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Effect of a nematicidal *Bacillus thuringiensis* strain on free-living nematodes. 3. Characterization of the intoxication process

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Summary – The toxicity of *Bacillus thuringiensis* is temperature sensitive. Incubation of *Caenorhabditis elegans* with nematicidal *B. thuringiensis* strains at 16, 20, and 25 °C shows that toxicity decreases as temperature declines. At 16 °C, toxicity is completely lost, while it is maximal at 25 °C. Toxicity is pH sensitive and is significantly reduced when nematodes are incubated with the weak bases NH_4Cl, chloroquine, acridine orange, methyl red, and neutral red. Based on these results, we proposed the hypothesis that the nematicidal factor is effectively internalized into the intestinal cells, a sharp deviation from the insecticidal *B. thuringiensis* toxins acting at the level of the brush border membrane. Although the absence of purified toxin prevents a more definitive elucidation of the mode of action, the results of this third and final part of this series of publications convincingly indicate that nematicidal *B. thuringiensis* do not hold the same promise as a biological control agent as the insecticidal *B. thuringiensis* strains.

Résumé – ***Action d'une souche nématicide de* Bacillus thuringiensis *sur les nématodes libres. III. Caractéristiques du processus d'intoxication*** – La toxicité de *Bacillus thuringiensis* est fonction de la température. L'incubation de *Caenorhabditis elegans* avec des souches de *B. thuringiensis* à 16, 20 et 25 °C montre que la toxicité décroît en même temps que la température. A 16 °C, la toxicité disparaît complètement, tandis qu'elle atteint son maximum à 25 °C. La toxicité, fonction du pH, diminue significativement lorsque les nématodes sont mis en incubation dans des bases faibles (NH_4Cl, chloroquine, acridine orange, rouge de méthyle, rouge neutre). A partir de ces résultats, il est possible d'avancer l'hypothèse que l'agent nématicide pénètre à l'intérieur des cellules intestinales, ce qui constitue une différence notable avec les toxines des souches insecticides de *B. thurengiensis* lesquelles agissent au niveau de la membrane en brosse. Bien que l'absence de toxine purifiée ne permette pas l'élucidation définitive de son mode d'action, les résultats exposés dans cette troisième, et dernière, partie de la série de publications traitant du sujet, apportent une indication convaincante du fait que les souches nématicides de *B. thurengiensis* ne peuvent tenir les mêmes promesses que les souches insecticides en tant qu'agent de contrôle biologique.

Key-words : *Bacillus thuringiensis*, *Caenorhabditis elegans*, mode of action, nematodes.

In two previous reports a detailed description was provided of the effect of nematicidal *B. thuringiensis* strains towards several nematodes and a detailed ultrastructural analysis was presented of the initial intoxication process in the nematode *Caenorhabditis elegans* (Borgonie *et al.*, 1996 *a, b*). Both studies indicated that there are large differences between the effect of *B. thuringiensis* on insects and on nematodes.

To compare the nematicidal and insecticidal mode of action of nematicidal *B. thuringiensis* strains, nematode intestinal cells were stained (Borgonie *et al.*, 1995) and the nematodes were incubated with toxic spore-crystal mixture. It was originally thought that the nematicidal mode of action would be identical to the insecticidal one in that the toxin would result in cell membrane pore formation, osmotic imbalance, and rupture (Knowles & Dow, 1993). If so, it was believed that the stain (present in the nematode intestinal cells) would be released and this possibility could be observed. Surprisingly, toxicity was lost after such treatment. Therefore, in this third part, factors influencing the intoxication process of three nematicidal *B. thuringiensis* strains against *C. elegans* (Leyns *et al.*, 1995) are characterized. Evaluation of *B. thuringiensis* as an effective nematicidal agent is discussed.

Materials and methods

Preparation of spore-crystal mixtures of *B. thuringiensis*, was as described earlier (Borgonie *et al.*, 1996 *a, b*).

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NEMATODES

The free-living nematode *Caenorhabditis elegans* var. Bristol was used. Monoxenic and axenic culture and the toxicity assays were done as described earlier (Borgonie *et al.*, 1996 *a, b*).

INCUBATION OF NEMATODES WITH WEAK BASES

Nematodes were washed off agar plates, rinsed three times in PBS, pH 7.2 and incubated in either acridine orange (Sigma), methyl red (Merck), neutral red (Merck), NH_4Cl, or chloroquine (Sigma). Acridine orange (AO), neutral red (NR) and methyl red (MR) were dissolved in PBS and sonicated extensively to hasten solubilization. Insoluble material was removed by centrifugation at 10 000 rpm for 1 min in a centrifuge. 50 µl of a mixed nematode culture was incubated with 50 µl of dye solution. The final concentrations were 10^{-3} % for AO and NR and 2.5 % for MR (concentrations are approximate since the small amounts lost during centrifugation were neglected). NH_4Cl and chloroquine were also dissolved in PBS and administered at final concentrations of 10 mM and 5 mM respectively (concentrations experimentally deduced). Incubation was done at 25 °C and lasted for 24 h. After this pre-incubation, the nematodes were washed three times in PBS and separated in two aliquots. One was then incubated with *B. thuringiensis* spore-crystal mixtures in the presence of each of the dyes or bases described above, at the given concentrations. The second aliquot was incubated with *B. thuringiensis* spore-crystal mixtures without additions. All incubations were at 25 °C; mortality was assayed after 24 h. Controls were performed using incubation without spore-crystal mixture in order to determine adverse effects.

Results

pH SENSITIVITY

Using pre-incubation with the weak bases acridine orange, methyl red, neutral red, NH_4Cl, and chloroquine, it was shown that toxicity was lost after such treatment (Figs 1, 2). The protective action was nearly completely reversible when NH_4Cl and chloroquine were removed from the incubation medium before spore-crystal mixture was added (Fig. 2). In the case of the vital stains the inhibition was irreversible (Fig. 1). Control experiments indicated that no mortality was caused by these substances. Feeding was periodically checked by observing pumping of the basal bulb.

TEMPERATURE SENSITIVITY

The temperature sensitivity was assayed by performing intoxication at three different temperatures (16, 20, 25 °C), which are all within the range of temperatures for *C. elegans* activity (16-25 °C). Toxicity progressively decreased as temperature declined (Fig. 3). If temperature was allowed to rise above 20 °C, after incubation at

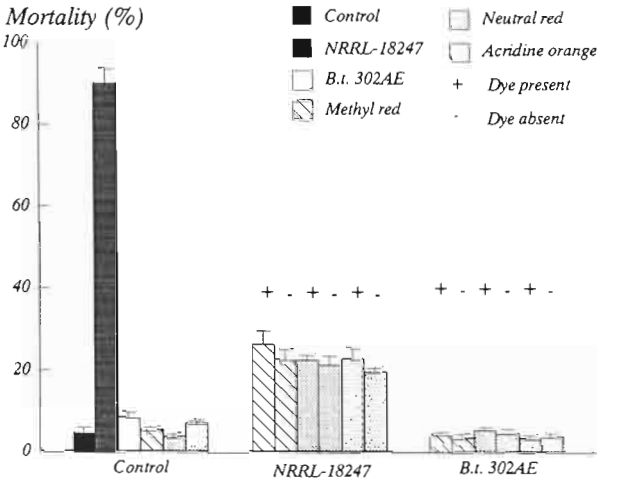

Fig. 1. *Influence of pre-incubations of young adult* Caenorhabditis elegans *with three different dyes on toxicity. Note that the protective effect remains present even in the absence of dye.*

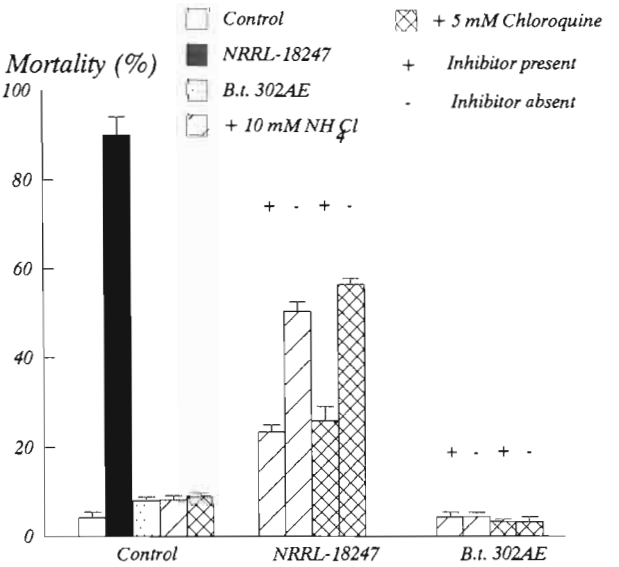

Fig. 2. *Influence on toxicity of pre-incubations of young adult* Caenorhabditis elegans *with weak bases. In contrast to Fig. 1, the protective effect is removed when the weak bases are absent. However, toxicity is not fully restored.*

16 °C, toxicity was restored (Fig. 4). Moreover, if nematodes were incubated with the toxic spore-crystal mixture for 24 h at 16 °C, followed by 4 h at 16 °C in PBS free of *B. thuringiensis* in order to allow removal of leftover spores and crystals from the nematode intestine, the mortality was partially restored if the incubation temperature was subsequently shifted to 25 °C for 24 h (Fig. 4).

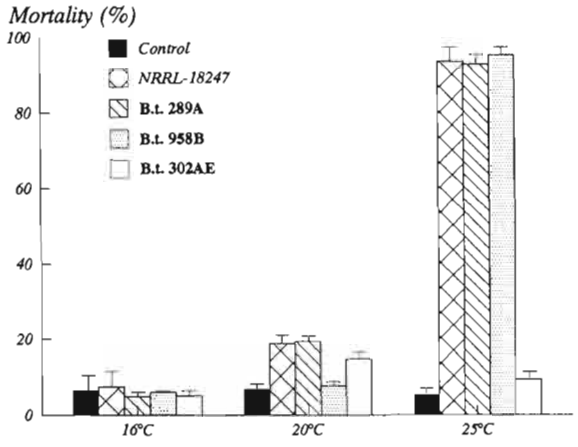

Fig. 3. *Temperature sensitivity of the intoxication process using three nematicidal and one non-nematicidal* Bacillus thuringiensis *strain on young adult* Caenorhabditis elegans.

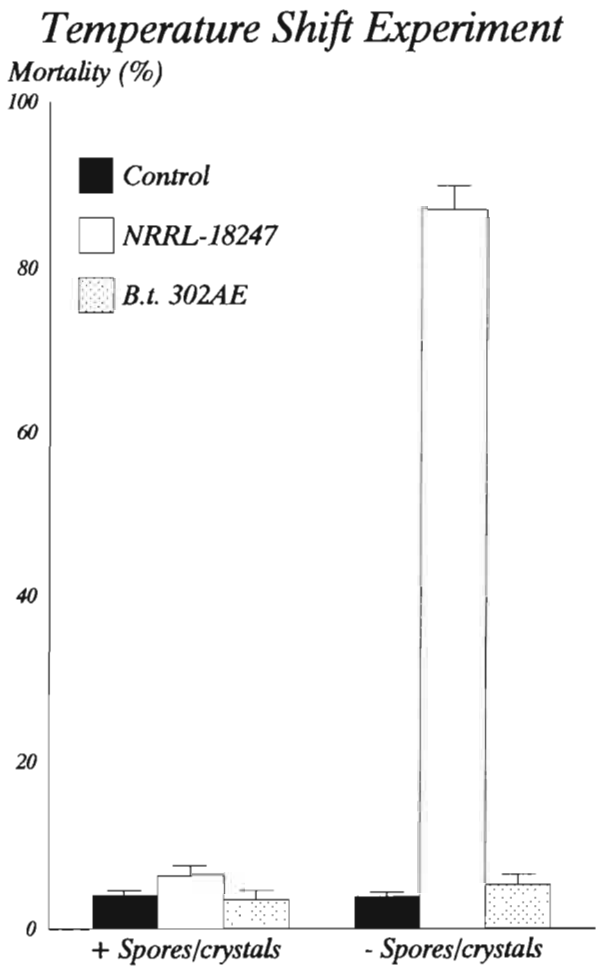

Fig. 4. *Effect of temperature shift on toxicity. Young adult* Caenorhabditis elegans *were first incubated with nematicidal strain NRRL-18247 for 24 h at 16 °C (A-C), then washed free of spores and crystals, and incubation temperature was shifted to 25 °C for 24 h after which mortality was determined.*

Discussion

Inhibition experiments

Experiments intended to determine the degree of similarity between the mode of action of the nematicidal toxic factor on nematodes and the insecticidal activity have unexpectedly revealed consistent, significant differences. Temperature shifts and weak bases have been used extensively in cell biology to inhibit intracellular transportation. Figure 5 gives a schematic overview of the sites of inhibition under consideration.

The use of vital stains was originally intended to monitor the stain as the intoxication process proceeded. Surprisingly toxicity was lost after such incubation. Incubation with weak bases resulted in significantly reduced toxicity as long as the weak bases remained present when a spore-crystal mixture was added. Incubation of cells with weak bases increases the pH of the endocytotic vesicle and has a variety of effects on receptor-mediated endocytosis (Maxfield, 1982). Effects reported include reduced rates of endocytosis (Maxfield *et al.,* 1979; Haigler *et al.,* 1980), inhibition of receptor recycling (Gonzalez-Noriega *et al.,* 1980; Tietze *et al.,* 1980, 1982), recycling of ligand-receptor complexes to the cell surface (Tietze *et al.,* 1980), and inhibition of toxicity of *Pseudomonas* exotoxin A (Olsnes *et al.,* 1991), diphtheria toxin (Mekada *et al.,* 1981; Sandvig & Olsnes, 1982; Olsnes *et al.,* 1991), and modeccin toxin (Sandvig & Olsnes, 1982).

Since it has been reported (Olliver-Bousquet, 1980; Antoine *et al.,* 1985) that chloroquine and NH_4Cl have wider effects than on endocytosis alone, it cannot be excluded that the loss of toxicity was due to factors other than neutralising pH (e.g., influence on gut physiology). Nevertheless, the use of the vital stains and acridine orange, also a weak base, makes this alternative explanation less likely. Several authors studied the influence of vital stains using TEM and reported that vital stains are found solidly associated with membranes of acidic vesicles (Canonico & Bird, 1961; Robbins *et al.,* 1964). The fact that the stain remained clearly visible in the intestinal cells of nematodes up to several days after nematodes had been rinsed was reported previously (Doncaster & Clark, 1964; Clokey & Jacobson, 1986) and supports a close association. Toxicity tests performed three days after the nematodes had been rinsed free of the stain also showed considerable loss of sensitivity to the spore-crystal mixture, thereby reducing the possibility that lumen related factors other than endocytotic vesicle pH might have been important.

Temperature shifts have been used in studies of the mode of action of toxins such as diphtheria (Draper &

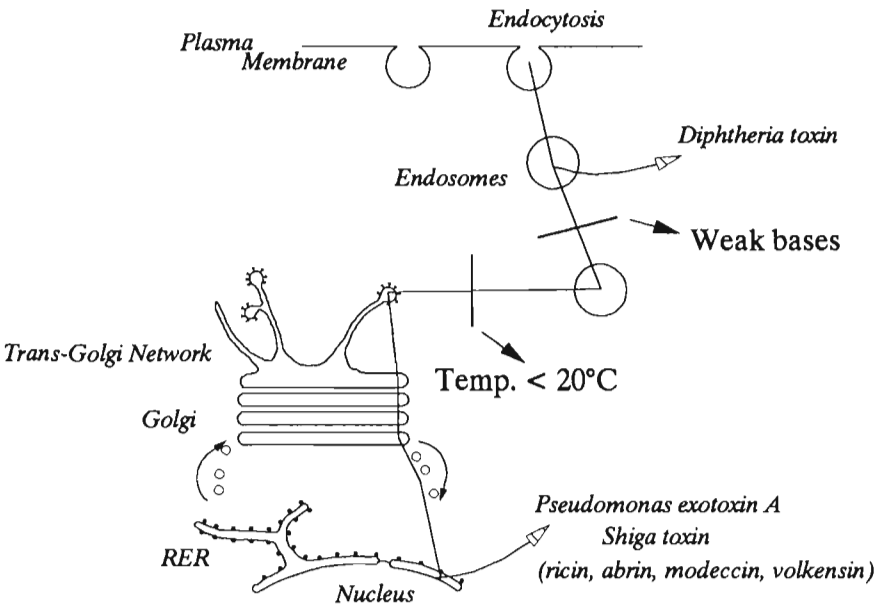

Fig. 5. *Schematic representation of the intracellular transport of several bacterial (diphtheria,* Pseudomonas *and Shiga toxin) and plant (ricin, abrin, modeccin, volkensin) toxins. The sites of interference of temperature and weak bases are indicated. The weak bases interfere by neutralizing the acid environment in intracellular organelles, a condition required by several toxins. Temperature decrease inhibits transport from late endosomes onward. However, the underlying mechanism of that inhibition is not yet understood.*

Simon, 1980; Sandvig & Olsnes, 1980), modeccin (Sandvig *et al.*, 1984), and ricin (Sandvig & Olsnes, 1979; Van Deurs *et al.*, 1987), and in a study of intracellular endocytotic pathways of internalized receptors or ligands (Dunn *et al.*, 1980). Although the underlying mechanism is not fully understood, it is assumed that a temperature lower than 20 °C causes an inhibition of vesicular transport at the level of the late endosome (Dunn *et al.*, 1980). It is striking that the intoxication process in *C. elegans* follows this pattern. Massive toxicity is observed at 25 °C, it is fluctuating at 20 °C, and a nearly complete protection is observed at 16 °C. This inhibition can be reversed by allowing the temperature to rise above 20 °C. The observation that, in the absence of spores and crystals after incubation at 16 °C, mortality can still be elicited by shifting the temperature to 25 °C strongly supports a temperature block being present.

Relevance of the inhibition experiments

Although it is tempting to conclude that loss of toxicity could be due to transport inhibition, too little is known about endocytosis and related transport pathways in nematodes to extrapolate to nematodes results obtained mostly with vertebrate cell lines. Therefore, the possibility of a more insect-like mode of action cannot be entirely excluded, i.e., a reduced fluidity of the cell membrane at 16 °C preventing toxin monomers diffusing in the membrane to form an oligomeric pore.

For some toxins, the observation of a time-lag reflects the time that toxin needs to reach its intracellular target. Although a time-lag is clearly present in the intoxication of the nematodes with nematicidal *B. thuringiensis* strains (Borgonie *et al.*, 1996 *b*), another possible explanation could be the result of suboptimal physiological conditions present (pH, ion concentration, etc.) in the intestinal lumen for the release/processing of the toxin, a phenomenon which does not necessarily indicate endocytosis.

However, we feel that the characteristics of the nematicidal activity deviate too much from the insecticidal one and, in our opinion, reflect a mode of action fundamentally different to that in insects.

Wider implications

The mode of action results reported here pertain to a free-living nematode and it remains to be determined to what extent these findings are applicable to the economically important plant-parasitic nematodes. Assuming that *B. thuringiensis* toxins could be successfully delivered to the gut of plant-parasitic nematodes, several potential problems are identified that might reduce the application efficiency of *B. thuringiensis* based toxin products to fight nematodes. First, the temperature sensitivity of the intoxication might considerably reduce the

areas of the world where such products can be used. Second, the continued success of the microbial nematicide *B. thuringiensis* will, at least in part, depend on the extent to which nematodes can develop resistance. The identification of resistant nematode strains does not necessarily indicate the potential for the emergence of resistant nematode strains in the field, which will be mostly dependent on the residual activity of *B. thuringiensis* based toxins. Since plant-parasitic nematodes only feed from inside the plant cells, transgenic plants expressing *B. thuringiensis* toxins will have to be used. Foliar applications (sprays), as used for insect applications, will have no effect whatsoever on nematodes. Therefore, the continued expression of *B. thuringiensis* toxins in transgenic plants is more likely to increase the selection pressure towards resistance. The relative ease with which mutant nematode strains were identified exhibiting reduced sensitivity to some of the *B. thuringiensis* strains (Borgonie *et al.*, 1996 *a*), may suggest that constitutive expression of *B. thuringiensis* nematicidal proteins in transgenic plants may lead relatively rapidly to nematode resistance in the field.

Although the absence of purified toxin to date prevents more detailed mode of action analysis (e.g., by using antibodies), the results reported here indicate that, although at least two of the three nematicidal *B. thuringiensis* strains very probably act identically, the mode of action of nematicidal *B. thuringiensis* strains is fundamentally different from that of insecticidal strains. In view of the temperature and pH sensitivity of the intoxication process, the possibility that the nematicidal factor is internalized in intestinal cells, in contrast to insecticidal cell membrane active *B. thuringiensis* toxins, should be considered.

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