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Recombinant cells of *Pseudomonas fluorescens*: a highly palatable encapsulation for delivery of genetically engineered toxins to subterranean termites (Isoptera: Rhinotermitidae)

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J. K. GRACE AND D. M. EWART. 1996. In the CellCap® process (Mycogen Corp., San Diego, CA), recombinant cells of the bacterium *Pseudomonas fluorescens* are induced to express the δ-endotoxin of *Bacillus thuringiensis* (Bt), then killed and fixed to encapsulate the δ-endotoxin. Two commercial agricultural formulations prepared by the CellCap® process were evaluated for palatability to the termite *Coptotermes formosanus*. The MVP® formulation, active against Lepidoptera, contained the *Ps. fluorescens*-encapsulated δ-endotoxin of Bt var. *kurstaki*. The M-Trak™ formulation, active against Coleoptera, contained the δ-endotoxin of Bt var. *san diego*. Papers treated with either formulation at concentrations as great as 1 g cm^{-3} were readily fed upon by *C. formosanus*. As expected, the two formulations tested were not significantly toxic to the termites, both having optimal activity at a pH range outside that of the termite gut. The palatability of the CellCap® formulations indicates that the host bacterium, *Ps. fluorescens*, is a suitable delivery system for genetically engineered termiticides.

INTRODUCTION

Bacillus thuringiensis Berliner (Bt) is a Gram-positive soil bacterium that produces insecticidal proteins. Numerous strains (also termed varieties or subspecies) of Bt have been isolated and cultured. These strains produce potent and specific proteins toxic to many different insects (Feitelson 1993; Tanada and Kaya 1993). As a result of this insecticidal activity and the ease with which Bt can be mass cultured, formulations containing different Bt strains have been used as microbial pesticides for many years.

Khan *et al.* (1977) isolated a strain of Bt from the termite *Bifiditermes beesoni* (Gardner) (Isoptera: Kalotermitidae). In laboratory studies, this isolate and other strains of Bt have been reported to be toxic to various termite species (Smythe and Coppel 1965; Khan *et al.* 1985). However, the instability of Bt in the exterior environment and soil (Leong *et al.* 1980; Bryant 1994) has been cited as limiting its potential for use in termite control (Logan *et al.* 1990). Another limiting factor in development of Bt formulations for termite control and other pest control applications is the requirement for ingestion of Bt toxins by the target insects.

The first insecticidal protein found in Bt was the delta-endotoxin (δ-endotoxin). This is considered to be the most widely studied of all entomocidal toxins (Tanada and Kaya 1993). The term 'δ-endotoxin' actually refers to the group of related crystal proteins (Cooper 1994). This and other Bt toxins have been the subjects of molecular engineering efforts to introduce toxin genes into plant tissues and to produce more environmentally-stable insecticide formulations (Feitelson 1993). An example of the latter effort is the CellCap® encapsulation process developed by Mycogen Corp. (San Diego, CA). In this process, a gene coding for the δ-endotoxin protein is removed from the Bt cell and incorporated into a plasmid. The plasmid is then inserted into the Gram-negative leaf-colonizing bacterium *Pseudomonas fluorescens* (Trevisan) Migula (Gaertner *et al.* 1993; Panetta 1993). *Pseudomonas fluorescens* was originally selected for this purpose due to its sensitivity to tetracycline and lack of pathogenicity to animals and plants (Gaertner *et al.* 1993). The recombinant *Ps. fluorescens* cells are grown in aerobic culture and induced to express the δ-endotoxin. The biotoxin-containing cells are then killed by addition of a fixative to the fermentation broth (Panetta

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1993). This fixation process results in both strengthening of the *Ps. fluorescens* cell walls and inactivation of proteolytic enzymes that can degrade the toxin (Panetta 1993). The fixed bacterial cells encapsulate and protect the δ-endotoxin from degradation without the possibility of dispersal or transfer of genetic material that would be associated with the use of a microbial pesticide containing live transgenic bacteria.

The search for effective bait toxin delivery systems is currently the most active area of subterranean termite control research. There are three basic approaches to toxin delivery. The first is aggregation or collection of foraging termites and topical application of the toxin to their cuticle. A second approach uses baits containing non-repellent or undetectable toxins. The third approach involves encapsulation of otherwise detectable or repellent insecticides. Encapsulation of otherwise repellent insecticides has been successful against agricultural pests and has been tried against termites (Schoknecht *et al.* 1994). There must be no leakage from the microcapsules and the toxin must not be released during the feeding process, but only upon ingestion. Clathrated toxins that are only released by the digestive process are reported to have shown promise (French 1994), but these have yet to be commercially exploited.

Efforts to develop termite baits have ignored the potential of genetically engineered organisms. The CellCap® system has been used by Mycogen to produce two formulations of *Ps. fluorescens* encapsulated Bt δ-endotoxin. These formulations are MVP®, which contains the δ-endotoxin of Bt var. *kurstaki* with activity against Lepidoptera, and M-Trak™, which contains the δ-endotoxin of Bt var. *san diego* with activity against Coleoptera (Panetta 1993). This encapsulation system has the potential to handle a wide variety of endotoxins well beyond the limited range currently in use. Formulations based upon the expression and encapsulation of Bt toxins active against other insects are in development (Feitelson 1993; Gaertner *et al.* 1993).

The present study was conducted to assess the palatability of the MVP® and M-Trak™ formulations to Formosan subterranean termites, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). The preference of subterranean termites for decayed substrates (Hendee 1934) indicates that they must regularly be consuming bacteria as a normal component of their diet (Levy *et al.* 1974; Margulis *et al.* 1986). This suggested that *C. formosanus* would accept the leaf-inhabiting *Ps. fluorescens* as an acceptable food component.

MATERIALS AND METHODS

Two concentrated CellCap® bio-insecticide formulations containing fixed recombinant *Ps. fluorescens* cells, MVP® (2117950) and M-Trak™ (4512782), were supplied to us by Mycogen Corp., San Diego, CA. The MVP® formulation contained 20% biomass (non-soluble components) and 11 418 mg of δ-endotoxin per ml. The M-Trak™ sample contained 13·5% biomass and 15 800 mg of δ-endotoxin per ml.

Aliquots of each CellCap® concentrate were diluted with distilled water to obtain solutions having δ-endotoxin concentrations of 1, 0·5, 0·1, 0·01 or 0·001 g cm^{-3}. Distilled water alone was used as a control. A Whatman filter paper disk was saturated with 0·2 ml of the test solution, placed in a glass Petri dish with 40 *C. formosanus* workers (undifferentiated individuals older than the third instar, as determined by size), and incubated for 14 d at 28°C in a dark, temperature-controlled cabinet. Termites were collected from an active field colony on the Manoa campus of the University of Hawaii immediately before their use in laboratory assays, using a collection technique described by Tamashiro *et al.* (1973). There were five replicates for each concentration of the two CellCap® formulations.

At the end of the 14-d exposure period, termite mortality was recorded, and the amount of filter paper consumed by the termites was estimated by placing the remaining portion of each disk over a template containing a grid of dots and counting the number of visible dots. A similar method of estimating termite feeding by measuring the surface area removed from paper disks was employed by Grace *et al.* (1986). Proportional paper removal and proportional mortality data were transformed by the arcsine of the square root, and subjected to analysis of variance (ANOVA, $\alpha \leqslant 0{\cdot}05$) (SAS Institute 1987).

RESULTS AND DISCUSSION

Termites fed readily on the papers treated with MVP® and with M-Trak™. No statistically significant decline in feeding was observed among the concentrations offered (Table 1). The highest surface concentration of MVP® to which the termites were exposed in this test exceeded the amount found necessary for complete control of diamondback moth in field studies by 160-fold (Gaertner *et al.* 1993). Thus, there was no evidence of any feeding deterrence induced by the presence of even relatively high concentrations of *Ps. fluorescens* cells on the surface of the filter papers. The palatability of the CellCap® formulations noted in this 14-d assay confirmed our qualitative observations of termite feeding at these same dosages during a preliminary 7-d exposure.

As expected, at least up to the maximum dosage tested, neither the δ-endotoxin of *B. thuringiensis* var. *kurstaki* nor that of *B. thuringiensis* var. *san diego* were toxic to the Formosan subterranean termite. However, there may be great potential for recombinant application of strains of Bt known to infect termites (Smythe and Coppel 1965; Khan *et al.* 1985) or, in a more indirect attack, those active against protozoa (Feitelson 1993) as bait toxicants for subterranean termites.

The palatability of the two formulations containing fixed

Table 1 Feeding and mortality of Formosan subterranean termites exposed for 14 d to filter papers saturated with formulations of *Bacillus thuringiensis* (Bt) δ-endotoxin encapsulated within fixed recombinant *Pseudomonas fluorescens* cells

CellCap® formulation	δ-endotoxin source (activity)	δ-endotoxin concentration (g cm^{-3})*	% paper eaten (S.D.)†	% termite mortality (S.D.)†
MVP®	Bt var. *kurstaki* (Lepidoptera)	1	56·4 (25·5)	16·0 (7·2)
		0·5	67·6 (13·1)	24·5 (8·7)
		0·1	56·4 (25·5)	17·0 (2·1)
		0·01	69·2 (11·5)	21·0 (10·7)
		0·001	66·4 (9·2)	25·5 (19·6)
M-Trak™	Bt var. *san diego* (Coleoptera)	1	66·4 (25·1)	14·0 (11·0)
		0·5	62·4 (29·8)	15·0 (13·7)
		0·1	66·8 (18·6)	10·0 (5·3)
		0·01	66·4 (17·2)	9·0 (5·2)
		0·001	50·4 (14·4)	9·0 (4·5)
Water controls		0	53·2 (21·8)	12·5 (3·5)

* Paper disks were saturated with 0·2 ml of aqueous solution.
† Means within columns do not differ significantly (ANOVA, $\alpha \leqslant 0{\cdot}05$).

Ps. fluorescens cells is a significant and encouraging result. Delivery of a toxicant or biological control agent in a matrix acceptable to foraging termites is a difficult and critical step in developing baits for termite control (cf. Delate *et al.* 1995). For example, the cyclic peptide destruxin E produced by the fungal pathogen *Metarhizium anisopliae* (Metsch.) Sorokin causes slow and steadily increasing mortality in termites forced to feed on this toxin (Grace 1995), but was found to be too repellent to be practical as a bait toxicant. However, if such microbial toxins could be expressed by recombinant bacteria and encapsulated by the CellCap® process, repellency would no longer be an issue. It is hoped that the results of this experiment will encourage others to subject more promising strains of Bt to this process, or to pursue other simply coded inclusions.

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