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Effect of nematicidal *Bacillus thuringiensis* strains on free-living nematodes. 1. Light microscopic observations, species and biological stage specificity and identification of resistant mutants of *Caenorhabditis elegans*

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Summary – Light-microscopic observations of the toxic action of *Bacillus thuringiensis* spore/crystals reveals that, in *Caenorhabditis elegans*, the intestine is destroyed in two stages over a period of 24 h. The anterior ring of four cells is the first and foremost target. Observations indicate that the intestine is the only tissue being destroyed. Screening of fourteen additional rhabditid nematode species against three nematicidal *B. thuringiensis* strains active against *C. elegans*, resulted in only one additional sensitive nematode species, and indicates a high species specificity of the nematicidal factor. However, in contrast to insect-specific *B. thuringiensis* toxins, the nematicidal toxin exhibits low developmental stage specificity against *C. elegans;* all developmental stages, including adult nematodes are sensitive. Moreover, sensitivity increases as development proceeds. Using ethyl methyl sulfonate induced mutagenesis two mutants of *C. elegans* have been recovered, exhibiting reduced sensitivity of up to 50 % against one of the nematicidal strains. Moreover, one of the mutants exhibited cross-resistance to a second nematicidal *B. thuringiensis* strain against which it was not screened. Preliminary data indicate that the reduced sensitivity in the mutants is not due to reduced pharyngeal pumping activity.

Résumé – ***Action de souches nématicides de* Bacillus thuringiensis *sur les nématodes libres. 1. Observation en microscopie optique, spécificité envers les espèces et les stades biologiques, identification de mutants résistants chez* Caenorhabditis elegans** – L'observation en microscopie optique de l'action toxique des spores/cristaux de *Bacillus thuringiensis* montre que, chez *Caenorhabditis elegans*, l'intestin est détruit en deux phases sur une période de 24 h. L'anneau antérieur de quatre cellules constitue la première cible. Les observations indiquent que les tissus intestinaux sont les seuls détruits. Le criblage de quatorze autres espèces de Rhabditides vis-à-vis de trois souches nématicides de *B. thuringiensis* actives contre *C. elegans* a démontré qu'une seule de ces espèces était sensible et indiqué une forte spécificité du facteur nématicide. Cependant, au contraire des toxines de *B. thuringiensis* douées de spécificité envers les insectes, les toxines nématicides ne montrent qu'une faible spécificité envers les différents stades de *C. elegans*, tous les stades, y compris les adultes, étant sensibles. De plus, la sensibilité s'accroît lors du processus de développement. Deux mutants de *C. elegans*, obtenus par action de l'éthyle-méthyle-sulfonate, montrent une sensibilité réduite de 50 % envers l'une des souches nématicides. En addition, l'un des mutants montre une résistance croisée à une deuxième souche nématicide de *B. thuringiensis* envers laquelle elle n'avait pas été testée. Des données préliminaires indiquent que cette réduction de la sensibilité chez les mutants n'est pas causée par une diminution de l'activité de pompage du pharynx.

Key-words : *B. thuringiensis, C. elegans,* mutants, specificity, toxicity, nematodes.

Bacillus thuringiensis Berliner is a Gram-positive bacterium commonly found in natural soils and insect-rich environments. Upon sporulation, *B. thuringiensis* produces one or more parasporal crystalline proteinaceous inclusions. Most of these crystal proteins or δ-endotoxins are toxic to larvae of lepidopteran, dipteran or coleopteran insects. This insecticidal activity is highly specific; individual toxins only kill the larvae of certain insect groups usually within a single insect order (Lambert & Peferoen, 1992; Knowles & Dow, 1993). Strains exhibiting toxicity against pathogenic protozoa, mites and nematodes have also been reported (Edwards *et al.*, 1989; Feitelson *et al.*, 1992).

The nematicidal activity of *B. thuringiensis* strains was mainly observed against eggs and, exceptionally, the first three juvenile stages of several animal-parasitic ne-

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matode species (Ciordia & Bizell, 1961; Bottjer *et al.*, 1985; Meadows *et al.*, 1989*a, b)* and the free-living nematodes *Caenorhabditis briggsae* and *Turbatrix aceti* (Bone *et al.*, 1985; Meadows *et al.*, 1990).

In contrast to the insecticidal activity of the δ-endotoxins, very little is known about the mode of action of the nematicidal *B. thuringiensis* strains. An increase in the permeability of the nematode egg-shell and a degeneration of the intestinal cells of the juvenile nematodes have been observed (Bone *et al.*, 1985, 1987; Bottjer & Bone, 1987; Wharton & Bone, 1989).

The current study reports on the species specificity of three nematicidal *B. thuringiensis* strains previously identified by screening against *Caenorhabditis elegans* (Leyns *et al.*, 1995). A detailed light-microscopic description is given of the effect of one nematicidal *B. thuringiensis* strain on the nematode *C. elegans*.

Materials and methods

Preparation of spore-crystal mixtures of *B. thuringiensis*

Two nematicidal (BTS0289A, BTS0958B) and one non-nematicidal (BTS0302AE) *B. thuringiensis* strains were obtained from the collection of Plant Genetic Systems NV (stored in 25 % glycerol at – 70 °C). One nematicidal (Edwards *et al.*, 1989) *B. thuringiensis* isolate (NRRL repository No. B-18247), obtained from Northern Research Laboratory, USDA, Peoria, IL, USA, was grown on 400 ml (Culturing Bacillus Isolates [CBI] medium: bacto-peptone 7.5 g; glucose 1 g; K_2HPO_4 4.35 g; distilled water to 1 l) in 2-l Erlenmeyer flasks on a rotary shaker (100 rpm) at 28 °C for 5 to 7 days. After adjustment to pH 7.2 and sterilisation at 120 °C for 20 min, two filter-sterilized salt solutions were added: $MgSO_4 \cdot 7H_2O$ 2.46 g; $MnSO_4 \cdot H_2O$ 0.04 g; $ZnSO_4 \cdot 7H_2O$ 0.28 g; $FeSO_4 \cdot 7H_2O$ 0.4 g; distilled water to 100 ml and $CaCl_2 \cdot 2H_2O$ 3.66 g; distilled water to 100 ml. Upon lysis, vegetative cells, spores and crystals were harvested by centrifugation at 3000 rotation per min (rpm), for 15 min. The pelleted particles, mainly consisting of spores and crystals were resuspended in phosphate buffered saline (PBS) (NaCl 8 g; KCl 0.2 g; Na_2HPO_4 1.15 g; KH_2HPO_4 0.2 g; distilled water to 1 l; pH 7-8) and stored in concentrations of $2 \cdot 10^9$ particles/ml at – 20 °C until use.

Nematodes

Fifteen available free-living nematode species belonging to three families within the Rhabditida were tested. Rhabditidae: *C. elegans* var. Bristol, *C. briggsae, Rhabditis oxycerca, Rhabditis teres, Rhabditis synpapillata, Rhabditis tripartita* and *Dolichorhabditis dolichura.* Panagrolaimidae: *Panagrolaimus superbus, Panagrellus redivivus.* Cephalobidae: *Acrobeloides maximus, Acrobeloides bodenheimeri, Acrobeloides nanus, Heterocephalobus pauciannulatus, Seleborca complexa* and *Chiloplacus* sp.

Monoxenic and axenic culture

All nematode cultures were sterilised using standard hypochlorite solution according to Sulston and Hodgkin (1988). Nematodes were cultured on *Escherichia coli* and generally handled according to Brenner (1974). Stock cultures were kept at 20 °C. *C. elegans* was cultured axenically according to Vanfleteren *et al.* (1990). Briefly, a mixture of 3 % (w/v) soy peptone, 3 % (w/v) yeast extract was autoclaved. Hemoglobin solution (0.1 %) was added aseptically afterwards. The hemoglobin solution was made by dissolving 5 g hemoglobin (SERVA) in 100 ml 0.1 M KOH and was autoclaved for no longer than 10 min. Culture in axenic medium was preferred for the study of the toxicity process using TEM in order to avoid mistaking *E. coli* for *B. thuringiensis.*

Synchronization of nematode development stages (Goh & Bogaert, 1991)

Eggs containing embryos in different developmental stages were collected by washing nematodes from 5 cm plates containing gravid females and eggs with 0.8 ml of M 9 buffer (22 mM KH_2PO_4, 33 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$). The suspension was mixed with 0.4 ml of alkaline hypochlorite (4 vol. 2 M NaOH; 3 vol. 14 % NaOCl) in an Eppendorf tube. After 8-10 min with occasional mixing the tube was spun in a centrifuge (1 min, 6000 rpm). The pellet was taken up in 0.4 ml of M 9 buffer and mixed with the same volume of ice-cold 2 M sucrose/100 mM NaCl. Distilled water (0.2 ml) was placed on top of the sucrose cushion and the eggs floated to the interface after 5 min centrifugation at 6000 rpm. The eggs with embryos were removed from the sucrose cushion with a Pasteur pipette and washed one in PBS. Eggs were collected by centrifugation and resuspended in M 9 buffer and left overnight to hatch. Hatched first stage juveniles (J1) do not develop further in the absence of food. J1 could then be tested or grown to the desired developmental stage.

Toxicity assay

Nematicidal activity of the spore-crystal mixtures was determined by adding 50 µl of the mixtures to wells of a 96 well microtiter plate (Falcon) containing 50 µl suspensions of 200 nematodes in PBS. Control wells consisted of 50 µl nematode suspensions in PBS supplemented with 50 µl PBS. The non-toxic strain *B. thuringiensis* 302AE served as a control. All nematode suspensions, including control treatments, contained tetracyclin (30 µg/ml) and chloramphenicol (30 µg/ml) to prevent germination and subsequent bacterial growth. Microtiter plates were incubated at 25 °C for 24 h after which nematicidal activity was determined by counting dead and living nematodes (nematodes were considered dead when they no longer reacted to prodding). Nematode mortality was expressed as the mean percentage of dead *versus* live nematodes. Each treat-

ment was replicated three times. When nematodes were not cultured axenically, they were washed off agar plates and washed three times in PBS. The PBS was aerated extensively to promote digestion of remaining bacteria, the nematodes were washed twice more and the bioassay was carried out as described above. No differences were observed in sensitivity towards nematicidal *B. thuringiensis* strains whether the nematodes were cultured axenically or not. Data were analysed using ANOVA and post hoc comparison using the computer software STATISTICA (Statsoft, Tulsa, OK, USA).

β-GALACTOSIDASE HISTOCHEMISTRY

The transgenic *C. elegans* strain (PC72ubIn5), generously made available by Dr. E. Stringham (formerly of the University of British Columbia, currently at the University of Gent, Belgium), carries an integrated array of the transgene *hsp16-lacZ* and the pRF4 plasmid (Stringham *et al.*, 1992; Stringham & Candido, 1993). The pRF4 plasmid carries the dominant *rol-6* allele, *su1006*, which encodes a mutant collagen that alters the body cuticle so that the animal is forced to roll onto its right side as it moves forward (Mello *et al.*, 1991). Expression of the β-galactosidase gene is heat inducible in all tissues except the gonad, probably due to a developmentally regulated program that overrides the heat inducibility of the *hsp-16* genes in the gonad (Stringham *et al.*, 1992).

It was hoped that tissues under stress as a result of the toxin would express the reporter gene. The transgenic nematode strain was incubated with the nematicidal *B. thuringiensis* NRRL-18247 and the non-toxic strain 302AE for 24 h. Samples were taken every hour and divided in two aliquots. One aliquot was prepared for X-gal staining after a 15 min recovery period, the second aliquot was subjected to a heat shock (33 °C, 2 h.) before recovery for 15 min and staining for X-gal. Staining was carried out by lyophilisation of the nematodes, followed by aceton fixation (3 min, – 20°C) and assaying for β-galactosidase activity with a histochemical stain containing 33 mM NaH_2PO_4; 166 mM Na_2HPO_4; 1 mM $MgCl_2$; 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$; 5 mM $K_3Fe(CN)_6$, SDS (40 μg/ml); 240 μg per ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Sigma); 0.6 % *N,N*-dimethylformamide; 75 μg/ml kanamycin sulfate (Fire, 1986; Fire *et al.*, 1990). To acertain that the transgenic nematode strain exhibited the same susceptibility as non-transgenic *C. elegans* towards the nematicidal *B. thuringiensis* strains, mortality of synchronised young adults was compared with Wild Type (WT). Mortality as determined for synchronised young adults was 95.7 % ± 1.2 % (n = 3) for the WT and 92.9 % ± 1.8 % (n = 3) for the *hsp16-lacZ* transgenic nematode strain. ANOVA analysis showed that this difference was not significant ($F = 4.98$; $P = 0.089$). All light microscopy was done using a Leitz microscope equipped with 2, 10, 40 and 63 × lenses. Micrographs were made using Kodak 160 T film and processed commercially. For light-microscopic observations, nematodes were fixed in freshly prepared 4 % paraformaldehyde overnight, mounted in 90 % glycerol/10 % PBS. The coverslip was sealed with clear nail polish. When using the *hsp16-lacZ* transgenic strain, nematodes were observed in the staining solution after being mounted as described above (overnight incubation in staining solution).

SCREENING FOR *B. THURINGIENSIS* RESISTANT MUTANTS

WT fourth stage juveniles (J4) were mutagenized by incubation in 0.25 % ethyl methyl sulfonate (EMS, SIGMA) as described by Brenner (1974). Mutagenized J4 were transferred to NGM (Brenner, 1974) agar plates seeded with *E. coli*, five animals per plate. The animals were allowed to reproduce for two generations at 20 °C. F2 progeny were screened for resistance by incubating mutagenized nematodes with *B. thuringiensis* strain NRRL-18247 for 48 h at 25°C. Since mortality is maximal from the J4 juvenile stage onwards, only adults that had survived the incubation were transferred to fresh NGM agar plates. Potential resistant nematode strains were tested three times. Testing of bacteria-stimulated pharyngeal pumping activity was done according to Horvitz *et al.* (1982). Individual young adult nematodes were placed on NGM agar plates containing a bacterial lawn of *E. coli* and after 10 min the rate of pharyngeal pumping was determined by direct observation. Temperature was 20 °C and illumination was kept to an absolute minimum since Avery and Horvitz (1990) reported higher pumping as a result of the heat generated by illumination.

Results

SPECIES SPECIFICITY OF THE NEMATICIDAL *B. THURINGIENSIS* STRAINS

Among the Rhabditidae tested only *C. elegans* and its sister species *C. briggsae* tested sensitive to the three nematicidal *B. thuringiensis* strains (Table 1). Other tested Rhabditidae were not sensitive, nor were nematode species belonging to the two other families.

NEMATODE STAGE SPECIFICITY OF THE NEMATICIDAL FACTOR

All *C. elegans* stages were sensitive, although to a different degree (Fig. 1). There was a gradual increase in sensitivity after each moult until the J4 stage, where maximum mortality was reached. Mortality remained at this level in young and old hermaphrodites. For further detailed study of the mode of action, one nematicidal *B. thuringiensis* strain (NRRL-18247) and one nematode species *(C. elegans)* were used.

LIGHT-MICROSCOPIC OBSERVATIONS

During the first 12-13 h after the start of feeding on the spore-crystal mixture, and accumulation of spores

Table 1. *Effect of four* Bacillus thuringiensis *strains on the mortality of fifteen species of Rhabditida.*

	Strains				
	NRRL-18247	*B.t.* 289A	*B.t.* 958B	*B.t.* 302AE*	Control
RHABDITIDAE					
C. elegans	77.4 ± 6.9	68.5 ± 4.6	67.1 ± 1.3	7.2 ± 2.5	1.2 ± 0.4
C. briggsae	58.7 ± 10.9	26.2 ± 1.7	81.7 ± 4.4	7.4 ± 2.8	0.9 ± 0.2
R. teres	4.2 ± 1	3.4 ± 0.6	5.8 ± 1.2	8.7 ± 0.9	2.5 ± 0.7
R. oxycerca	3.5 ± 2.0	8.5 ± 1.2	6.9 ± 1.8	7.2 ± 4.3	3.8 ± 1.6
R. synpapillata	5.2 ± 1.0	6.3 ± 0.9	5.2 ± 3	4.3 ± 1.9	2.9 ± 1.0
R. tripartita	4.3 ± 1.2	3.7 ± 0.7	5.1 ± 1.1	4.8 ± 1.1	3.6 ± 0.7
D. dolichura	3.4 ± 0.7	3.6 ± 0.3	2.3 ± 1.1	1.3 ± 0.5	1.9 ± 0.1
PANAGROLAIMIDAE					
P. redivivus	6.8 ± 1.5	9.6 ± 4.4	5.9 ± 3.2	1.1 ± 1.0	4.4 ± 2.2
P. superbus	1.6 ± 1.3	3.1 ± 2.7	3.1 ± 1.1	3.0 ± 1.3	1.7 ± 1.4
CEPHALOBIDAE					
A. maximus	4.0 ± 0.6	3.6 ± 0.4	3.8 ± 1.0	4.3 ± 0.4	3.3 ± 0.3
A. bodenheimeri	4.2 ± 0.8	5.2 ± 1.5	3.8 ± 4.4	2.0 ± 4.6	4.7 ± 1.6
A. nanus	1.9 ± 0.3	8.2 ± 0.7	4.4 ± 1.2	5.9 ± 2.1	5.1 ± 3.3
H. pauciannulatus	4.4 ± 0.9	4.7 ± 1.3	4.4 ± 1.3	4.4 ± 0.6	4.9 ± 0.2
S. complexa	5.1 ± 4.5	4.3 ± 1.7	3.1 ± 3.4	2.8 ± 2.4	2.4 ± 0.4
Chiloplacus sp.	7.0 ± 2.0	8.9 ± 0.5	5.7 ± 0.7	7.6 ± 0.7	5.8 ± 1.7

* = 302AE is a non-toxic strain; control : nematodes incubated in PBS only; mixed (200 nematodes) nematode cultures containing all developmental stages were tested for 24 h. at 25 °C; nematode mortality was expressed as the mean ± standard deviation (n = 3) percentage of dead *versus* live nematodes.

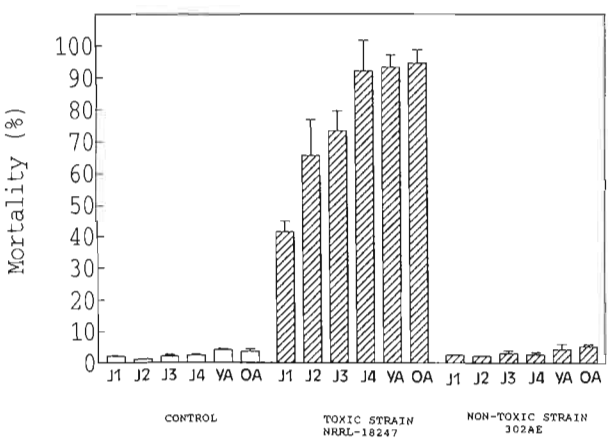

Fig. 1. *Effects of nematicidal strain NRRL-18247 and non toxic strain on different* Caenorhabditis elegans *developmental stages. A-F : Controls (nematodes + PBS); G-L : Nematodes + NRRL-18247; M-R : Nematodes + non-toxic strain 302AE. (Incubation lasted for 24 h at 25°C; J1, J2, J3, J4 : first, second, third and fourth juvenile stage respectively; YA, OA : young and old adults respectively).*

and crystals was observed in the anterior intestinal lumen. This accumulation sometimes resulted in a buildup of spores and crystals in the anterior direction, causing accumulation of spores and crystals in the basal and sometimes the median bulb. Accumulation of spores towards the posterior direction was extremely rare. This buildup in the lumen and the resulting pressure on the anterior intestinal cells forced the nuclei in a more posterior position. From 12-13 h onward there was a significant decrease of motility. Only occasional movements by the head were observed. The nematodes still react when touched on the head with a needle. Beyond 12 h, initially the remainder of the intestine began to deteriorate along the longitudinal axis. In a final stage, when the nematodes failed to respond to any stimulus, accumulated spores may germinate resulting in complete colonization of the nematode body as has been described earlier (Borgonie *et al.*, 1995*c*). Except for the destruction caused by germination, the damage of the nematicidal strain was limited to the intestinal cells only.

This sequence of events is supported and best visualised by observations of the stained nuclei in the *hsp16-lacZ* transgenic *C. elegans* strain used (Fig. 2). Although it was hoped that feeding on the toxic *B. thuringiensis*

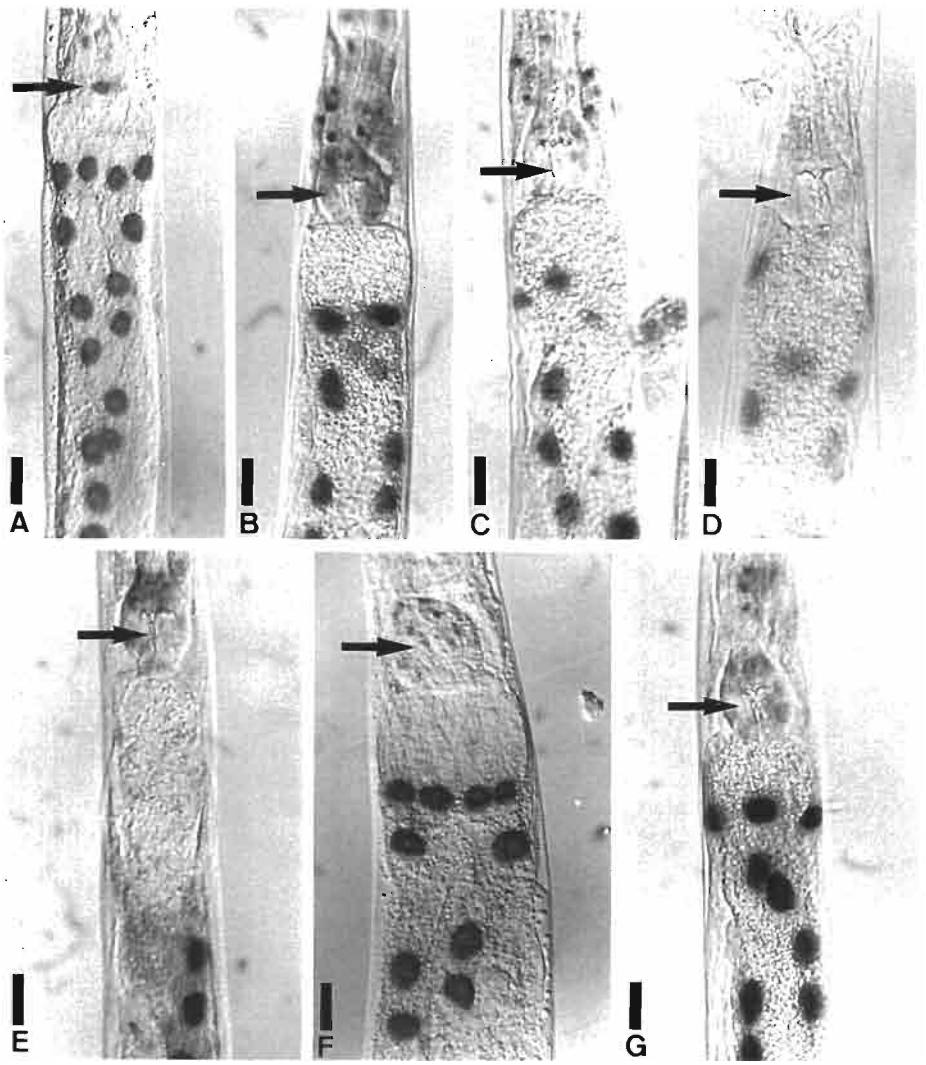

Fig. 2. *Progressive destruction of intestine as result of the transgenic* Caenorhabditis elegans *containing the* hsp-16-lacZ *transgene with nematicidal* Bacillus thuringiensis *strain NRRL-18247. Visualisation of nuclei after a 2-h heat shock followed by β-galactosidase histochemistry. Incubation time- A : + 1 h; B : + 10 h; C : + 13 h; D : + 15 h; E : + 18 h; F-G : controls; F : + 24 h (PBS only); G : + 24 h, with non-nematicidal strain* B. thuringiensis *302AE. Arrow indicates position of basal bulb.* (Bars = 20 μm).

strains would elicit a stress response, enabling identification of tissues and/or cells being affected, feeding of the transgenic strain with toxic or non-toxic *B. thuringiensis* did not elicit the stress response. Nevertheless, the transgenic strain proved valuable in visualizing the intestinal nuclei. Analysis of the nuclei stained after incubation with *B. thuringiensis,* followed by a 2-h heat shock, confirmed that in the first 12 h of incubation only the four anterior cells were destroyed (Fig. 2A-C). After the first 12 h of incubation, the blue stain in the nuclei, gradually faded away until no more stain can be observed (Fig. 2D-E). At the end of this first stage, nuclei in the remainder of the intestine, or nuclei in other tissue, still exhibited strong, localized staining (Fig. 2C). Beyond 12 h the stain in the more posteriorly situated intestinal nuclei underwent the same fading process (Fig. 2E). Before the onset of germination, stain was still readily visible in non-intestinal tissue. No loss of nuclear staining was observed when the transgenic nematode strain was incubated in PBS without any *B. thuringiensis* strain (Fig. 2F) or incubated with the non-toxic *B. thuringiensis* strain 302AE (Fig. 2G).

Control experiments indicated that no mortality was caused by these substances. Feeding was periodically

assayed by observing pumping of the basal bulb.

MUTANT SCREENING

To facilitate analysis of the nematicidal mode of action, three screens were carried out for resistant nematode mutants using *B. thuringiensis* strain NRRL-18247 and the nematode *C. elegans*. To date, two mutants (*Btr-1* and *Btr-2 : B. thuringiensis* resistant) have been recovered that exhibited significantly reduced sensitivity to the nematicidal *B. thuringiensis* strain (Fig. 3). Reduced sensitivity can be the result of many factors, one of which is a reduced pumping activity of the basal bulb, leading to a reduced ingestion of the spores and crystals. Comparison of pumping rates between WT and mutants did not reveal reduced pumping activity in the two mutants. On bacterial lawns, WT (n = 10) pumped 253 times per min (± 19), *Btr-1* (n = 10) 266/min (± 23) and *Btr-2* (n = 10) 264/min (± 28). ANOVA analysis shows no significant differences for pharyngeal pumping between WT and both mutant strains (F = 0.192; $P \leq 4.0413$). Although all the mutants were screened against one nematicidal *B. thuringiensis* strain, one of the mutants exhibited cross-resistance to a second nematicidal *B. thuringiensis* strain 289A but not to the third nematicidal strain 958B.

Discussion

Data on the effects of *B. thuringiensis* var. *israelensis* on *Trichostrongylus colubriformis* indicate that the juvenile stages are sensitive and that the sensitivity decreases as development proceeds (Bone *et al.*, 1985). This observation is comparable to insects but not with *C. elegans*. Bone *et al.* (1985) furthermore indicated that the nematicidal and insecticidal factor are probably not the same, but that the nematicidal agent most likely acts at the level of the cellular membrane. Bottjer and Bone (1987) reported ovicidal action of *B. thuringiensis israelensis* against *T. colubriformis* and that degeneration of the intestine took place in juveniles.

In this study, light-microscopic observations show that the toxicity process of *C. elegans* occurs in two distinct phases. The first phase is characterized by the gradual destruction of the anterior intestinal ring of four cells immediately posterior to the pharynx. Damage to the remainder of the intestine or to non-intestinal tissues, if present, is not visible. The presence of strong nuclear localized stain when using the *hsp16-lacZ* transgenic nematode strain furthermore indicates that the nuclei in the four cells maintain their structural integrity up to 12 h after incubation. Since the stress response was only elicited after a heat shock, the presence of nuclear localized stain indicates that the ring of four cells retains at least some transcriptional and translational activity up to that point, notwithstanding the severe cellular damage. Afterwards the stain becomes weaker and diffuses at the edges of the nucleus indicative of degradation of the nuclear membrane. The presence of diffuse stain about 12 h after incubation is an indication that the nuclei remained active during the beginning of the heat shock, but that nuclear integrity was lost as result of a combination of the severe cellular damage combined with the effects of the ensuing heat shock in a decaying cell.

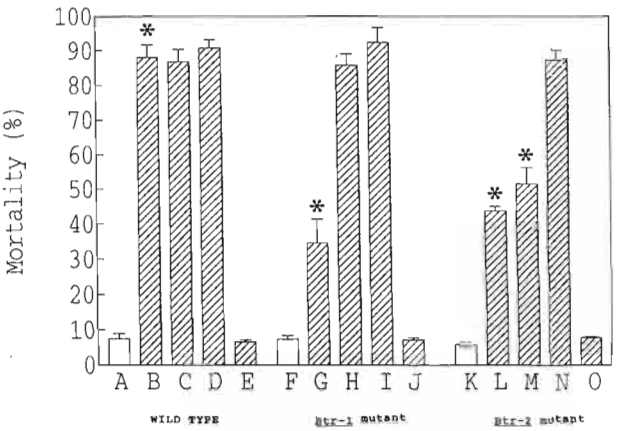

Fig. 3. *Effects of different* Bacillus thuringiensis *strains on the mortality of Wild Type and two EMS induced mutants of* C. elegans. *A, F, K : control; B, G, L :* Bt *NRRL-18247; C, H, M :* Bt *289A; D, I, N :* Bt *958AB; E, J, N :* Bt *302AE; for EMS induced mutants. (Data marked with an asterisk are significantly different – P < 0.001 – from the Wild Type for each* Bt *strain).*

During the second phase, beyond 12 h, the remainder of the intestine degrades. Other non-intestinal tissues do not exhibit signs of damage. Considering the extent of time before the first effects were visible in the rest of the intestine, it is unlikely that this degradation of the remainder of the intestine is the result of the activity of the toxin, but is probably the result of the decay of the dead nematode.

When the toxicity process in *C. elegans* is compared with that reported for insects (Gill *et al.*, 1992; Knowles & Dow, 1993) some similarities are observed, but considerable differences are evident. It is the low stage specificity of the nematicidal toxin, with increasing toxicity as development proceeds, that attains maximum toxicity in the adult nematode. In insects, the insecticidal factor is highly specific to larval stages and toxicity decreases as development proceeds (Knowles & Dow, 1993). In analogy with insects, the nematicidal toxin exhibits the same high species specificity. The underlying reason for this high specificity cannot be interpreted adequately in the absence of the purified toxin. In view of the results already obtained with insects, several hypotheses are possible. Specificity could be the result of the necessity of a receptor for the toxin, as is the case for insects (Hofmann *et al.*, 1988*a, b*; Van Rie *et al.*, 1989, 1990). This could explain not only the species specificity but the highly restricted intestinal site of action as well. If a receptor is found to be involved in toxicity then it must be expressed at all developmental stages in nematodes.

Another possibility could be the necessity of specific enzymes in the intestinal lumen for the release and/or processing of the toxin. Although the anterior most ring of four intestinal cells are different ultrastructurally and biochemically (Borgonie *et al., 1995a, b*), it is unclear why the predominant toxic action is directed against these four cells.

Another important difference between nematicidal and insecticidal endotoxins is that the toxicity process in nematodes occurs very slowly in comparison with *B. thuringiensis* strains active against lepidopteran insects or mosquito-active *Bacillus sphaericus,* where the first signs of toxicity are visible within minutes. A considerably slower action has been reported for *B. thuringiensis* var. San Diego on the cottonwood leaf beetle (Bauer & Pankratz, 1992), where the first effects are visible after 2 h. The authors speculated that this slower action might be the result of different characteristics of the insect gut and/or the toxin as compared to Lepidoptera (Bauer & Pankratz, 1992).

Therefore, identification of resistant mutant strains will be of considerable benefit for mode of action studies. Moreover the identification of a nematode strain exhibiting cross-resistance to another *B. thuringiensis* strain, for which it was not screened, indicates that at least a part of the underlying toxic mechanism is common to both these nematicidal *B. thuringiensis* strains.

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