BIOLOGICAL CONTROL OF *ELDANA SACCHARINA* USING CLONED *BACILLUS THURINGIENSIS* GENES. I. CLONING OF DELTA-ENDOTOXIN DNA SEQUENCES FROM *B. THURINGIENSIS* 234

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Abstract

A gene bank of *Bacillus thuringiensis* isolate number 234 was made in the positive selection cloning vector pEcoR252. The library was probed with a ^{32P} labelled 4,2 kb DNA fragment coding for the delta-endotoxin protein of *B. thuringiensis* HD1. In this manner, the partial DNA homology known to exist among different delta-endotoxin genes (*tox*) allowed us to detect 11 clones carrying *B. thuringiensis tox* DNA sequences. The protoxin crystal protein produced by *B. thuringiensis* 234 was isolated using density gradient centrifugation through Urografin 60%. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of solubilized pure *B. thuringiensis* 234 crystals was used to determine the protoxin's approximate molecular weight of $1,30 \times 10.^5$ Antibodies were raised against the SDS-PAGE purified 130 kDa protein band and are currently being used for the immunological screening of delta-endotoxin production by clones of the *B. thuringiensis* 234 gene library carrying the *tox* DNA sequences.

The project is a contribution to the biological control programme of the stalk borer *Eldana saccharina* Walker.

Introduction

Bacillus thuringiensis is a Gram positive bacterium which, during sporulation, produces a highly toxic secondary metabolite, the delta-endotoxin protein (Tox), with a very specific activity spectrum (for a review see Aronson *et al*[1]). Insecticides formulated with *B. thuringiensis* are not harmful to predatory insects or to other animals, including man, therefore providing an effective and safe environmental control. Although commercial production of *B. thuringiensis*-based biopesticides began more than a quarter of a century ago in the USA, the molecular genetics of delta-endotoxin production have been elucidated only recently. Crystal protein genes from a variety of subspecies have been cloned and expressed (Schnepf and Whiteley;[9] Whiteley et al;[11] Held et al,[6] among others), making the use of cloned delta-endotoxin genes a feasible alternative to traditional biocontrol using B. thuringiensis-based formulations.

The disadvantages of *B. thuringiensis* formulations are that (i) the spores and crystals are inactivated by prolonged exposure to UV radiation; (ii) spores and crystals are effective only against surface feeding insects or the surface feeding stages of boring and burrowing insects, (this being particularly important in the control of *Eldana saccharina* Walker) and finally, (iii) spores and crystals show low field persistence. The results presented here form part of a project aimed at generating a self-perpetuating biological insecticide likely to aid in overcoming the difficulties so far met in the control of *E. saccharina.*

The intention is to introduce a *B. thuringiensis tox* gene highly toxic to *E. saccharina* into a suitable native fluorescent pseudomonad which is a good colonizer of borings in sugarcane. Constitutive expression of *tox* by the recombinant *Pseudomonas* strain may provide a more effective means of control because of the potential to overcome the limited persistence of *B. thuringiensis* formulations in the field. In addition, colonization of holes and cracks would render the pesticide available for ingestion in regions otherwise unreached. A total DNA gene library of *B. thuringiensis* isolate number 234 (isolated at the South African Sugar Association Experiment Station) was made and, using *in situ* colony DNA-DNA hybridizations, 11 clones bearing *tox* DNA sequences were detected. Antibodies to the electroeluted 130 kDa delta-endotoxin produced by *B. thuringiensis* 234 were raised in rabbits in order to immunologically assay delta-endotoxin production by the recombinant strains.

Materials and Methods

Bacterial strains and plasmids

B. thuringiensis isolate number 234, a natural isolate highly toxic to *E. saccharina,* was obtained from the SASA Experiment Station at Mount Edgecombe for the construction of the gene library. Plasmid pEcoR252, a positive selection vector containing the *E. coli EcoRI* gene under the control of the Lambda rightward promoter and the ampicillin resistance gene and origin of replication of pBR322, and its host strain, E. coli LK111 were provided by Plant Genetic Systems in Ghent, Belgium. Plasmid pES1 (pBR322 carrying the tox gene from B. thuringiensis subsp, kurstaki) was procured from the American Type Culture Collection.

DNA techniques

Extraction of plasmid and total DNA was carried out using the alkaline lysis method as described by Maniatis *et al.*[8] Restriction enzymes (Boehringer Mannheim) were used according to the manufacturer's instructions. Sucrose gradient density centrifugation of partially digested *B. thuringiensis* 234 DNA and litigations were carried out according to Maniatis *et al.*[8] Transformations were done using the method described by Chung and Miller.[2] Colony hybridizations were performed following Grunstein and Hogness,[4] using a ^{32p} radioactively labelled *tox* probe prepared using the Random Primed Labeling Kit (Boehringer Mannheim), following the manufacturer's instructions. The *tox* fragment was obtained from a *HpaI-PstI* total digestion of pES1 followed by agarose gel electrophoresis and purification of the 4,2 kb band using the Geneclean™ kit (B10 101, USA) following the manufacturer's instructions.

Protein isolation and Immunoelectroblotting

Delta-endotoxin crystals from *B. thuringiensis* isolate number 234 were isolated from cultures grown on nutrient agar (Biolab) for 48–72 h at 30°C, using density gradient centifugation through Urografin 60% (Schering) following the method of Gonzalez *et al.*[3] Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the isolated crystals was according to Hames.[5] Antibodies to the electroeluted 130 kDa delta-endotoxin protein were raised in rabbits following procedures described by Hudson and Hay.[7] Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets and the immunological detection of them was carried out according to Towbin *et al.*[10]

Results

Construction of a total DNA library from B. thuringiensis *234 and detection of clones carrying* tox *DNA sequences.*

Total DNA from *B. thuringiensis* 234 was partially digested with *Sau*3A and subjected to density gradient centifugation in order to discriminate DNA fragments in the 4,5–10,5 kb size range. The resulting DNA was ligated to pEcoR252 opened at its BglII site and used to transform E. coli LK111.

The average size of the insert DNA, estimated from *Eco*R1 digestions of plasmid DNA, was approximately 7,2 kb. Transformants were screened for *tox* DNA sequences by *in situ* colony hybridization using a ^{32P}-labelled *tox* DNA sequence from pES1 as a probe. Eleven clones carrying insert DNA sequences at least partially homologous to the *tox* gene of *B. thuringiensis* subsp. *kurstaki* HD1 were detected. The clones, bearing the recombinant pEcoR252-*B. thuringiensis tox* DNA plasmids are referred to as pGH 1 to 11 in this work. The sizes of their inserts, estimated by analysis *Eco*R1 and *Pst*I restriction patterns, were found to range from 4,6 to 11,0 kb (results not shown).

Isolation of the delta-endotoxin crystal and immunological detection of toxin production

Since Southern blotting cannot establish whether a gene is functional, recombinant clones must be assayed for expression, in our case production of the delta-endotoxin protein. Immunoelectroblotting of cell extracts, using antibodies raised against the delta-endotoxin of *B. thuringiensis* 234, provide a valuable means of detecting delta-endotoxin production.

The delta-endotoxin crystals produced by *B. thuringiensis* 234 during sporulation were isolated using three rounds of density gradient centrifugation through Urografin 60%. The apparent molecular weight of the delta-endotoxin produced by *B. thuringiensis* 234 was determined, by SDS-PAGE, to be approximately 130 kDa (Figure 1). The faint protein bands migrating in front of the delta-endotoxin probably represent products of degradation. Alternatively, it is possible that the *B. thuringiensis* 234 delta-endotoxin crystal cannot be isolated to electrophoretic purity using Urografin density gradients. To ensure antigen purity during the raising of antibodies the delta-endotoxin protein was electroeluted from a SDS-PAGE of *B. thuringiensis* 234 delta-endotoxin crystals after density gradient isolation.

Immunoelectroblotting of cell extracts from E. coli LK111 carrying plasmids pGH 1 to 11 showed that pGH 1, 4, 5, 6 and 10 produced proteins reactive with antibodies directed

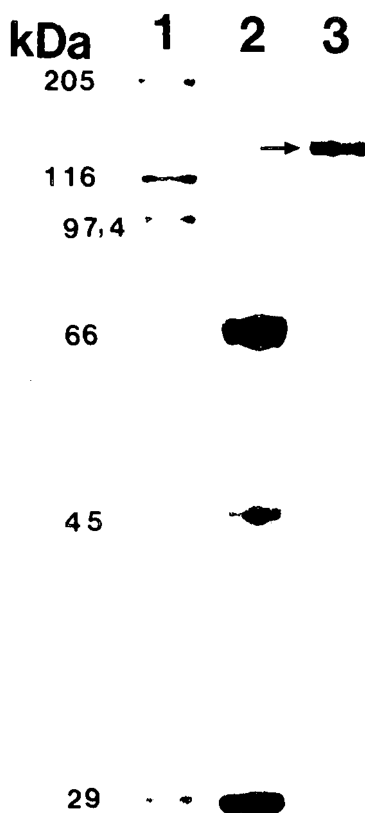

FIGURE 1 SDS-PAGE of solubilized *B. thuringiensis* 234 delta-endotoxin crystals. Lane 1: molecular weight markers carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase B, β-galactosidase and myosin. Lane 2: carbonic anhydrase, ovalbumin and bovine serum albumin. Lane 3: solubilized *B. thuringiensis* 234 delta-endotoxin crystals. The position of the delta-endotoxin is indicated with an arrow.

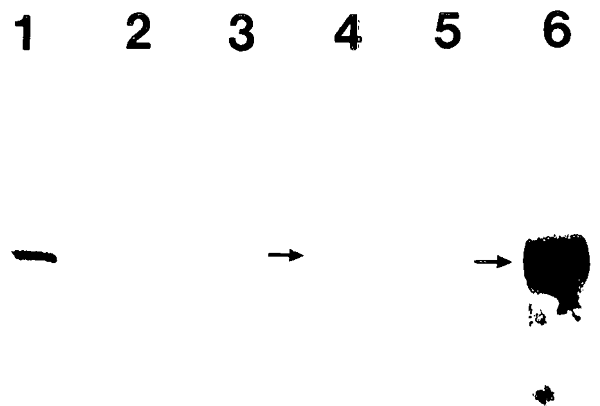

FIGURE 2 Immunological detection of delta-endotoxin after electrotransfer of cell extract proteins separated by SDS-PAGE to a nitrocellulose sheet. Cell extracts from: (lane 1), *E. coli* LK111 (pGH 10); (lane 2), *E. coli* LK111 (pGH 11); (lane 3), *E. coli* LK111; (lane 4), *E. coli* LK111 (pES1); (lane 5), *E. coli* K514 (pEcoR252); (lane 6), *B. thuringiensis* 234. Protein bands reactive with anti-*B. thuringiensis* 234 delta-endotoxin antibodies are indicated with arrows.

against the delta-endotoxin of *B. thuringiensis* 234. Results are shown for pGH 10 (Figure 2). The *E.coli* LK111 (pES1)-encoded delta-endotoxin exhibited cross reaction with the anti- *B. thuringiensis* 234 antiserum. As expected, there was no immunological cross reaction between the antibodies directed against the *B. thuringiensis* delta-endotoxin and protein products, of similar molecular weight to that of the toxin, produced by the host strain (*E. coli* LK111 and/or the proteins encoded by plasmid pEcoR252.

Discussion

B. thuringiensis isolate number 234 was chosen for our work due to its high toxicity towards *E. saccharina* (S Jacobs, personal communication). In addition, since the strain is a regional isolate, the environmental implications of using recombinant strains bearing *B. thuringiensis* 234 delta-endotoxin genes are minimized.

More than one delta-endotoxin gene can be present in the same *B. thuringiensis* strain (see Aronson *et al*[1]). In addition, more than one of these different delta-endotoxin genes can be functional. Consequently, the same *B. thuringiensis* strain can produce more than one toxin which in turn may differ in toxicity. There is, however, considerable DNA homology among different delta-endotoxin genes. This made possible the use of a *B. thuringiensis* subsp. *kurstaki* HD1 *tox* DNA probe to screen the *B. thuringiensis* 234 DNA library for delta-endotoxin DNA sequences and in this manner detect 11 clones carrying *tox* DNA sequences.

Analysis of restriction digestion patterns of pGH 1-11 using *Eco*R1 and *Pst*I (data not shown) suggests that *B. thuringiensis* 234 has two, possibly three different *tox* genes. Further restriction pattern analysis is underway in order to discriminate the genes unambiguously before proceeding to their characterization.

Results of immunoelectroblotting of cell extracts using anti- *B. thuringiensis* 234 endotoxin antibodies showed that the recombinant clones E.coli LK111 bearing plasmids pGH 1, 4, 5, 6 and 10 bear a B. thuringiensis 234 tox gene expressed in E.coli. In contrast, plasmids pGH 2, 3, 7, 8, 9 and 11 although giving a positive reaction in colony DNA-DNA hybridization, did not produce delta-endotoxin, probably reflecting a lack of sufficient genetic information for adequate expression. Since, as previously mentioned, the same *B. thuringiensis* strain can produce more than one delta-endotoxin, and *B. thuringiensis* 234 appears to have more than one *tox* gene, toxicity tests on *E. Saccharina* underway at the SASA Experiment Station will be instrumental in determining which clone(s) produces the most potent delta-endotoxin(s).

Following the identification of the *B. thuringiensis* 234 *tox* gene encoding the most potent delta-endotoxin it will be subcloned in the broad host range cloning vector pDER405 which has been shown to be conjugally transferable to a native fluorescent *Pseudomonas* strain proven to be a good colonizer of sugarcane surfaces (S Jacobs, personal communication). We expect that the combination of good colonizing capacity and constitutive expression of a highly toxic delta-endotoxin gene from *B. thuringiensis* 234 will render the resulting recombinant fluorescent *Pseudomonas* strain an effective biocontrol agent.

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