Studies on *Bacillus thuringiensis* strains isolated from Swedish soils: insect toxicity and production of *B. cereus*-diarrhoeal-type enterotoxin

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At moderate concentration, 23 of 40 strains of *Bacillus thuringiensis* isolated from Sweden were toxic to *Trichoplusia ni* and five were toxic to *Aedes aegypti*. Five of the strains were toxic to *Diabrotica undecimpunctata* at high concentration, two were toxic to *Heliothis virescens* at low concentration and five produced thuringiensin (formerly called β-exotoxin). No strain was toxic towards the beet armyworm *Spodoptera exigua* at low concentration. Twenty-three of the strains produced a *B. cereus*-diarrhoeal-type enterotoxin.

**Key words:** *Bacillus cereus*-diarrhoeal-type enterotoxin, *Bacillus thuringiensis*, Coleoptera, Diptera, Lepidoptera, thuringiensin.

Development of resistance in insects to most, if not all, chemical insecticides, together with increasing environmental consciousness has resulted in an intensified search for alternative insect-control agents. Among biological-control agents for insects, *Bacillus thuringiensis* is an ideal organism for large-scale commercial production (Lüthy et al. 1982). During sporulation, *B. thuringiensis* produces protein inclusions (called δ-endotoxin) which kill larvae of various Lepidoptera (Heimpel 1967; Angus 1968), Diptera (De Barjac 1978; Abdel-Hameed et al. 1990) and Coleoptera (Krieg et al. 1983; Herrnstadt et al. 1986). In addition, some *B. thuringiensis* strains produce thuringiensin (formerly called β-exotoxin) which has potential in controlling flies (Diptera) (Carlberg 1986). These findings have led to the commercial development of *B. thuringiensis* preparations for use in controlling both agricultural pests and vectors of human and animal disease. Such preparations have been effectively used to control the target insects, with no adverse effects on the environment (Lüthy 1980). However, Carlson & Koletz (1993) recently showed that some strains of *B. thuringiensis* produce a protein that cross-reacts with antibodies against *B. cereus*-diarrhoeal-type enterotoxin. This emphasises the need to screen *B. thuringiensis* strains carefully for use as insecticides. However, considering the everyday use of *B. thuringiensis* insecticides and the sequence of events necessary for *B. cereus* to cause food poisoning (i.e. holding food for long periods at temperatures where growth and enterotoxin production occur), the likelihood of an enterotoxin-producing strain of *B. thuringiensis* causing food poisoning is remote.

Landén et al. (1994) assessed the distribution, frequency and diversity of *B. thuringiensis* in Southern Sweden. As a result of this survey, 40 strains of *B. thuringiensis* were isolated. The present study is on the qualitative toxicity of these strains against various insect species and on their production of *B. cereus*-diarrhoeal-type enterotoxin.

**Materials and Methods**

**Bacteria and Growth Media**

The 40 strains used, isolated from soils of Southern Sweden by Landén et al. (1994), were classified into the different biochemical groups of *B. thuringiensis* according to Martin & Travers (1989). The strains were maintained as lyophilized cultures and subcultured routinely on L-agar slants composed of (g/l):
tryptone (Difco), 10; yeast extract (Difco), 5; NaCl, 5; and agar (Difco), 15. LB-medium (L-agar without the agar), Brain Heart Infusion (Oxoid, UK) and modified Conner & Hansen's medium, [composed of (g/l): KH₂PO₄, 5.0; K₂HPO₄, 5.0; MgSO₄·7H₂O, 0.05; MnSO₄·H₂O, 0.03; FeSO₄·7H₂O, 0.04; CaCl₂·7H₂O, 0.05; Na₂(H₂)PO₄·4H₂O, 1.5; glucose, 1.0; and Casamino acids (Difco), 10] were used as appropriate. All media were adjusted to pH 7 before sterilization.

Insects and Bioassays

Trichoplusia ni was reared according to Shorey & Hale (1965), except that formaldehyde, ascorbic acid and inhibitor solution were omitted from the synthetic food. The B. thuringiensis were grown on a rotary shaker at 180 rev/min at 30°C in 250-ml Erlenmeyer flasks, each containing 30 ml LB-medium, until sporulation and cell lysis were essentially completed (4 to 5 days). Growth, sporulation and cell lysis were monitored using phase-contrast microscopy and production of the parasporal inclusions was tested by the crystal-sampling method of Swift and Swift (1962). Cultures were centrifuged at 2000 g for 5 min and the pellets were pooled and resuspended in LB-medium to give 10⁶ c.f.u./ml. Three ml of this suspension were mixed with 30 ml of the whole culture broth was mixed with a suitable artificial diet in microtitre plates with V-shaped wells. For the toxicity tests against mosquitoes, the bacteria were grown on modified Conner & Hansen's medium, [composed of (g/l): KH₂PO₄, 5; K₂HPO₄, 5; MgSO₄·7H₂O, 0.05; MnSO₄·H₂O, 0.03; FeSO₄·7H₂O, 0.04; CaCl₂·7H₂O, 0.05; Na₂(H₂)PO₄·4H₂O, 1.5; glucose, 1.0; and Casamino acids (Difco), 10] were used as appropriate. All media were adjusted to pH 7 before sterilization.

Results and Discussion

Toxicity against Different Insect Species

Of the 40 isolates tested, 23 showed toxicity against T. ni (Lepidoptera) larvae and five of these were also toxic to A. aegypti fourth-instar larvae (Table 1). Four of the five strains toxic to the mosquitoes were confirmed to be B. thuringiensis H-14 strains by serotyping. Toxicity to mosquitoes and T. ni is not one of the recognised features of B. thuringiensis H-14 strains; the present conflicting results were probably due to differences between the assay system used for T. ni in the present study and other conventional commercial assays. More spores were used and inhibitors of bacterial growth were omitted from the food recipe in the present study. Accordingly, the spores germinated in the food, producing other virulence factors beside the δ-endotoxin and these may have affected the toxicity results (Lövgren et al. 1990). This assay was chosen for the initial screening to ensure that strains with other specificities would not be inadvertently missed.

For toxicity tests against the tobacco budworm, H. virescens (Lepidoptera), and the beet armyworm, S. exigua (Lepidoptera), standardized food recipes including growth inhibitors were used. The results revealed that two of the 23 strains showed potent activity against T. ni, were also toxic to the larvae of H. virescens (Table 1) but none of the 40 isolates tested showed toxic activity against S. exigua. The small number of strains apparently active against H. virescens and S. exigua may be largely due to the low concentration of bacteria used; in addition, the assays used probably only revealed the toxicity caused by the δ-endotoxin.

Five strains were toxic to the southern corn rootworm D. undecimpunctata (Coleoptera) at high concentration (Table 1). Of these five, three were also toxic to T. ni (Lepidoptera). One of these three (strain 3:2 C) showed potent toxic potential activity against both insects. Preliminary results showed that this strain is an exceptionally potent one. Further studies are being carried out to determine its activity spectrum against other coleopteran and lepidopteran species.

Assays for enterotoxin were made using a B. cereus-diarrhoeal-type enterotoxin reversed-passive latex agglutination kit (B.C.E-1RPLA; Oxoid, UK), in microtitre plates with V-shaped wells. For each strain, dilutions of the culture filtrate were made in two rows. The first well of each row contained 25 µl of the undiluted culture filtrate, the second a 1:2 dilution, the third a 1:4 dilution and the fourth 25 µl of the diluent. Anti-enterotoxin-sensitized latex suspension was added to each well in the first row (25 µl/well) and the control latex was added to each well in the second row (25 µl/well). The contents of each well were mixed and the covered plates were kept at room temperature for 24 h. The plates were then examined for agglutination against a black background.

Production of B. cereus-diarrhoeal-type Enterotoxin

A loopful from a slant culture was used to inoculate 5 ml of Brain Heart Infusion in a 50-ml culture flask (Nuncow, Denmark). The cultures were grown on a rotary shaker at 180 rev/min at 37°C for 18 h, centrifuged, filtered through 0.2-mm pore low-protein-binding filters, and the resultant filtrates tested for enterotoxin.