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Construction of a Bioinsecticidal Strain of *Pseudomonas fluorescens* Active against the Sugarcane Borer, *Eldana saccharina*

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A *cryIA(c)* gene was cloned from a native *Bacillus thuringiensis* strain showing activity against the sugarcane borer, *Eldana saccharina*. The sequence of the cloned gene was very similar to that of the *B. thuringiensis* subsp. *kurstaki* HD-73 *cryIA(c)* gene. The gene was introduced into an isolate of *Pseudomonas fluorescens*, capable of colonizing sugarcane, on two broad-host-range plasmids, pDER405 and pKT240, having copy numbers of 13 and 28, respectively. By using the Omegon-Km vector, the *cry* gene was introduced into the chromosome of *P. fluorescens* isolate 14. Bioassays on eldana larvae showed that the strain carrying the gene integrated into the chromosome was as toxic as one carrying it on pKT240. Glasshouse trials indicated that sugarcane treated with *P. fluorescens* 14::Omegon-Km-*cry* were more resistant to eldana damage than untreated sugarcane was.

Many strains of *Bacillus thuringiensis* produce crystalline inclusions during sporulation which contain proteins exhibiting highly specific insecticidal activity (19). The inclusions dissolve in the larval midgut, releasing one or more insecticidal proteins called δ-endotoxins. Most are protoxins which are proteolytically converted into smaller toxic polypeptides. The activated toxins appear to generate pores in the midgut epithelium cells of susceptible insects, thus disturbing the osmotic balance. The cells swell and lyse, resulting in larval death. In some instances, specific high-affinity binding sites have been shown to exist in the midgut epithelial cells of susceptible insects, which may explain the specificity of the toxins (19, 43).

Eldana saccharina Walker (Lepidoptera: Pyralidae) is an endemic species in Africa, normally found in wetlands. Although *E. saccharina* has become a major pest of sugarcane in South Africa, its natural hosts are the larger members of the Cyperacea such as *Cyperus inmensus*, in which it feeds preferentially in the rhizome (2). Land management practices, including the clearing of riverine woodland, have created adequate environments for Cyperacea, and this has enabled *E. saccharina* to extend its host range. In sugarcane, the larvae bore into the stalks and can cause considerable crop loss. It was decided to screen local isolates of *B. thuringiensis* for activity against *E. saccharina* larvae and develop a biological control agent.

A number of approaches have been employed to use δ-endotoxins in biological control of agricultural pests. Formulations of *B. thuringiensis* crystals and spores have been used for more than two decades, but problems due to their instability in the environment have been encountered (23, 48). More recently, the cloning of insecticidal crystal protein genes and their expression in plant-associated bacteria (28, 29, 45) or transgenic plants (8, 31, 42) have provided an alternative strategy. As monocotyledonous plants such as sugarcane are difficult to transform, it was decided to introduce the δ-endotoxin gene into a bacterium able to colonize sugarcane.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains of *B. thuringiensis* were isolated from soil samples around insect-infested sugarcane and from dead *E. saccharina* larvae by growth on PEMBA medium (polymyxin-pyruvate-egg yolk-mannitol-bromothymol blue agar [20]). The strains and plasmids used are described in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (27), and ampicillin (100 μg/ml) was used to select for transformants. *Pseudomonas fluorescens* strains were isolated from sugarcane by growth on King's medium B (22) and confirmed by Analytab Products tests, using the API 2ONE identification strips. Spontaneous nalidixic acid- and rifampin-resistant mutants were isolated.

Laboratory toxicity bioassays. Two-week-old *E. saccharina* larvae were fed on an artificial insect diet in which different concentrations of freeze-dried bacteria were incorporated (5). Larvae were incubated in plastic 32-cell trays for 5 days at 30°C, after which mortality was recorded.

Purification of the δ-endotoxin. δ-Endotoxin crystals from *B. thuringiensis* isolate 234 were isolated from cultures grown on nutrient agar for 48 to 72 h at 30°C, using gradient centrifugation through Urografin 60% (Schering) as described by Gonzalez et al. (15).

Isolation of DNA from *B. thuringiensis* isolate 234. Total chromosomal and plasmid DNA was isolated from 50 ml of an overnight culture of isolate 234 grown in nutrient broth at 30°C with vigorous agitation (170 rpm) in a rotary shaker. The cells were pelleted and resuspended in 1.5 ml of 50 mM Tris-HCl–100 mM EDTA–20% (wt/vol) sucrose (pH 8.0). To this was added 14 mg of lysozyme, 40 μg of RNase A, and 400 μg of proteinase K. Two milliliters of a 1% Sarkosyl solution in 75 mM EDTA (pH 8.0) was added by blowing through the cell suspension, which was incubated at 37°C until a clear lysate developed (approximately 30 min). This was subjected to CsCl (refractive index of 1.40, density of 1.74 g/ml) ultracentrifugation without ethidium bromide for 16 h at 185,000 × *g*. The

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype or phenotype	Reference
Strains		
E. coli LK111	*thr-1 leu-6 thi-1 supE44 tonA2* r_K^- m_K^+ *lacI*^{q} *lacZ* δM15 *lacY1*	49
E. coli HB101	*hsdS20 recA13 proA2 rpsL20*	27
E. coli S17-1	*recA thi pro* hsR$^-$M$^+$ <RP4:2-tc:Mu:Km:Tn7>Tp^r Sm^r	37
P. fluorescens 14	Rif^r Nal^r	This work
Plasmids		
pRK2013	Km^r *mob*$^+$ *tra*$^+$	11
pKT240	Km^r Ap^r Sm^r	4
pDER405	Cm^r Tc^r	32
pJFF350	Km^r	10
pEcoR252	Ap^r	26
pUC19	Ap^r	44
pSUP204	Cm^r Km^r	38
pES1	Ap^r HD-1 *cry*$^+$	34

tubes were drained from the bottom, and fractions showing increased viscosity, indicative of the presence of DNA, were pooled, dialyzed against TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1) and once with water-saturated ether.

Construction and screening of a *B. thuringiensis* isolate 234 genomic library. *B. thuringiensis* isolate 234 DNA was partially digested with endonuclease *Sau*3AI and fractionated on a sucrose density gradient (27). The DNA fragments ranging from 6 to 10 kb in length were pooled and ligated with pEcoR252 which had been digested with endonuclease *Bgl*II. The ligated DNA was used to transform competent *E. coli* LK111 cells. Transformants were selected on LB agar containing ampicillin (100 μg/ml). A total of 5,300 transformants were screened by colony hybridization. Colonies were prepared for DNA hybridization on nitrocellulose membranes as described by Grunstein and Hogness (16). A ^{32}P-labelled 2.1-kb fragment of pES1 was used as a probe. The fragment was labelled by random priming with [α-^{32}P]dCTP, using the Boehringer Mannheim random primer labelling kit as instructed by the manufacturer. Hybridization was carried out by the method of Church and Gilbert (9), using low-stringency conditions (65°C, 1 M Na$^+$ in the absence of formamide).

Immunological detection of δ-endotoxin production. Cell extracts were prepared from 1.5-ml stationary phase cultures by pelleting, resuspension in 100 μl of denaturing loading buffer (25), and boiling for 10 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25). Electrophoretic transfer of the proteins to nitrocellulose and their immunological detection were carried out as described by Towbin et al. (40). Polyclonal antibodies to the *B. thuringiensis* 234 δ-endotoxin electroeluted from SDS-PAGE were raised in rabbits as described by Garvey et al. (12), using incomplete Freund's adjuvant (6). The antigen was obtained by electroelution of the 130- to 135-kDa proteins from SDS-PAGE of purified δ-endotoxin crystals.

Molecular techniques. Molecular techniques were performed as described by Ausubel et al. (3).

Nucleotide sequencing. DNA fragments were subcloned into pUC19, and sequencing was performed by the chain termination method of Sanger et al. (33), using a Sequenase kit (United States Biochemical Corp.). The nucleotide and deduced amino acid sequences were analyzed by using an IBM XT computer and the Genetics Computer Group program of the University of Wisconsin.

Colonization assays. Three-month-old sugarcane plants were dipped in stationary-phase cultures of *P. fluorescens* strains containing 1 drop of Tween 80 per 50 ml of culture. Plants were harvested at various time intervals by being cut off at ground level, weighed, cut into pieces, and shaken vigorously on a wrist-action shaker in sterile flasks containing glass beads and sterile water for 5 min. Bacteria were enumerated by plating on King's medium B containing nalidixic acid (100 μg/ml) and rifampin (50 μg/ml).

Plasmid stability. Plasmid-carrying strains were grown to stationary phase in LB medium (27) containing the appropriate antibiotic (ampicillin, 40 μg/ml; chloramphenicol, 300 μg/ml; kanamycin, 20 μg/ml; or tetracycline, 20 μg/ml) and diluted 10^{-6} in LB without antibiotics. Growth of the cultures to stationary phase was achieved in 20 generations. Several cycles of dilution and growth were performed, and samples were plated at various times on LB media with and without antibiotics.

Plasmid copy number determination. The method for determining plasmid copy number was based on the quantification of a ^{32}P-labelled probe binding to a specific DNA sequence present in the plasmids. This sequence was also integrated, as a single copy, into the chromosomal DNA of a control strain. *Pseudomonas* cells were grown in 50 ml of LB medium at 28°C with vigorous agitation (170 rpm) in a rotary shaker, harvested at early stationary phase, and resuspended in 1.5 ml of 50 mM Tris-HCl–100 mM EDTA–20% (wt/vol) sucrose (pH 8.0). DNA was extracted as described above for isolate 234. The DNA was digested to completion with *Bam*HI, for which there are no recognition sites within the δ-endotoxin gene. The DNA preparations were then extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1) and three times with water-saturated ether. The digested DNA preparations were quantified spectrophotometrically (3). Dilutions were then made, and 5-μl aliquots of each were dot blotted in triplicate onto nitrocellulose membranes (Amersham Hybond) as instructed by the manufacturer. The 3.7-kb *Nde*I fragment which carries the δ-endotoxin gene was labelled with [α-^{32}P]dCTP, using the Boehringer Mannheim random primer labelling kit as instructed by the manufacturer. Hybridization was carried out by the method of Church and Gilbert (9). For the quantification of bound probe, the membrane areas corresponding to the different dilutions were cut out and placed in individual scintillation vials containing 5 ml of scintillation cocktail (Filter-Count; Packard). The radioactivity of the samples, estimated as counts per minute, was then determined with a Packard scintillation counter.

Quantification of toxin production. An enzyme-linked immunosorbent assay (ELISA) based on antigen competition was designed as described by Harlow and Lane (17). Purified δ-endotoxin was dissolved as described by Wie et al. (47). Antigen dilutions were made in carbonate buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$ [pH 9.6]). Cell extracts for the quantification of δ-endotoxin from recombinant *P. fluorescens* strains were prepared from three replicate cultures as described by Schnepf and Whiteley (34), with the following modifications. Cell pellets were resuspended directly in 4 M urea–0.285 M 2-mercaptoethanol–0.05 M $NaHCO_3$ and disrupted in an Aminco French pressure cell. The resulting suspensions were dialyzed twice against 100 volumes of 3 mM $NaHCO_3$–7 mM 2-mercaptoethanol (pH 9.0) for a total of 16 h at 4°C. The suspensions were then centrifuged at 100,000 × *g*, and the pellet was discarded. Protein content was determined by the biuret assay (18).

Microtiter plates were coated with antigen (200 μl per well) for 1 h at 37°C and blocked with 0.5% (wt/vol) casein in phosphate-buffered saline (PBS; 0.1 M NaCl, 8 mM $NaHPO_4$, 2 mM KCl, 1 mM KH_2PO_4 [pH 7.4]) for 2 h at 37°C. Microtiter plates were washed three times with 0.05% Tween 20 in PBS after the blocking as well as the antibody-antigen competition and second antibody reactions. Antibody reactions, carried out in blocking solution, were incubated at 37°C for 1 h. Goat anti-rabbit horseradish peroxidase-immunoglobulin G conjugate (Sigma) diluted 1:3,000 was used as the second antibody. After addition of the substrate, A_{405} was measured with a Bio-Tek ELISA reader.

Effect on *E. saccharina* of sugarcane inoculated with *P. fluorescens* 14::Omegon-Km-*cry*. Six-month-old sugarcane plants grown in pots in the glasshouse were sprayed with 100 ml of a suspension of either *P. fluorescens* 14 or *P. fluorescens* 14::Omegon-Km-*cry* at 2 × 10^9 CFU/ml. After 2 weeks, each plant was inoculated with 300 *E. saccharina* eggs placed by hand behind a leaf sheath at the base of the stalk. Stalks were sampled 4 weeks after egg placement, and larval numbers and the number of internodes that had been bored were recorded.

RESULTS

Cloning of the δ-endotoxin gene of *B. thuringiensis* isolate 234. More than 50 local isolates of *B. thuringiensis* were subjected to screening assays on *E. saccharina* larvae, and isolate 234 was identified as a potential candidate for the isolation of a *cry* gene. Crystals isolated from *B. thuringiensis* isolate 234 were bipyramidal, and the δ-endotoxin had an apparent M_r of 135,000 (results not shown).

As it was not known whether the δ-endotoxin was encoded by the chromosome or by a plasmid in isolate 234, total DNA was extracted. Partial *Sau*3AI digests were performed, and fragments in the size range of 6 to 10 kb were ligated to *Bgl*II-digested pEcoR252 and transformed into *E. coli* LK111. A gene library consisting of 5,300 clones with an average insert size of 8.2 kb was obtained. This was screened by colony hybridization with a ^{32}P-labelled 2.1-kb *Pvu*II fragment from pES1 as a probe, as *B. thuringiensis* subsp. *kurstaki* HD-1, from which pES1 was derived (36), also showed some toxicity toward eldana larvae (results not shown). However, in order to optimize hybridization, low-stringency conditions were used. Twelve clones which carried insert DNA sequences at least partially homologous to the *cryIA*(*a*) crystal protein gene of HD-1 were identified. Of these, five produced proteins which reacted with the antibodies raised against the toxin purified from isolate 234 (Fig. 1A). All five were found to produce a δ-endotoxin with an apparent M_r of 135,000. Toxicity bioassays of the 12 clones were carried out, and the results confirmed that the cell extracts from the 5 positive clones were toxic to *E. saccharina* (results not shown). Plasmid pGH37 was chosen for further analysis.

Determination of the DNA sequence of the *cry* gene encoded by pGH37. A restriction map of pGH37 was generated, on the basis of which a number of subclonings and deletions were performed to determine the location of the *cry* gene (Fig. 2). Subcloning of the 10-kb *Bam*HI fragment into pEcoR252 generated pGH37-1. Deletion of the 3.3-kb *Sma*I-*Nru*I fragment of pGH37 generated pGH37-D1. Both of these plasmids expressed the δ-endotoxin gene, as determined by immunoelectroblot analysis (Fig. 2). Subcloning of the 5.9-kb *Bam*HI-*Sph*I fragment from pGH37-D1 into pUC19 yielded clones producing the δ-endotoxin (results not shown). This located the gene to the 5.3-kb fragment of pGH37 from the *Nru*I site to the *Sph*I site. To sequence the *cry* gene, the *Bam*HI-*Xho*I and *Xho*I-*Sph*I fragments of pGH37-D1 were cloned in both orientations into pUC19. Comparisons between the DNA and

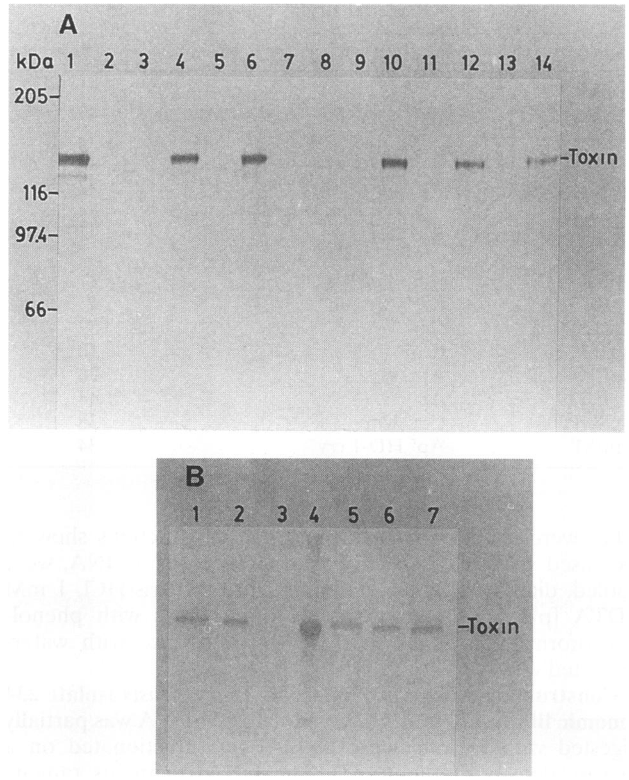


FIG. 1. Immunoelectroblot analysis of recombinant *E. coli* and *P. fluorescens* clones. (A) Clones from the *B. thuringiensis* 234 library. Lanes: 1 to 12; *E. coli* LK111 carrying pGH1 to pGH11 and pGH37, respectively; 13, *E. coli* K514λ(pEcoR252); 14, *E. coli* LK111(pES1). (B) Lanes: 1, *P. fluorescens* 14(pDER405-*cry*); 2, *P. fluorescens* 14(pKT240-*cry*); 3, *P. fluorescens* 14; 4, *E. coli* S17-1(pJFF350-*cry*); 5 to 7, *P. fluorescens* 14::Omegon-Km-*cry* clones 1, 2, and 3, respectively.

deduced amino acid sequences with those of other δ-endotoxin genes showed that the isolate 234 *cry* was almost identical to that found in *B. thuringiensis* subsp. *kurstaki* HD-73, *cryIA*(*c*) (1). There were only four different nucleotides, at positions 978 (A to C), 981 (G to T), 1102 (T to G), and 1020 (T to C), but these did not lead to any amino acid changes. However, Southern hybridization analysis of *Hin*dIII-digested DNA from isolate 234 and HD-73, probed with a 0.7-kb *cry* fragment of pES1, showed that they are different strains (Fig. 3). The *cry* gene, an allele of *cryIA*(*c*), will shortly be given a number by the Cry Gene Nomenclature Committee.

Isolation of sugarcane-colonizing *P. fluorescens*. Colonization studies showed that a number of isolates of *P. fluorescens* were able to survive on sugarcane. Isolate 14 was selected as one of the strains which, after 60 days, showed only a decrease in titer from 8 × 10^7 to 9 × 10^5 CFU per plant despite a 42% increase in plant mass. This corresponded to a decrease from 1 × 10^7 to 8 × 10^4 CFU/g of fresh mass. None of the other isolates tested showed more efficient colonization.

Plasmid stability in *P. fluorescens* isolate 14. Plasmids pDER405, pKT240, and pSUP204 have been shown to replicate in various pseudomonads (4, 32, 37). They were introduced into isolate 14 by triparental conjugation, using *E. coli* HB101(pRK2013) as the mobilizing strain (37). Stability assays showed that pDER405 was stable over at least 100 generations, pKT240 was stable over 50 generations, and pSUP204 was extremely unstable (Fig. 4).

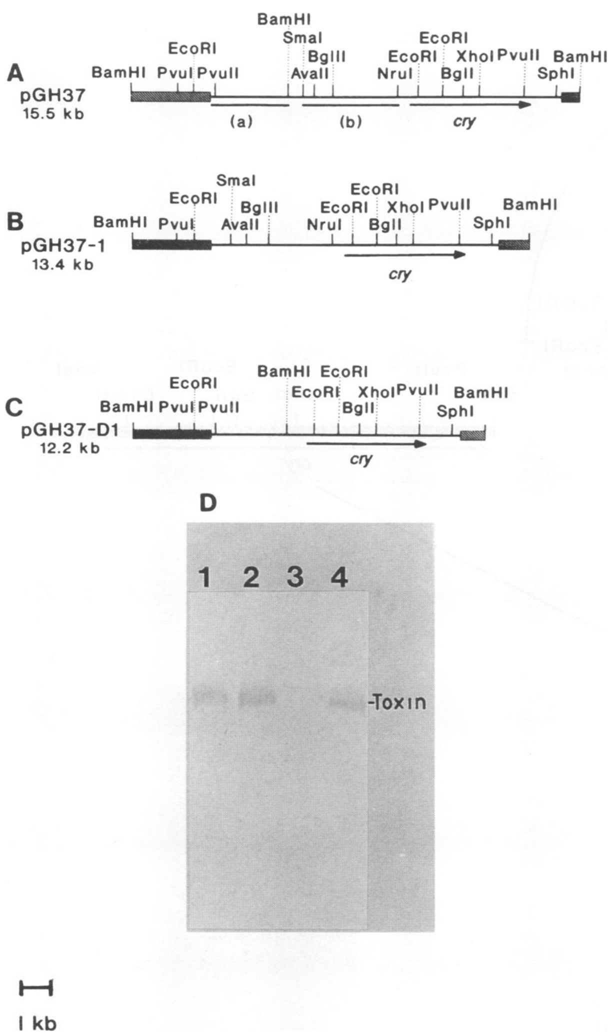


FIG. 2. Localization of the δ-endotoxin gene of pGH37. (A) Restriction map of pGH37. (B) pGH37-1 was generated by subcloning the 10-kb *Bam*HI fragment from pGH37 into pEcoR252, resulting in the deletion of fragment a. (C) Deletion of the 3.3-kb *Sma*I-*Nru*I fragment from pGH37 generated pGH37-D1, resulting in the deletion of fragment b. (D) Immunoelectroblotting analysis. Lanes: 1, 2, and 4, *E. coli* LK111 carrying pGH37-1, pGH37-D1, and pGH37, respectively; 3, *E. coli* K514λ(pEcoR252).

Construction of *P. fluorescens* cry^+ strains. The construction of pDER405-*cry* and pKT240-*cry* is shown in Fig. 5. In the first construct, the 6.7-kb *Bam*HI fragment of pGH37-D1, carrying the *cry* gene, was cloned into the *Bam*HI site of pDER405. In the second construct, the same fragment was made blunt and cloned into the *Hpa*I site of pKT240. The plasmids were introduced into isolate 14 by triparental conjugation, and the resultant strains were found to express the *cry* gene (Fig. 1B).

DNA sequence analysis of the *cry* gene showed that it was carried on a 3.7-kb *Nde*I fragment (Fig. 6). This fragment was cloned into the *Nde*I site of the integration vector, pJFF350, which carries the interposon Omegon-Km (10) (Fig. 5A and B). pJFF350-*cry* was transformed into *E. coli* S17-1 and conjugally mobilized into isolate 14, selecting for Km^r exconjugants. As the plasmid cannot replicate in this host, kanamycin selects for integration of the Omegon-Km-*cry* cassette into the chromosome. During transposition, the two *Eco*RI sites,

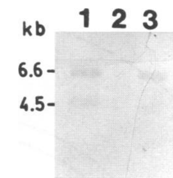


FIG. 3. Southern blot analysis of *B. thuringiensis* DNA digested with *Hin*dIII. The internal 732-bp *Eco*RI fragment from pES1 (F fragment) was used as a probe. Lanes: 1, isolate 234; 2, empty; 3, *B. thuringiensis* subsp. *kurstaki* HD-73.

located at the outer ends of the 28-bp inverted repeats of Omegon-Km-*cry* in pJFF350-*cry*, do not transpose. Therefore, all single-copy integrations of the Omegon-Km-*cry* cassette will carry the internal 0.7-kb *Eco*RI fragment and two different flanking *Eco*RI fragments larger than the 0.9- and 5.9-kb fragments of pJFF350-*cry* (Fig. 5C). Southern blot analysis was performed on *Eco*RI-digested DNA from exconjugants probed with the 3.7-kb *Nde*I fragment from pGH37-D1, carrying the δ-endotoxin gene (Fig. 5D). Size differences indicated that each of the single-copy integrations occurred at different sites on the chromosome. Western blot (immunoblot) analysis confirmed the expression of the *cry* gene in these exconjugants (Fig. 1B). *P. fluorescens* isolate 14 carrying pDER405-*cry*, pKT240-*cry*, and Omegon-Km-*cry* were all toxic to *E. saccharina* larvae (Fig. 7). Quantification of δ-endotoxin production in triplicate cultures by using ELISA indicated that it represented 2.2% (standard deviation [SD], 0.196%), 3.5% (SD, 0.185%), and 3.7% (SD, 0.153%) of the total dissolved protein in isolate 14 carrying pDER405-*cry*, pKT240-*cry*, and Omegon-Km-*cry*, respectively.

Plasmid copy number determination in *P. fluorescens* isolate 14. In experiments to determine plasmid copy number, twofold dilutions of standardized amounts of total DNA from *P. fluorescens* 14::Omegon-Km-*cry*, *P. fluorescens* 14(pKT240-*cry*), and *P. fluorescens* 14(pDER405-*cry*) were dot blotted, hybridized with the ^{32}P-labelled probe, and exposed to X-ray film (Fig. 8). There was lack of detectable hybridization of the probe to the negative control, *P. fluorescens* 14. For the remaining treatments, an increase in the signal intensity, which correlated to increasing amounts of dot-blotted DNA, was observed. In addition, similar amounts of dot-blotted DNA from the different strains resulted in different signal intensities, indicating differences in copy numbers. In a second experiment, dilutions of total DNA from the control isolate 14, as

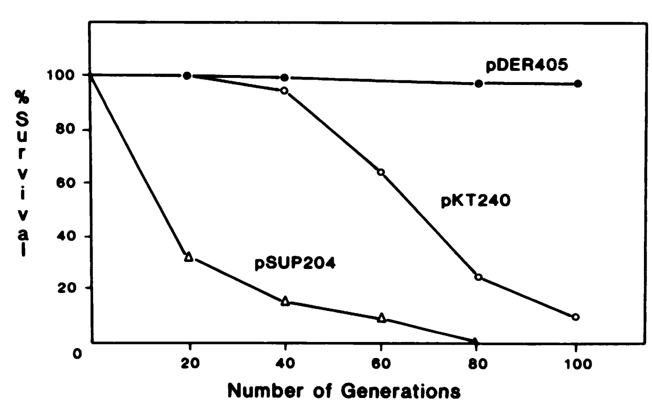
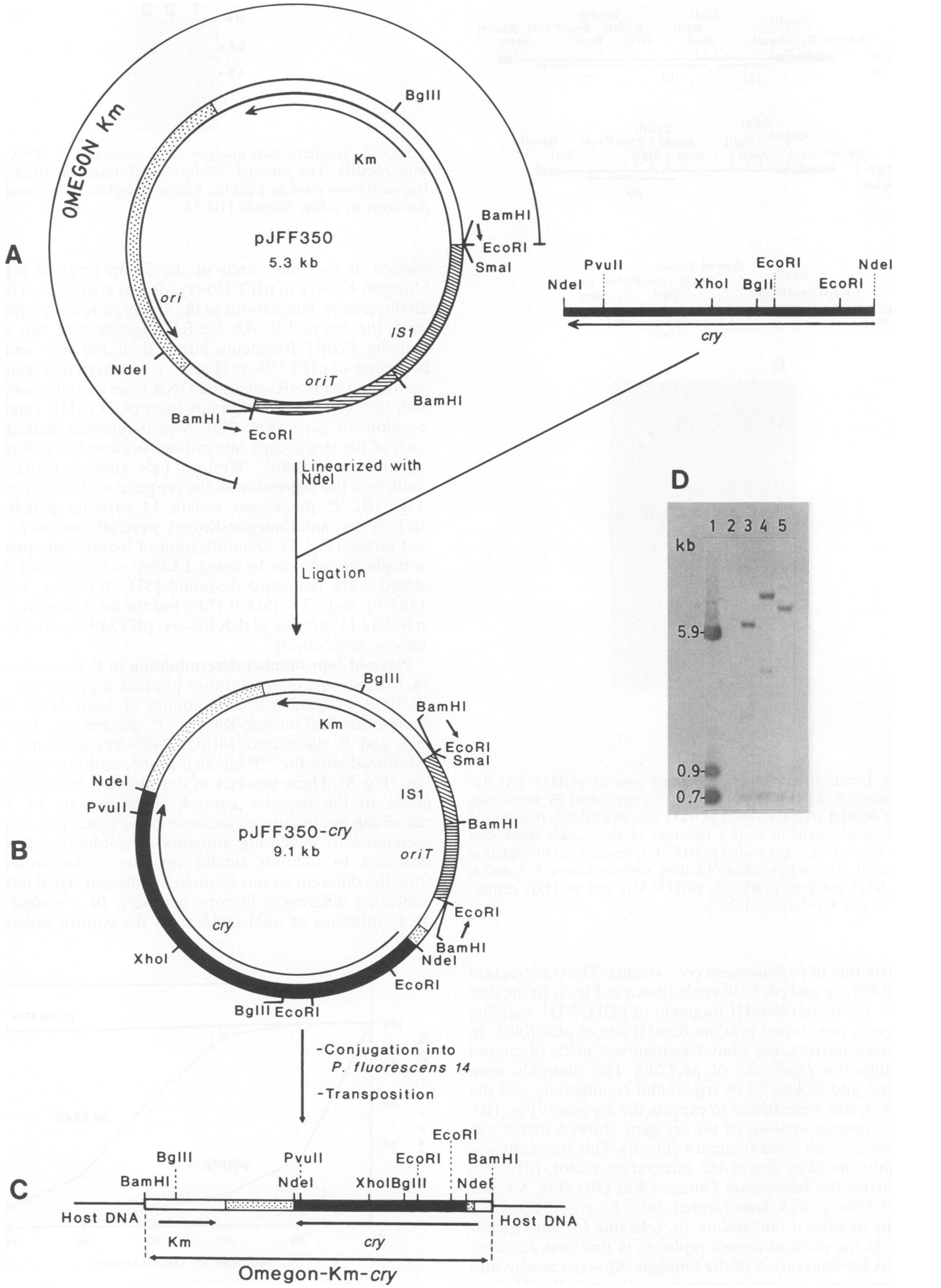


FIG. 4. Plasmid stability in *P. fluorescens* 14.


A
OMEGON Km
BglII
Km
BamHI
EcoRI
SmaI
pJFF350
5.3 kb
ori
IS1
NdeI
oriT
BamHI
BamHI
EcoRI
PvuII
EcoRI
NdeI
NdeI
XhoI
BglI
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cry
Linearized with
NdeI
Ligation
BglII
BamHI
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SmaI
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pJFF350-cry
9.1 kb
oriT
EcoRI
cry
BamHI
XhoI
NdeI
EcoRI
BglII EcoRI
-Conjugation into
P. fluorescens 14
-Transposition
EcoRI
BglII
PvuII
EcoRI
BamHI
BamHI
NdeI
XhoIBglII
NdeI
C
Host DNA
Host DNA
Km
cry
Omegon-Km-cry
D
1 2 3 4 5
kb
5.9
0.9
0.7

FIG. 5. Construction of pJFF350-*cry* and generation of *P. fluorescens* 14::Omegon-Km-*cry* strains. (A) The 3.7-kb *Nde*I fragment from pGH37-D1 was cloned into the *Nde*I site of pJFF350. (B) The resulting pJFF350-*cry* was conjugally transferred into *P. fluorescens* 14, in which transposition of the Omegon-Km-*cry* cassette (C) was selected for on kanamycin-supplemented media. (D) Single-copy integration was verified in three Km^r clones, producing δ-endotoxin, by Southern blot analysis. Chromosomal DNA digested with *Eco*RI was probed with the 3.7-kb *Nde*I fragment bearing the δ-endotoxin gene from pGH37-D1. Lanes: 1, pJFF350-*cry*; 2, *P. fluorescens* 14; 3 to 5, *P. fluorescens* 14::Omegon-Km-*cry* clones 1, 2, and 3, respectively. ⊢⊣, the transposable Omegon-Km cassette (the small arrows represent the 28-bp inverted repeats of Omegon); ■, *cry* gene; ▨, pBR322 *ori* segment; ▧, *ori*T from RP4; ▧, IS*1*; □, segments of Omegon and the Km^r gene of Tn*5*.

well as from the recombinant *cry*$^+$ strains, were dot blotted and hybridized with the ^{32}P-labelled probe, and the bound probe was then quantified by liquid scintillation analysis. For each strain, the resulting counts per minute was plotted against the DNA concentration, and the region of the curve conforming to a linear relationship [correlation coefficients of 0.97, 0.98, and 0.95 for *P. fluorescens*::Omegon-Km-*cry*, *P. fluorescens* (pDER405-*cry*), and *P. fluorescens*(pKT240-*cry*), respectively] was used in plasmid copy number determinations. In the case of *P. fluorescens* 14(pDER405-*cry*) and *P. fluorescens* 14 (pKT240-*cry*), plotted values of total DNA blotted were adjusted to account for the plasmid DNA contribution. For this, on the basis of information on chromosomal genetics in the genus *Pseudomonas* (21, 30, 46), the genome size of *P. fluorescens* 14 was assumed to be 2.4 × 10^9 Da. As *P. fluorescens* 14::Omegon-Km-*cry* carries a single copy of the *cry* gene per cell, the number of copies of the gene, and hence the copy number, is the ratio between the counts per minute from the strain carrying either plasmid and the counts per minute of *P. fluorescens* 14::Omegon-Km-*cry*. These ratios were 28 (SD, 3.6) for pKT240-*cry* and 13 (SD, 2.2) for pDER405-*cry*.

Effect of *P. fluorescens* 14::Omegon-Km-*cry*-inoculated plants on *E. saccharina*. As the toxicity of isolate 14::Omegon-Km-*cry* was similar to that of the strain carrying pDER405-*cry* and pKT240-*cry*, it was used in glasshouse trials. Apart from the *cry* gene being stably integrated into the chromosome in this strain, this approach is more acceptable from a biosafety consideration, as the *cry* gene is not on a mobilizable plasmid. A comparison of the number of eldana larvae recovered and the damage to stalks between plants sprayed with isolate 14 and 14::Omegon-Km-*cry* is shown in Table 2.

DISCUSSION

The *cry* δ-endotoxin gene of *B. thuringiensis* isolate 234, whose product is toxic to *E. saccharina* larvae, is almost identical to that in *B. thuringiensis* subsp. *kurstaki* HD-73, indicating that this gene is widely spread. Southern hybridization analysis of *Hin*dIII digests of the DNA of the two strains showed that whereas HD-73 carries only the 6.6-kb *cryIA*(*c*) gene, isolate 234 carried, in addition to the *cryIA*(*c*) gene, the 4.5-kb *cryIA*(*a*) gene. Whether the latter gene of isolate 234 contributes to the toxicity of isolate 234 to eldana larvae is being investigated.

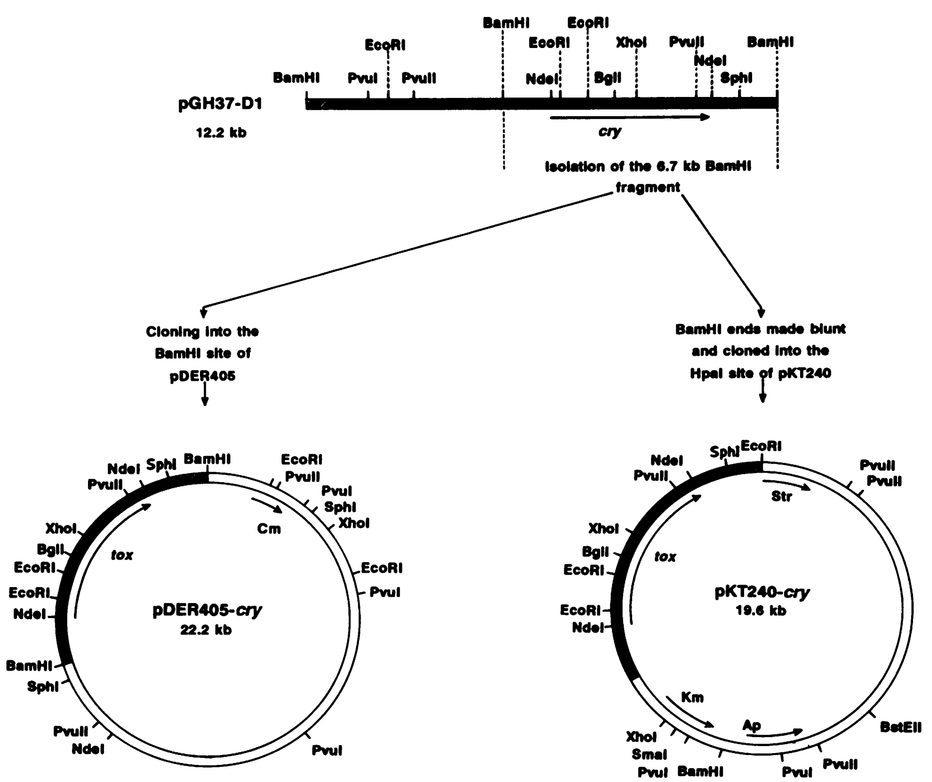

FIG. 6. Construction of pDER405-*cry* and pKT240-*cry*. The 6.7-kb *Bam*HI fragment of pGH37-D1 was cloned into the *Bam*HI site of pDER405 and, after being made blunt, into the *Hpa*I site of pKT240.

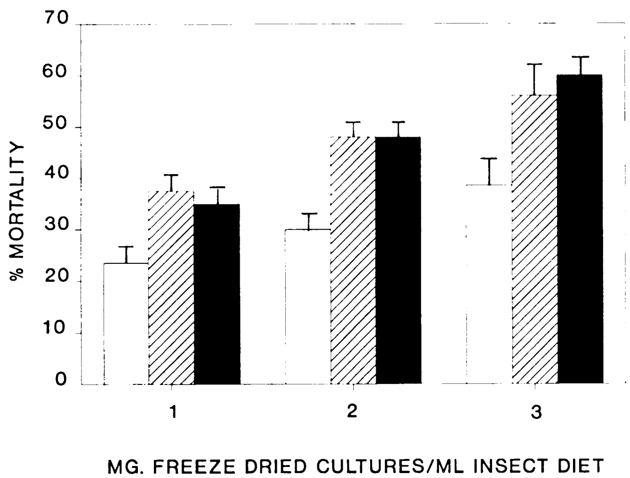

FIG. 7. Toxicity of *P. fluorescens* 14(pDER405-*cry*) (□), *P. fluorescens* 14(pKT240-*cry*) (▨), and *P. fluorescens* 14::Omegon-Km-*cry* (■) against *E. saccharina* larvae. Results are means of three replicates. Bars above the histograms represent SDs.

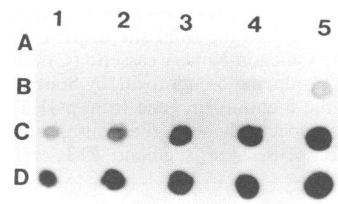

FIG. 8. Hybridization of dot-blotted DNA to a ^{32}P-labelled 3.7-kb *Nde*I fragment bearing the *cryIA*(*c*) δ-endotoxin gene from isolate 234. Rows: A, negative control strain *P. fluorescens* 14; B, *P. fluorescens* 14::Omegon-Km-*cry*; C, *P. fluorescens* 14(pDER405-*cry*); D, *P. fluorescens* 14(pKT240-*cry*). Columns 1 to 5 represent decreasing amounts of dot-blotted total DNA (250, 125, 62.5, 31.25, and 15.6 ng, respectively).

Nearly all of the bacteria isolated from sugarcane were found to be *Pseudomonas* strains, and *P. fluorescens* isolate 14 colonized sugarcane effectively. Plasmids pDER405, pKT240, and pSUP204 were considered as potential vectors for the introduction of the *cry* gene into isolate 14. However, the former two were chosen because of the plasmid stability of pDER405 and the higher copy number of pKT240 (13 and 28, respectively).

As horizontal spread of the *cry* gene could occur when it is carried on a mobilizable plasmid, we integrated the gene into the chromosome. Other workers have integrated *cry* genes into root-colonizing pseudomonads and *Agrobacterium radiobacter*, using a transposon Tn*5*-mediated system (28, 29), or by integration dependent on recombination between homologous DNA sequences (41, 45). In one study on the stability of the *cry* gene integrated by the latter mechanism, the gene was lost in planta approximately 40 weeks after inoculation and in vitro after 50 to 60 days of growth under nonselective conditions (41).

We used the artificially generated interposon Omegon-Km (10) to integrate the *cry* gene into the chromosome of isolate 14. The Omegon module consists of the Ω interposon, flanked with synthetic inverted 28-bp ends of IS*1*, which can transpose if IS*1* gene products are supplied. Omegon-Km is carried on plasmid pJFF350, which has an origin of transfer allowing mobilization into gram-negative bacteria. The disabled IS*1* element on pJFF350 cannot itself transpose but enables transposition of the Omegon-Km module. Thus, *P. fluorescens* carrying the *cry* gene in the chromosome is stably *cry*$^+$.

Southern blot analysis of isolate 14 carrying the *cry* gene integrated into the chromosome showed that the gene could be integrated at single sites. It was of interest that a strain carrying the integrated gene was as toxic to *E. saccharina* as a strain carrying the gene on pKT240 was, despite the fact that the copy number of pKT240 in isolate 14 is 28. It is possible that the increased expression of the *cry* gene integrated into the chromosome was due to the deletion of 1.4 kb of DNA 5′ to the gene which occurred during the cloning of the 3.7-kb *Nde*I *cry* fragment into pJFF350. Schnepf et al. (36) found that a region of *B. thuringiensis* DNA located between 87 and 258 bp upstream from the transcription initiation site of a *cryIA*(*a*) gene caused reduced transcription in *E. coli*. This regulatory sequence was AT rich (82%) and contained a region of dyad symmetry between bases −258 and −176 (35), although the significance of these features is not known. Two AT-rich regions of dyad symmetry occur upstream of the *Nde*I site of the isolate 234 *cry* gene and were removed during the subcloning into pJFF350. Thorne et al. (39) also found that the deletion of more than 70 bp upstream from a similar *cry* gene resulted in a 20- to 50-fold increase in the accumulation of toxin in *E. coli*.

Support for our hypothesis that increased expression after integration into the chromosome is due to the deletion of the upstream region comes from a previous experiment in which we cloned the entire 6.7-kb *Bam*HI fragment carrying the *cry* gene and the upstream region into pJFF350 and integrated it into the chromosome of isolate 14. No detectable toxin was found upon Western blot analysis (data not shown).

Glasshouse trials to determine whether *P. fluorescens* 14::Omegon-Km-*cry* could protect sugarcane from eldana larvae showed that there was a decrease in the presence of larvae and consequent damage of approximately 60% after 4 weeks compared with the control strain. These results are promising. A further improvement to the biocontrol strain, in which the *cry* gene will be cloned downstream of the efficient *tac* promoter (13) and the construct will be introduced into the

TABLE 2. Eldana damage to sugarcane[a]

Treatment	No. of eldana larvae/pot[b] (mean ± SD)	% of internodes damaged/pot (mean ± SD)	No. of eldana larvae/stalk (mean ± SD)	% of internodes damaged/stalk (mean ± SD)
None	3.6 ± 1.3	33.5 ± 8.6	2.2 ± 0.8[c]	30 ± 7.3
P. fluorescens 14::Omegon-Km-*cry*	0.9 ± 0.5	10.9 ± 4.5	0.6 ± 0.2[d]	12.5 ± 4.9

[a] Bacterial suspensions were sprayed onto 6-month-old sugarcane plants 2 weeks before inoculation with *E. saccharina* eggs. Stalks were sampled 4 weeks later.
[b] Twenty-seven pots used for each treatment.
[c] Fifty-eight stalks tested.
[d] Forty-six stalks tested.

chromosome, is under way. In addition, the potential of an obligate sugarcane endophyte, *Acetobacter diazotrophicus* (7), as a recipient for the *cry* gene is being investigated.

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