

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 unreachable. 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 ligations 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 centrifugation 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 centrifugation 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 *Bgl*III 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

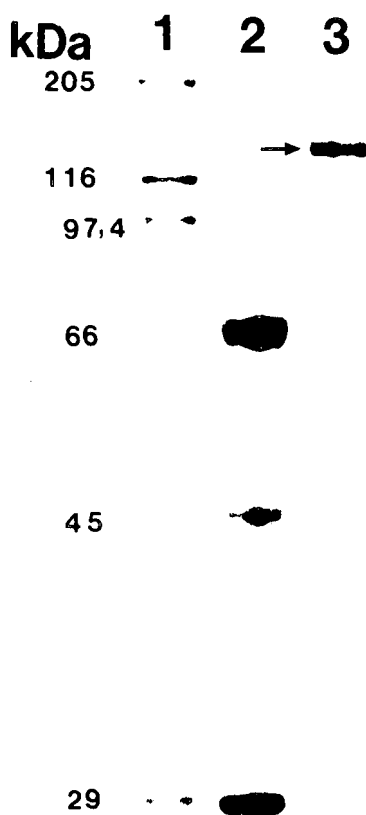


FIGURE 1 SDS-PAGE of solubilized *B. thuringiensis* 234 delta-endotoxin crystals. Lane 1: molecular weight markers carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase B, β -galactosidase and myosin. Lane 2: carbonic anhydrase, ovalbumin and bovine serum albumin. Lane 3: solubilized *B.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

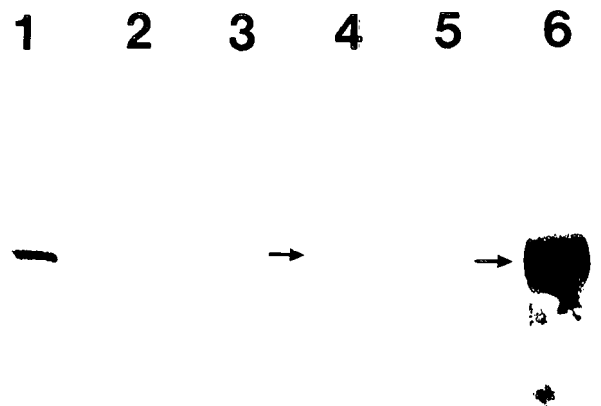


FIGURE 2 Immunological detection of delta-endotoxin after electrotransfer of cell extract proteins separated by SDS-PAGE to a nitrocellulose sheet. Cell extracts from: (lane 1), *E. coli* LK111 (pGH 10); (lane 2), *E. coli* LK111 (pGH 11); (lane 3), *E. coli* LK111 (pES1); (lane 4), *E. coli* K514 (pEcoR252);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