Lane 1 shows the RAPD banding pattern obtained with control *E. coli* (B21) DNA and lane 2 shows a negative control. In each case, 5 µl of the amplified DNA mixture was added to each well of a 2% agarose gel which was then subjected to electrophoresis at 16V/10 m amps for 16 hours.

In some instances, the RAPD technique has proved useful in helping to resolve uncertainties in the identification of insect species. This is illustrated by examining lanes 3-5, Figure 1. Lanes 3 and 4 show the RAPD product from two Lesser Yellow Underwings (*Noctua comes*) where the species classification was certain. For the third individual collected (lane 5), the species

classification was not certain and the RAPD analysis suggests that it is probably not *N. comes*. This is currently under further investigation.

Amplification of virus-specific sequences

Testing for the presence of latent or occult baculoviruses in apparently healthy individuals requires a sensitive detection method, as we have shown previously that conventional methods, for example Southern blotting and DNA hybridisation, are inadequate (Hughes *et al.*, 1993). Figure 2. demonstrates that PCR amplification of the virus specific polyhedrin gene sequences can be used as a reliable, sensitive and rapid method for the detection of latent virus sequences that may be present in all stages of the insect life cycle. Amplification of the polyhedrin gene was chosen as sequencing data has revealed that this gene is highly conserved between all baculovirus isolates so far examined. In Figure 2 we demonstrate the feasibility of this approach by testing two populations of *Mamestra brassicae* larvae. One population has been previously demonstrated to harbour an occult or latent virus infection (MbLC) and the other strain being free of detectable virus (MbWT) (Hughes *et al.*, 1993; 1994). Both insect populations are maintained on semi-synthetic diet under laboratory conditions.

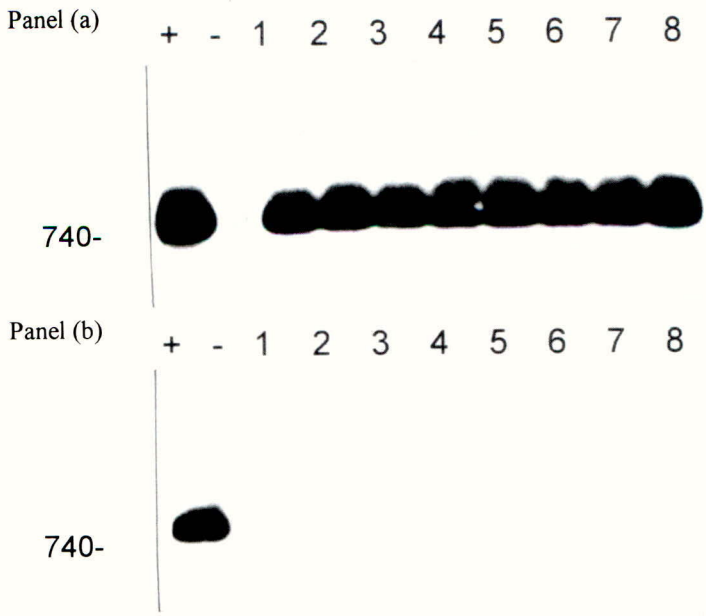

Figure 2. Autoradiograph showing PCR amplification of polyhedrin-specific DNA from MbLC (panel A) and MbWT (panel b). All stages of insect development are shown: eggs (lane 1), neonate larvae (lane 2), second instar (lane 3), third instar (lane 4), fourth instar (lane 5), fifth instar (lane 6), pupae (lane 7) and adults (lane 8). A positive control of MbNPV DNA is shown (lane +). No DNA was added in the negative control (lane -). Amplified DNA (one tenth of total) was resolved in a 1% agarose gel and tranferred to a nylon membrane before probing with radiolabelled polyhedrin-specific DNA.

Figure 2, panel (a) shows that polyhedrin-specific sequences were detected in DNA extracted from adults, pupae, larvae and eggs. No polyhedrin-specific sequences have ever been detected in DNA samples extracted from MbWT (panel b). Studies are currently underway to test the insects collected from the four sites in Oxfordshire (Table 1) for the presence of latent or occult baculovirus polyhedrin gene sequences. The DNA extraction and PCR detection methods described here will enable us to complete the first detailed survey of indigenous, sub-lethal baculovirus infections in natural insect populations. These data will form a useful base which can be used in the risk assessment of releasing natural or genetically-modified baculoviruses into the environment.

ACKNOWLEDGEMENTS

This work was supported by a grant from the UK Natural and Environmental Research Council to LAK, TS and RDP.

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PLASMID TRANSFER BY THE INSECT PATHOGEN *BACILLUS THURINGIENSIS* IN THE ENVIRONMENT

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ABSTRACT

Transfer of the plasmid pBC16 between *Bacillus thuringiensis* subsp. *tenebrionis* and an acrystalliferous streptomycin resistant strain of *Bacillus thuringiensis* subsp. *kurstaki* HD1 was studied *in vitro*, in soil and in insects. Plasmid transfer (maximum frequency 10^{-2} transconjugants per donor cell) occurred in laboratory broths at between 20-37°C and pH 6-9, conditions relevant to soil and insect environments. However, no gene transfer was detected in soil release experiments and in insects on leaf discs. In soil, the formation of spores may have been a contributing factor in the lack of plasmid transfer. In insects gene transfer was probably inhibited by competition for nutrients from the insect gut microbiota. The importance of these findings to the release of genetically modified *B. thuringiensis* strains and *B. thuringiensis* ecology will be discussed.

INTRODUCTION

Bacillus thuringiensis is a Gram positive, spore forming bacterium that produces insecticidal crystal protein toxins during sporulation. It is commonly isolated from a range of environments, including insects, soil, stored grain dust and leaves from deciduous and coniferous trees (Martin & Travers, 1989; Meadows *et al.*, 1992; Smith & Couche, 1991). The insecticidal crystal protein genes are usually found on large self-transmissible plasmids (Gonzalez *et al.*, 1982; Whiteley & Schnepf, 1986). The systems of genetic transfer of *B. thuringiensis* have been well studied with transduction, conjugation and transformation all having been reported (Heierson *et al.*, 1987; Lecadet *et al.*, 1992; Koelher & Thorne, 1987; Naglich & Andrews, 1988). The movement of some large self-transmissible plasmids has been found to occur at such a high frequency in certain strains that transfer can be detected without the need for a selectable marker (Aronson & Beckman, 1987). However, in most cases, the movement of conjugative plasmids is not so frequent and needs to be monitored using small mobilizable plasmids containing a selectable marker. One of the most commonly used plasmids for this purpose is pBC16 which is mobilizable and contains a gene for tetracycline resistance (Bernhard *et al.*, 1978).

The proposed release of genetically modified microorganisms (GMMOs) into the environment has necessitated studies into the fate of GMMOs and their genes (Lilley *et al.*, 1994). In the natural environment conjugation is an important process by which GMMOs may disseminate specific genes to other bacteria. Consequently, many studies have been conducted into the

effect of environmental parameters on plasmid transfer, mostly focused on *Pseudomonas* spp. (Bale *et al.*, 1987; Fry & Day, 1990; Rochelle *et al.*, 1989; Venables *et al.*, 1995). However, only relatively few studies have examined the environmental parameters required for plasmid transfer between *B. thuringiensis* strains (Haack *et al.*, 1996). In addition, during the evolution of *B. thuringiensis* strains, gene transfer is a likely route by which strains now harbouring multiple toxin genes could have developed. By studying gene transfer processes in the environment the evolutionary mechanisms that have resulted in the large diversity of toxin genes found in present day strains may become clearer.

MATERIALS AND METHODS

The strains used in this work were *B. thuringiensis* subsp. *tenebrionis* containing plasmid pBC16 and an acrystalliferous streptomycin resistant mutant of *B. thuringiensis* subsp. *kurstaki* HD1. The strains were cultured in brain heart infusion (BHI) broth at 30°C for 18 hours. For in vitro studies, $5x10^7$ cfu were used to inoculate 50 m brain heart infusion broth (10^6 cfu m^{-1}). To study the effect of temperature on gene transfer the broths were incubated at 15°C to 42°C. To study the effect of pH on gene transfer, BHI broths containing 0.5 M phosphate buffer (pH6-8), 0.5 M maleic acid buffer (pH 5-7) and 0.5 M Tris-HCl buffer (pH7.5-9.5) were used. All cultures were incubated at 30°C. Samples were taken after 6 hours incubation.

To study gene transfer in soil, 2 kg of soil was collected from HRI Wellesbourne, Warwick, UK. This soil has been characterised as a sandy loam, Wick series no.1. The soil was sieved (2 mm) and air dried to 4% water content. *B. thuringiensis* cells from a BHI broth culture grown for 18 hours at 30°C were washed in sterile distilled water and added to 100 g dry wt. soil to a final density of 10^7 cfu g^{-1} dry wt. The soil mosture content was adjusted to give a matric potential of -33 KPa. The inoculated soil samples were placed at both 18°C and 30°C. Samples of 1 g dry wt. soil were taken on 0, 1, 7, 21 and 28 days.

Gene transfer was studied in larvae of *Phaedon cochleriae* on Chinese cabbage leaves. Spore/crystal mixes of donor and recipient strains were prepared from nutrient agar plates that had been grown for 5 days at 30°C. Spores and crystals were resuspended in 0.25 strength Ringer's solution containing 0.1% v/v ETALFIX to a density of 10^8 cfu ml^{-1}. To one leaf disc (35 mm. diam.) placed abaxial surface down on 2% w/v water agar, 50 µl of sample was spread and left to air dry. Finally, 30 larvae were placed on to the leaf disc and incubated at 25°C, with a relative humidity of 80%. Samples of 3 pooled insects were taken after 4 hours (live insects) 1, 2, and 3 days (dead insects).

All experiments were performed in triplicate. Each sample was diluted 10 fold in 0.25 strength Ringer's solution and 0.1 ml plated on nutrient agar containing tetracycline (25 µg ml^{-1}), streptomycin (50 µg ml^{-1}), or tetracycline and streptomycin for the detection of donor, recipient and transconjugants respectively. Soil samples were vortexed at maximum speed for 10 seconds, and insects homogenised, prior to dilution. The plates were incubated at 30°C for 48 hours and those with between 30-300 colonies present were counted.

RESULTS

Plasmid transfer was detected over the range 20-37°C with an optimum of 30°C, and over a wide pH range of (pH 6 - 9). The highest frequency of gene transfer was 10^{-2} transconjugants per donor. Transconjugants were allowed to sporulate and observed under the microscope but none were found to contain crystal toxins. PCR amplification using *cryIII* primers also indicated the absence of the toxin gene in these strains.

In soil, no gene transfer was detected and the released cells quickly (7 days) formed spores which remained detectable for over 100 days at a level of 10^3 cfu g^{-1} soil. In insects, $5x10^3$ spores/crystals of *B. thuringiensis* subsp. *tenebrionis* per mm^2 leaf was found to be sufficient to kill the young larvae used in this study. Within the dead larvae, the number of *B. thuringiensis* donor and recipient cells were present at a level of 10^3 cfu $larvae^{-1}$. Over the three day study no transconjugants were detected in dead insects. The numbers of non-*B. thuringiensis* bacteria present within the dead insects remained at a level of 10^6 cfu $larvae^{-1}$.

DISCUSSION

The plasmid pBC16 was stably maintained in *B. thuringiensis* subsp. *tenebrionis* and could be transferred to *B. thuringiensis* subsp. *kurstaki* HD1 cry^- Sm^r at a high frequency. In *B. thuringiensis* subsp. *tenebrionis* the *cryIIIA* gene has been shown to reside on a large 90MDa plasmid (Adams *et al.*, 1994). Since no transconjugants contained the crystal toxin gene this plasmid either does not co-transfer with pBC16 or is non-transferable. The temperature and pH ranges over which *B. thuringiensis* subsp. *tenebrionis* is able to transfer pBC16 are not significantly different from those found in the environment. It would therefore be expected that such physical parameters are not restrictive to gene exchange.

Although *B. thuringiensis* is commonly applied as a spore/crystal suspension to foliage, a significant proportion of the spores may reach the soil. Spore forming bacteria present in soil samples have been shown to act as recipients for *B. thuringiensis* plasmids (Jarrett & Stephenson, 1990). The added cells rapidly declined in numbers and those surviving formed spores. No plasmid transfer was detected (detection frequency limit $1x10^{-4}$ transconjugants per donor cell) which agrees with the results of previous studies of plasmid transfer between *Bacillus* spp. in soil (van Elsas *et al.*,1987). However the report of Haack *et al.*, (1996) demonstrated that the conjugal transfer of *Tn*916 could be detected between *B. subtilis* and *B. thuringiensis* in non-sterile sandy soil at a frequency of $6.2x10^{-5}$ transconjugants/donor.

In the laboratory, insect larvae have been shown to be a potential 'hot spot' for gene exchange between *B. thuringiensis* strains. For example Jarrett & Stephenson, (1990) observed plasmid transfer between *B. thuringiensis* strains in the dead larvae of the susceptible Lepidopteran insects *Galleria mellonella* and *Spodoptera littoralis*. The transfer frequency in *G. mellonella* feeding on an artificial diet was similar to that achieved in broth. Larger variation between individual larvae and decreased transfer frequency was detected in *S. littoralis* feeding on cotton leaves. In contrast, in our study no plasmid transfer was detected between *B. thuringiensis* strains in *Phaedon cochleriae* larvae (Coleoptera) feeding on Chinese cabbage leaves. This may be due to the inability of the *B. thuringiensis* spores to germinate, outgrow and compete with the natural microbiota of the insect gut.

ACKNOWLEDGEMENTS

This work was funded by the Biotechnology and Biological Science Research Council (BBSRC).

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PCR DETECTION OF *BACILLUS THURINGIENSIS* TOXIN GENES IN THE ENVIRONMENT

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ABSTRACT

The distribution of *Bacillus thuringiensis* toxin genes in natural samples was determined by polymerase chain reaction amplification of extracted DNA. Two methods of DNA extraction were developed, the first involved direct lysis of cells and spores using a bead beater followed by DNA purification, the second allowed germination of spores in nutrient media and lysis by chemical treatments prior to purification. Detection of *B. thuringiensis* toxins was performed using a combination of 18 primer pairs that allowed the detection of Lepidopteran active genes (12 pairs), Dipteran active genes (2 pairs) and Coleopteran active genes (4 pairs). From DNA isolated from soil only coleopteran active genes were detected, these were found in 4 of the 12 different soils sampled. The products were cloned and sequence analysis indicated 81-95% similarity to the coleopteran active *cryIIIA* gene. From leaf samples five of the nine samples taken were positive for either Lepidopteran or Dipteran active toxins, sequence analysis confirmed each product as similar to known *B. thuringiensis* toxin genes (75-100% similarity). To confirm that this method was appropriate for the detection of *B. thuringiensis* in the environment, soil samples that had been inoculated with a known *B. thuringiensis* strain in previous years were sampled. These results have further confirmed that this method was capable of detecting *B. thuringiensis* toxin genes in natural samples. The spatial location and distribution of toxin genes in natural bacterial populations using this method is under investigation.

INTRODUCTION

Bacillus thuringiensis is a Gram positive bacterium that during sporulation produces a proteinaceous crystal toxin. The toxins studied from a large number of strains have been shown to be active against many insects, predominately those belonging to Lepidoptera, Coleoptera and Diptera. Many *B. thuringiensis* toxin genes have been sequenced and this information has been used to sub-divide them according to their % similarity (Hofte & Whiteley, 1989). The main toxin types are CryI, II, III and IV, however the list is extending rapidly and many more new *cry* gene types have been described. In traditional strain searches

many environmental samples are plated on to selective media, or heat treated to isolate colonies which after further characterization are found to be predominantly *Bacillus thuringiensis* (Martin & Travers, 1989; Meadows *et al.*, 1992). This approach can be restrictive and strains that have *cry* toxin genes which can not grow on the selective media, or strains that are not present in the sample as spores, can be missed. In addition, the detection limit for *B. thuringiensis* in samples with a high population of other bacillus strains can be poor. The aim of this work was to use PCR amplification of DNA to screen environmental samples to determine if *cry* genes similar to known toxin genes could be detected in samples, and if sequence analysis of cloned PCR products could be used to determine their identity.

MATERIALS AND METHODS

Two methods of DNA extraction were developed, the first involved the direct lysis of cells and spores using a bead beater followed by DNA purification, the second allowed germination of spores in nutrient media and lysis by chemical treatments prior to purification. In the indirect approach, 10 g of soil was placed in 500 ml of nutrient broth and incubated at 30 °C for 18 h. The cells were pelleted by centrifugation at 5000 x *g* for 15 min and the DNA extracted using the QIAGEN tip-500 method. The only modification to the manufacturers instructions was the incorporation of 0.1 mg ml^{-1} of Lysozyme in buffer P1 followed by incubation for 1 hour at 37 °C. After purification, the DNA was ethanol precipitated and re-suspended in 1 ml of 1 mM Tris-HCl pH 8.0. For DNA isolation directly from samples, 10 g soil was placed in 50 ml 0.5 M NaCL, 100 mM Tris-HCl pH 7.5 and 20 g of 0.1 mm diam. glass beads were added. The sample was subjected to 3 rounds of bead beating (Biospec Products) for 1 min each. The sample was centrifuged at 14,000 x *g* for 30 min and the supernatant applied to a QIAGEN tip-500 column. The DNA was purified using the QIAGEN tip-500 system. DNA extraction was performed from leaves in an identical fashion with 10 g w/w starting material.

PCR amplification was performed using a Hybaid omnigene thermocycler with tube control. Two programs were used: A, 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min; and B, 95 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 2 min. A total of 30 cycles were completed and samples run on a 1 % w/v agarose gel to determine the presence of amplified product. Samples were amplified in 100 µl final volume containing 0.1 mM of each dNTP, 3.3 nM of each primer, 1 x reaction buffer, and 2 u of Dynazyme (Flowgen). Detection of *B. thuringiensis* toxins was performed using a combination of 18 primer pairs (Carozzi *et al.*, 1991) that allowed the detection of Lepidopteran active genes (12 pairs), Dipteran active genes (2 pairs) and Coleopteran active genes (4 pairs). Target DNA extracted from samples was included in the PCR mix at three concentrations 10 µl, 1 µl and 0.1 µl.

PCR products were TA cloned into the vector PCR™II (Invitrogen) and sequenced using primers designed 50 bp upstream of the cloning site. Automated sequencing (Applied Biosystems) was performed at The University of Liverpool Sequencing Unit.

RESULTS

From DNA isolated from soil only Coleopteran active genes were detected, these were found in 4 of the 12 different soils sampled. No Lepidopteran or Dipteran active toxin genes were detected in soil. On leaves only Lepidopteran and Dipteran active toxin genes were detected in 5 out of the 9 samples taken. The products were cloned and 50 inserts selected for sequence analysis. Analysis of the Coleopteran active toxin sequences indicated that they had 81-95% similarity to the Coleopteran active *cryIIIA* gene. From leaf samples five of the nine samples taken were positive for either Lepidopteran or Dipteran active toxins, sequence analysis confirmed that each product had a high degree of similarity to known *B. thuringiensis* toxin genes (75-100% similarity).

DISCUSSION

The DNA that was extracted from cells in soil and on leaves was found to have sufficient purity for PCR amplification. Pre-culture of the cells was found to give a greater proportion of samples with positive results. Since Gram positive species have not been shown to enter a non-culturable but viable state, the use of a culture step prior to DNA extraction was not considered to be a problem. A range of toxin genes were detected in the soil and on the leaf samples tested. Coleopteran toxins were primarily found in soil samples and Dipteran and Lepidopteran toxins on leaves. The limited number of samples used in this preliminary set of data does not confirm that this type of pattern is expected for other soil and leaf samples. Sequence analysis of the PCR products indicated that not all the genes were identical to those previously sequenced and available in the databases. The PCR method described here now offers the potential to identify which samples can contain new, or a selective sub-group of toxin genes to initiate a strain search. Further if this method is combined with colony isolation and DNA hybridization specific *cry* toxin genes present within a strain could be targeted. In addition, since new toxin sequences will be identified by this approach, directed mutagenesis of an existing toxin gene to the new sequence could be used to create cultures with new insecticidal activity without entering into a strain search.

ACKNOWLEDGEMENTS

We would like to thank Angela Benfold for running the automated DNA sequencing facility. We also acknowledge the financial support of the MAFF and BBSRC for this work.

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