Screening of the nematicidal activity of the bacterium *Bacillus thuringiensis* Berliner on the free-living nematode *Caenorhabditis elegans*

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Summary. The nematicidal activity of 68 *Bacillus thuringiensis* Berliner serotypes on *Caenorhabditis elegans* was investigated. Toxicity was determined by transferring a 100 µl aqueous suspension containing twenty *C. elegans* juveniles onto an agar plate containing a sporulated, crystal-producing culture of *B. thuringiensis* as the source of food. The *B. thuringiensis* 407 Cry-, a crystal-minus strain, was included as a non-toxic control. The number of eggs, juveniles and adults was determined after 96 hours of incubation at 25°C. For the *B. thuringiensis* 407 Cry- strain, the average numbers of *C. elegans* juveniles and adults, and *C. elegans* eggs were 62/plate, and 22/plate, respectively, after the 96-hour incubation period. A total of six *B. thuringiensis* serovars caused significantly high nematicidal activity and a total of 13 serovars, caused a significantly high reduction in the production of eggs. Six *B. thuringiensis* serovars had a significant effect in both assays. These were var. *thuringiensis, cameroun, tolworthi, darmstadiensis, toumanoffi* and *fukuokaensis*. Whereas four of these synthesize the wide-spectrum β-exotoxin and one produce a Cyt protein, var. *cameroun* produces neither.

Key words: *Bacillus thuringiensis*, *Caenorhabditis elegans*.

Bacillus thuringiensis Berliner is a sporulating Gram-positive bacterium commonly found in soils. Upon sporulation, it produces a parasporal inclusion body, the crystal. The crystal is made of proteins, the crystal (Cry) and the cytolytic (Cyt) proteins, also called β-endotoxins. Some *B. thuringiensis* strains exhibit specific insecticidal properties against Lepidopteran, Dipteran or Coleopteran insect pest larvae. The toxicity is caused by the Cry and Cyt proteins (Höfte & Whiteley, 1989; Beegle & Yamamoto, 1992). The mode of action of the Cry proteins in susceptible insects has been partly deciphered (Gill *et al.*, 1992; Schnepf *et al.*, 1998). To be active, the crystal must be dissolved in the alkaline insect midgut. Inactive Cry proteins are released and are proteolytically cleaved by specific proteases in the insect midgut. The activated toxin binds to specific receptors at the surface of the midgut epithelial cells. Pore formation is induced, the cells start swelling and burst open, the midgut becomes non-functional and the insect dies.

In addition to the Cry and Cyt proteins, some *B. thuringiensis* strains produce a β-exotoxin, a heat-stable nucleotide analogue, often referred to as thuringiensin. It interferes with RNA biosynthesis which results in broad-spectrum toxicity against invertebrates and vertebrates (Lecadet & de Barjac, 1981).

Today more than 50,000 *B. thuringiensis* strains are kept in various collections worldwide (Sanchis *et al.*, 1996). They have been classified, based on H-serotyping, the immunological reaction to the bacterial flagellar antigens, into at least 82 serovars (Lecadet *et al.* 1999). The phylogenetic relationships between serovars has been inferred by ribotyping (Joung & Côté, 2001a, 2001b). At least 250 Cry toxins have now been characterized (Crickmore *et al.*, 1998, 2002). Often, novel *B. thuringiensis* strains are screened for insecticidal activity against a very limited number of insect pests, usually selected Lepidopteran, Dipteran and Coleopteran larvae, only to be met with negative results. Given the wide diversity of the *B. thuringiensis* strains and the diversity of the Cry proteins, and given their mode of action, it

appears reasonable to hypothesize that some could express novel toxicity against new targets.

Plant parasitic nematodes, such as *Meloidogyne* spp. (root-knot nematode), *Aphelenchoides* spp. (foliar nematode) and *Pratylenchus* spp. (root lesion nematode), cause major damage on corn, potato, soybean, vegetable crops, *etc.* (Nickle, 1991). Their control rests on the use of chemical fumigants and nematicides (Dropkin, 1980). They appear as good targets amenable to microbial control with *B. thuringiensis*. The latter is a normal soil bacterium that could be sprayed where plant parasitic nematodes are found. Alternatively, if desirable, specific *B. thuringiensis* cry genes could be expressed in transgenic plants.

Few studies have been conducted on the use of *B. thuringiensis* against plant parasitic nematodes. As might have been expected because of the wide-spectrum action of the toxin, some β-exotoxin + *B. thuringiensis* strains have proven toxic against *Meloidogyne* species (Prasad *et al.*, 1972; Ignoffo & Dropkin, 1977; Devidas & Rehberger 1992). Some other assays were conducted with *B. thuringiensis* on *Meloidogyne* species with no mention on the presence or absence of β-exotoxin (Chahal & Chahal, 1991; Zuckerman *et al.*, 1993). A small number of assays were conducted with some *B. thuringiensis* strains and commercial formulations on various nematodes, including free-living (*Caenorhabditis* species and *Turbarix aceti*) (Bone *et al.*, 1985, 1988; Borgonie *et al.*, 1996; Meadows *et al.*, 1990) and zoo-parasitic nematodes (*Trichostrongylus*, *Nippostrongylus*) (Bottjer *et al.*, 1985; Meadows *et al.*, 1989a, 1989b). In most studies, eggs were most susceptible to nematicidal activities.

The aim of the present study was to assay the potential nematicidal activity of a much wider spectrum of *B. thuringiensis* strains and serovars. A total of 68 different *B. thuringiensis* serovars were tested on a model nematode selected for its ease of use, *Caenorhabditis elegans*.

MATERIAL AND METHODS

Culture of *Caenorhabditis elegans*. *Caenorhabditis elegans* strain BC00842 was obtained from D. L. Baillie, Simon Fraser University, Vancouver, B.C., Canada. It was grown on nematode growth medium (NGM) (Brenner, 1974) agar plate seeded with *Escherichia coli* strain HB-101 as the food source, and maintained at 25°C.

Synchronous populations of juvenile nematodes (J2-J3) were obtained as follows: *C. elegans* were washed off NGM plates with water, rinsed three times with water and then washed three times with 5 ml of antibiotic solution (tetracyclin 20 μg/ml and ampicilin 100 μg/ml), 4 minutes each, to remove *E. coli*. The inoculum was recovered in a beaker and transferred in a sterile tube. The nematode suspension was allowed to settle for 4 minutes. *C. elegans* adults, which are heavier than juveniles, settled more quickly at the bottom of the tube, while juveniles (J2-J3) remained in the middle of the supernatant. After these 4 minutes, a sample of the middle of the suspension was transferred in a counting plate to determine the two volumes of inoculum required to contain 20 and 100 second-stage juvenile nematodes.

Culture of *Bacillus thuringiensis*. The 69 *Bacillus thuringiensis* strains used in this study are listed in Table 1. They include 68 different serovars, varieties based on serotyping, and *B. thuringiensis* strain 407 Cry-. The 68 serovars were obtained from H. de Barjac and M.-M. Lecadet, Laboratoire des Bactéries Entomopathogènes, Institut Pasteur, Paris, France. *Bacillus thuringiensis* strain 407 Cry- was obtained from D. Lereclus, Unité de biochimie microbienne, Institut Pasteur. They were grown in 2YT nutrient broth (tryptone 16 g; yeast extract 10 g; sodium chloride 5 g; and bi-distilled water to 1L) in a rotary shaker (New Brunswick Scientific Model C-25, Edison, NJ, USA), at 180 rpm, 30°C, overnight. Each *B. thuringiensis* strain was, afterwards, striated, with ≈1.5 cm spacing between striations, on a T-3 agar plate (tryptone 3 g; tryptose 2 g; yeast extract 1.5 g; sodium phosphate 1 M pH 6.8 50 ml; $MnCl_2$ 4 H_2O 0.05M 1 ml; agar 15 g and bi-distilled water to 1L) and incubated at 30°C for five days to induce sporulation and crystal formation.

Effect of sporulated *Bacillus thuringiensis* strains on the viability of *Caenorhabditis elegans* under laboratory conditions. A total of 68 *B. thuringiensis* serovars were tested against *Caenorhabditis elegans* in *in vitro* bioassays. Twenty *C. elegans* juveniles (J2), contained in 100 μl of the inoculum, were transferred on the center of a T-3 agar plate, previously striated with a *B. thuringiensis* strain as source of food, and previously incubated at 30°C during five days to induce sporulation and crystal formation, as described above. All agar plates were incubated at 25°C for four days to allow feeding of *C. elegans* on a sporulating, crystal-producing, *B. thuringiensis* strain. After four days, the numbers of eggs, living juveniles (J2-J3) and adults (J-4) were determined under a stereo-microscope. Each *B. thuringiensis* strain was assayed in two separate experiments with four replications (four agar plates) each, for a total of eight replicates. Two

Table 1. List of *Bacillus thuringiensis* strains and serovars used in this study. H-serotype, serovar and strain number are given. Nematicidal activity, effects on the reduction in the production of eggs and presence of the wide-spectrum β-exotoxin are summarized. - no activity, + high activity, ++ highest activity.

H-serotype	Serovar	Strain	Nematicidal	Reduction of Eggs	β-Exotoxin
		407 cry-	-	-	-
1	*thuringiensis*	T01 001	++	++	+
2	*finitimus*	T02 001	-	-	-
3a, 3b, 3c	*kurstaki*	T03A 001	+	+	-
3a, 3c	*alesti*	T03 001	-	-	-
3a,3d	*sumiyoshiensis*	T03B 001	-	-	-
3a,3d,3e	*fukuokaensis*	T03C 001	++	++	-
4a,4b	*sotto*	T04 001	-	-	-
4a,4c	*kenyae*	T04B 001	-	-	-
5a,5b	*galleriae*	T05 001	-	-	-
5a,5c	*canadensis*	T05A 001	+		
6	*entomocidus*	T06 001			
7	*aizawai*	T07 001	+		
8a,8b	*morrisoni*	T08 001		+	+
8a,8c	*ostriniae*	T08A 001	+		
8b,8d	*nigeriensis*	T08B 001	+	++	
9	*tolworthi*	T09 001	++	++	+
10a,10b	*darmstadiensis*	T010 001	++	++	+
10a,10c	*londrina*	T010A 001	+	+	-
11a,11b	*toumanoffi*	T011 001	++	++	+
11a,11c	*kyushuensis*	T011A 001	-	-	-
12	*thompsoni*	T012 001	+	-	-
13	*pakistani*	T013 001	-	-	-
14	*israelensis*	T014 001	+	-	-
15	*dakota*	T015 001	-	-	-
16	*indiana*	T016 001	+	++	-
17	*tohokuensis*	T017A 001	+	-	-
18a,18b	*kumamotoensis*	T018 001	-	-	-
18a,18c	*yosoo*	T018A 001	+	++	-
19	*tochigiensis*	T019 001	-	-	-
20a,20b	*yunnanensis*	T020 001	-	-	-
20a,20c	*pondicheriensis*	T020A 001	-	+	-
21	*colmeri*	T021 001	+	+	-
22	*shandongiensis*	T022 001	+	++	-
23	*japonensis*	T023 001	-	+	-
24a,24b	*neoleonensis*	T024 001	+	-	-
24a,24c	*novosibirsk*	T024A 001	+	+	-
25	*coreanensis*	T025 001	-	-	-
26	*silo*	T026 001	+	++	-
27	*mexicanensis*	T027 001	+	+	-
28a,28b	*monterrey*	T028 001	-	-	-
28a,28c	*jegathesan*	T028A 001	+	-	-
29	*amagiensis*	T029 001	-	-	-
30	*medellin*	T030 001	-	+	-
31	*toguchini*	T031 001	-	-	-
32	*cameroun*	T032 001	++	++	-
33	*leesis*	T033 001	-	-	-
34	*konkukian*	T034 001	-	-	-
35	*seoulensis*	T035 001	-	-	-
36	*malaysiensis*	T036 001	-	-	-

Table 1 (continued). List of *Bacillus thuringiensis* strains and serovars used in this study. H-serotype, serovar and strain number are given. Nematicidal activity, effects on the reduction in the production of eggs and presence of the wide-spectrum β-exotoxin are summarized. - no activity, + high activity, ++ highest activity.

H-serotype	Serovar	Strain	Nematicidal	Reduction of Eggs	β-Exotoxin
37	*andaluciensis*	T37 001	-	-	-
38	*oswaldocruzi*	T38 001	-	-	-
39	*Brasiliensis*	T39 001	-	-	-
40	*huazhongensis*	T40 001	-	-	-
41	*Sooncheon*	T41 001	-	-	-
42	*jinghongiensis*	T42 001	-	-	-
43	*guiyangiensis*	T43 001	-	-	-
44	*higo*	T44 001	+	++	-
45	*roskildiensis*	T45 001	+	-	-
46	*chanpaisis*	T46 001	-	-	-
47	*wratislaviensis*	T47 001	+	+	-
48	*balearica*	T48 001	+	+	-
49	*muju*	T49 001	+	+	-
50	*navarrensis*	T50 001	-	-	-
51	*xiaguangiensis*	T51 001	+	-	-
52	*kim*	T52 001	+	-	-
53	*asturiensis*	T53 001	+	++	-
54	*poloniensis*	T54 001	-	-	-
55	*palmanyolensis*	T55 001	+	-	-

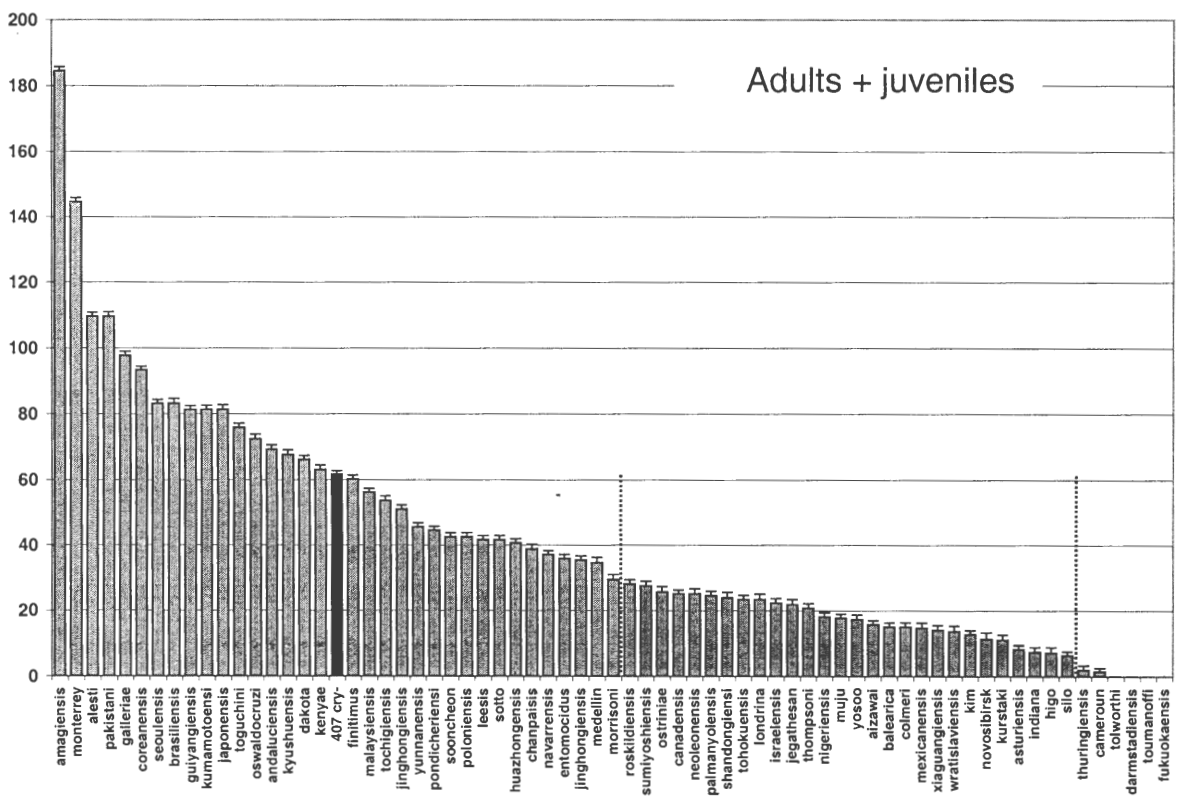

Fig. 1. Nematicidal activity of *Bacillus thuringiensis* serovars. The average number of juveniles and adults present on Petri dishes containing a sporulated, crystal-producing culture of *B. thuringiensis*, 4 days after treatment. The two vertical dotted lines separate three statistically different groups of *B. thuringiensis* serovars.

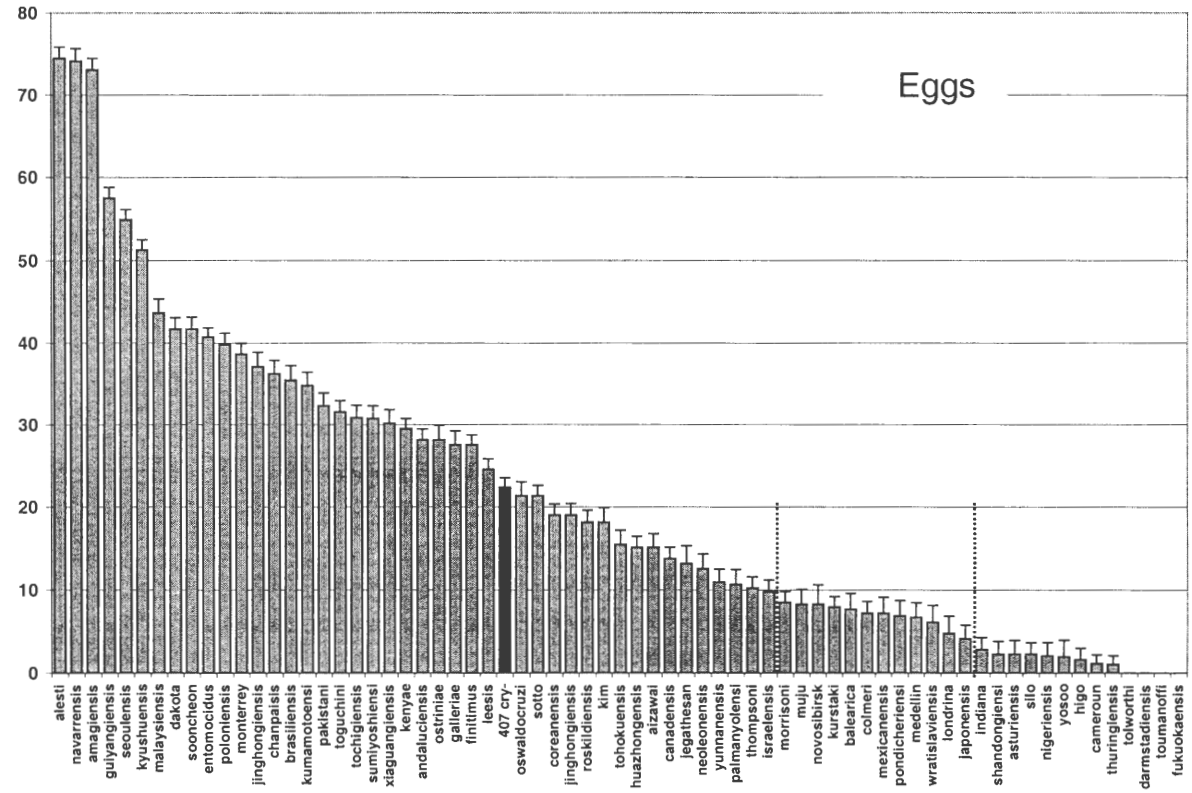

Fig. 2. Reduction in the production of eggs by *Bacillus thuringiensis* serovars. The average number of eggs present on Petri dishes containing a sporulated, crystal-producing culture of *B. thuringiensis*, 4 days after treatment. The two vertical dotted lines separate three statistically different groups of *B. thuringiensis* serovars.

additional bacterial strains, *Escherichia coli* HB101 and *B. thuringiensis* 407 Cry-, a crystal-minus strain, were used as non-toxic 'negative' controls.

Statistical analysis. For all experiments, data were transformed using ($\log_{10}$ [x+1]) or (arcsin (sqrt (x)) before statistical analysis. Data were analyzed by the analysis of variance and general linear model (GLM) procedures (SAS Institute Inc. 1988). Waller's test was used to compare treatments when the analysis of variance showed significant differences among means (P=0.05). Data are expressed as means ± standard error.

RESULTS

As shown in Fig. 1, a total of six *B. thuringiensis* serovars, when fed to *Caenorhabditis elegans* in in vitro bioassays, caused significantly high nematicidal activity as measured by the absence or near absence of juveniles and adults on the Petri dishes 4 days after treatment. These were var. *thuringiensis, cameroun, tolworthi, darmstadiensis, toumanoffi* and *fukuokaensis*. A total of 28 more serovars caused significant nematicidal activity when compared to the *B. thuringiensis* 407 Cry-, non-toxic control strain. These results are summarized in Table 1.

Likewise, as shown in Fig. 2, a total of 13 *B. thuringiensis* serovars, when fed to *Caenorhabditis elegans* in in vitro bioassays, caused a significantly high reduction in the production of eggs as measured by the absence or near absence of eggs on the Petri dishes 4 days after treatment. These were the same six serovars indicated above, var. *cameroun, thuringiensis, tolworthi, darmstadiensis, toumanoffi* and *fukuokaensis*, with the addition of var. *indiana, shandongiensis, asturiensis, silo, nigeriensis, yosoo* and *higo*. A total of 12 more serovars caused a significant reduction in the production of eggs when compared to the *B. thuringiensis* 407 Cry-, non-toxic control strain. These results are summarized in Table 1.

Of the six *B. thuringiensis* serovars that caused significantly high nematicidal activity, and consequently, an absence or near absence in the production of eggs, four synthesize the wide-

spectrum β-exotoxin (summarized in Table 1). These are var. *thuringiensis, tolworthi, toumanoffi* and *darmstadiensis*. The other two varieties, *cameroun* and *fukuokaensis*, are β-exotoxin -. Serovar *fukuokaensis*, however, produces a Cyt protein, Cyt2Ba3. No Cyt proteins are known in var. *cameroun*.

DISCUSSION

The current study was a first thorough screening of the potential nematicidal activity of *B. thuringiensis*. We assayed 68 different *B. thuringiensis* serovars on *C. elegans*. Our aim, at the onset of the current study, was to determine whether a strain from a *B. thuringiensis* serovar could exhibit nematicidal activity using *C. elegans* as a model nematode. Our longer-term goal would be to develop a *B. thuringiensis* strain, or alternatively a Cry protein, for the control of plant parasitic nematodes. Because plant parasitic nematodes are difficult to grow and assay in the lab, *C. elegans* was chosen in the current assay because it is easy to use, has a short generation time, feeds on bacteria and can be easily assayed in Petri dishes. It is easily amenable to a large screening program as the one carried here. Although the present discussion is focused on *C. elegans*, our longer-term goal should be kept in mind. It is not certain that results obtained with *C. elegans* can be transposed directly to plant parasitic nematodes. Serovars toxic to *C. elegans* may turn out not to be toxic to plant parasitic nematodes. Conversely, serovars that could be toxic to plant parasitic nematodes may not be nematicidal to *C. elegans* and would remain undiscovered in the current study. Our study was also limited in that only one *B. thuringiensis* strain per serovar was assayed. Different strains from the same serovar might have yielded different results. In addition, only bacterial cultures were assayed here. To reveal the potential nematicidal activity of some *B. thuringiensis* strain, the crystal may first need to be treated in vitro. Such treatments might include crystal solubilization in an alkaline environment to release the protoxins. This could be followed by an enzymatic activation using one or more proteolytic enzymes, trypsin, chymotrypsin, proteinase K, and other proteases, to generate different peptidic fragments with potentially different biological activities. Yet, despite these limitations, the present study generated very valuable results. It presents the clear benefit of identifying several *B.* thuringiensis serovars expressing nematicidal activity and/or reduction in the production of eggs and deserving of further studies.

Of the six *B. thuringiensis* serovars that gave the highest nematicidal activity, four produce the β-exotoxin. We cannot rule out that *C. elegans* mortalities recorded in the presence of β-exotoxin + *B. thuringiensis* strain could have also been caused not only by the β-exotoxin but also by the β-endotoxins. Assays with pure Cry toxin would help clarify this point. Nonetheless, the development of these *B. thuringiensis* serovars into commercial formulations for the eventual control of plant parasitic nematodes appears unlikely. Most countries will not register *B. thuringiensis* products containing β-exotoxin because of its wide-spectrum activity (Glare & O'Callaghan, 2000). Var. *fukuokaensis* also gave the highest nematicidal activity. However, it does produce a Cyt protein, Cyt2Ba3. In vitro, Cyt proteins express a wide-spectrum cytolytic activity (Thomas & Ellar, 1983). In vivo, the cytolytic activity has been shown in Dipterans (Höfte & Whiteley, 1989) and Coleopterans (Federici & Bauer, 1998). Whether the Cyt protein is responsible for the nematicidal activity is unknown. It would be interesting to assay pure Cyt2Ba3 on *C. elegans*. Var. *cameroun* is the sixth serovar of the group that showed the highest nematicidal activity. It is known to produce at least two Cry toxins, CryC35 and CryC53. It would now be interesting to assay these pure Cry proteins on *C. elegans*. Several other *B. thuringiensis* serovars showed greater nematicidal activity than that shown by *B. thuringiensis* 407 Cry- on *C. elegans*. In some cases, the Cry protein complement is known but this is not the case for each. It would certainly be interesting to assay pure Cry and Cyt proteins from these.

Some *B. thuringiensis* serovars did not cause the highest *C. elegans* mortality but showed significant reduction in the production of eggs. From a biological control point of view, given that the aim would be to control plant parasitic nematodes, these serovars would be interesting by hampering the reproduction of the nematode. Again, assays with pure Cry and Cyt proteins would be warranted.

In addition to the *B. thuringiensis* serovars that showed highest *C. elegans* mortality or highest reduction in the production of eggs, several more serovars showed significantly high nematicidal activity. Some of these may better express their nematicidal activity on different target nematodes or following different in vitro treatements.

The present work was useful in pinpointing the *B. thuringiensis* serovars that exhibit nematicidal activity against *C. elegans*. It should be followed by

the development of assay systems on economically important plant parasitic nematodes. Assays could be done first with the *B. thuringiensis* strains identified here and, second, with the purified, solubilized, enzymatically-treated crystal, or with cloned enzymatically-cleaved Cry and Cyt proteins.

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Bélair G. and Côté J.-C. Выявление нематицидной активности у *Bacillus thuringiensis* Berliner с помощью скрининга на культурах свободноживущей нематоды *Caenorhabditis elegans*.

Резюме. С помощью биотестирования на *Caenorhabditis elegans* исследована нематицидная активность 68 серотипов *Bacillus thuringiensis* Berliner. Токсичность определяли перенося 100 µl водной суспензии с 20-ю личинками *C. elegans* на поверхность агаризованной среды, содержащей в качестве пищи для нематод образующие споры и кристаллы культуры *B. thuringiensis*. Не образующий кристаллов штамм *B. thuringiensis* 407 Cry- был использован в качестве нетоксичного контроля. Число яиц, личинок и взрослых нематод определяли через 96 часов инкубации при 25°C. После 96-часовой инкубации на культуре *B. thuringiensis* 407 Cry- в среднем формируется 62 экземпляра личинок или взрослых нематод и 22 яйца этих нематод. Шесть сероваров *B. thuringiensis* вызывают значительную смертность нематод, а 13 сероваров приводили к существенному снижению числа образующихся яиц. Шесть сероваров (*thuringiensis, cameroun, tolworthi, darmstadiensis, toumanoffi* и *fukuokaensis*) показали активность как в снижении числа активных нематод, так и числа яиц. Четыре штамма из этих шести способны к синтезу β-экзотоксина, один штамм продуцирует Cyt-белок, но еще один из этих активных штаммов (var. *cameroun*) не способен к синтезу веществ двух этих групп.