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EFFICACY OF *CLOSTRIDIUM BIFERMENTANS* SEROVAR *MALAYSIA* ON TARGET AND NONTARGET ORGANISMS

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ABSTRACT. *Clostridium bifermentans* serovar *malaysia* (*C.b.m.*) is highly toxic to mosquito larvae. In this study, the following aquatic nontarget invertebrates were treated with high *C.b.m.* concentrations (up to 1,600-fold the toxic concentration for *Anopheles stephensi*) to study their susceptibility towards the bacterial toxin: *Planorbis planorbis* (Pulmonata); *Asellus aquaticus* (Isopoda); *Daphnia pulex* (Cladocera); *Cloeon dipterum* (Ephemeroptera); *Plea leachi* (Heteroptera); and *Eristalis* sp., *Chaoborus crystallinus, Chironomus thummi,* and *Psychoda alternata* (Diptera). In addition, bioassays were performed with mosquito larvae (*Aedes aegypti, Anopheles stephensi,* and *Culex pipiens*). *Psychoda alternata* larvae were very susceptible, with LC_{50}/LC_{90} values comparable to those of mosquito larvae (about 10^3–10^5 spores/ml). The tests with *Chaoborus crystallinus* larvae showed significant mortality rates at high concentrations, but generally not before 4 or 5 days after treatment. The remaining nontarget organisms did not show any susceptibility. The investigation confirms the specificity of *C.b.m.* to nematocerous Diptera.

INTRODUCTION

For several years, 2 larvicidal bacteria, *Bacillus thuringiensis* Berliner var. *israelensis* (*B.t.i.*) and *Bacillus sphaericus* Neide, have been used successfully for mosquito and blackfly control all over the world. Nevertheless, in consideration of the increasing resistance to chemical insecticides and the need for alternative control agents (Anonymous 1987), there has been an ongoing search for new mosquitocidal pathogens (Thiery et al. 1992b).

About 3 years ago, a *Clostridium bifermentans* Weinberg and Séguin strain toxic to mosquito and blackfly larvae was isolated from soil samples of a Malaysian mangrove swamp. Further characterization and the existence of a specific H-antigen allowed it to be individualized as a serovar—*malaysia* (*C.b.m.*). It is the first strictly anaerobic mosquitocidal pathogen that has been characterized (de Barjac et al. 1990).

The larvicidal activity of *C.b.m.* is closely linked to the sporulation stage and is partly due to the existence of parasporal inclusion bodies (Charles et al. 1990, de Barjac et al. 1990). The toxic proteins have not yet been clearly characterized, but they are very different from *B.t.i.* and *B. sphaericus* crystal toxins (Nicolas et al. 1993).

The determination of the host range shows a specificity for larvae of the Culicidae and the Simuliidae. Susceptibility varies according to the mosquito species, with *Anopheles* species being the most susceptible, followed by *Culex* and all strains of *Aedes aegypti* (Linn.) larvae, which are about 10 times less sensitive than *Anopheles*. The LC_{50} (48 h) ranges from 5×10^3 to 2×10^5 cells/ml (Thiery et al. 1992b). Larvae of *Simulium* species seem to be less susceptible (de Barjac et al. 1990). Toxicity was not found for lepidopteran, coleopteran, and dipteran (Brachycera) larvae or for *Biomphalaria glabrata* Say snails (Gastropoda) (Thiery et al. 1992b). The safety for vertebrates was demonstrated by a classical series of tests recommended by the World Health Organization (Thiery et al. 1992a).

Our objective was to extend the investigations concerning specificity and thus environmental compatibility of the bacterial toxin(s) by studying possible effects of *C.b.m.* on several nontarget organisms that inhabit the larval habitats of mosquitoes and would, therefore, be directly concerned by mosquito control operations. The selection of the nontarget organisms was made in respect to their significance and frequency in the habitat. Another aspect of selection was the representation of different systematic groups of organisms and different feeding strategies.

MATERIAL AND METHODS

Bacterial culture: The *C.b.m.* was grown under anaerobic conditions as described in Nicolas et al. (1990) and was stopped at t_8 of sporulation (16 h culture). A lyophilized preparation of this whole culture (70% cells contained spores, spore density: 1.6×10^8 spores/ml) was used for all assays. The sample was suspended in deionized water prior to the start of the tests. The suspension was either used immediately or frozen in aliquots at −20°C to prevent a loss of toxicity. Appropriate concentrations were gained by further dilution of the whole culture.

Bioassays of mosquito larvae: a) Culicini larvae: Laboratory-reared larvae of *Ae. aegypti* (strain Bora-Bora) and *Culex pipiens* Linn. were

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Table 1. Larvicidal activity of *Clostridium bifermentans* ser. *malaysia* on Culicidae larvae and on other Nematocera (Diptera).

Species	*n*	LC_{50} (24 h)[1]	LC_{90} (24 h)	LC_{50} (48 h)	LC_{90} (48 h)
Aedes aegypti	600	20.2 ± 11.3[2]	143 ± 81	10.9 ± 7.9	42.7 ± 20.8
(Bora-Bora)		3.2 ± 1.8[3]	22.9 ± 13	1.7 ± 1.2	6.9 ± 3.4
Anopheles stephensi	420	1.6[3,4]	7.8	0.5	1.8
An. stephensi	525	0.7 ± 0.2[3,5]	1.6 ± 0.3	0.4 ± 0	1.1 ± 0.1
Culex pipiens	600	92.3 ± 19.9[2]	263.3 ± 89.6	65 ± 26	195 ± 55
		14.7 ± 3.1[3]	42.0 ± 14.5	10.6 ± 4.4	31 ± 9
Psychoda alternata	350	8.0 ± 3.1[2]	52.0 ± 27	2.0 ± 1.9	7.3 ± 6.2
		1.3 ± 0.5[3]	8.3 ± 4.2	0.3 ± 0.3	1.2 ± 1.0
Chaoborus crystallinus	300			3,577 ± 359	22,355 ± 956[2,6]
				572 ± 58	3,577 ± 153[3]

[1] Mean of 3 experiments ± SE ($P < 0.05$).
[2] Lethal concentration expressed in 10^{-5} × dilution of *C.b.m.* whole culture.
[3] Lethal concentration expressed in 10^4 × spores/ml.
[4] Result of one experiment.
[5] Results obtained by Thiery et al. (1992b) ($P < 0.05$).
[6] Mean of 2 experiments ± SE ($P < 0.05$), LC values on 7th day.

tested against the above-mentioned preparation at dilutions of 0.5, 1, 2, 4, 8, 16, and 33 × 10^{-4} (spores/ml ranging from 8 × 10^3 to 5.3 × 10^5). Twenty-five early 4th-instar larvae were placed in plastic cups containing 150 ml of the appropriate bacterial suspension. Three cups were prepared per dilution and tests were run in triplicate at 24 ± 2°C. Three cups served as controls. Mortality was determined 24 and 48 h after application of the *C.b.m.* preparation. The LC_{50} and LC_{90} values ($P < 0.05$) were calculated by log probit analysis by use of a computer program (Raymond 1985).

b) Anophelini larvae: Three cups containing 20 early 4th-instar larvae of *Anopheles stephensi* Liston were prepared per dilution (series of 6 dilutions; range 7 × 10^{-6} to 2.2 × 10^{-4}, corresponding to 1.1 × 10^3 to 3.5 × 10^4 spores/ml), 3 cups served as controls. The test was run once.

Nontarget organisms: The following species served as test organisms: *Planorbis planorbis* Linn. (Pulmonata); nymphs of *Cloeon dipterum* Linn. (Ephemeroptera); *Plea leachi* MacGregor (Heteroptera); *Asellus aquaticus* Linn. (Isopoda); *Daphnia pulex* De Geer (Cladocera); larvae of *Eristalis* sp. (Brachycera, Diptera); and 2nd–4th-instar larvae of *Chaoborus crystallinus* De Geer, *Chironomus thummi s.l.*, and *Psychoda alternata* Say (Nematocera, Diptera). The animals were collected from their natural habitats, transferred to the laboratory, and allowed to adapt to laboratory conditions (20°C; day–night rhythm: 14: 10 h) for 1 or 2 days. *Psychoda alternata* larvae remained in their substrate until the beginning of the test, then they were washed out with small-meshed sieves.

Toxicity tests: The LC_{90} value (48 h) calculated for *Anopheles stephensi* (1.1 × 10^{-4} dilution = 1.76 × 10^4 spores/ml) served as a basis for the choice of the bacterial concentrations used for nontarget organisms: for *Planorbis planorbis, Cloeon dipterum, Plea leachi, Asellus aquaticus, Dapnia pulex,* and *Eristalis* sp.: 3.5 × 10^6 spores/ml (= 200-fold concentration); for the larvae of *Chironomus thummi s.l.:* 4.4 × 10^6 spores/ml (= 250-fold concentration); for *Chaoborus crystallinus:* up to 2.8 × 10^7 spores/ml (= 1,600-fold concentration), and finally, for *Psychoda alternata,* concentrations varying between 10^3 and 10^5 spores/ml. To exclude nonspecific effects based on the activity of exotoxins or metabolite toxins, autoclaved *C.b.m.* material was used in some tests.

Twenty to 25 individuals were placed in each of the test dishes containing 100–150 ml water (mixture of original and spring water) and the appropriate *C.b.m.* concentration. Two to 3 replicates were prepared for each concentration; a respective number served as a control. Small amounts of food were added meeting the special requirements of the organisms (Tetra Min, plant material, or prey organisms, i.e., *Daphnia pulex*). Experiments were conducted at 20°C. The duration of the experiment was up to 2 wk, observations were made every 1 or 2 days. Tests were run in duplicate or triplicate. Test results were interpreted by calculation of the percent mortalities observed. Deviations between control and treated batches were checked for their significance by means of multiple range tests according to Duncan (1955).

Psychoda alternata: LC_{50}/LC_{90} tests were carried out with this species due to its susceptibility towards *C.b.m.* Dead and living animals were

Table 2. Larvicidal activity of *Clostridium bifermentans* ser. *malaysia* on nontarget organisms.

Species	Order	*n*	Dose (spores/ml)	Test (days)	% mortality ± SE[1] Test	Control
Planorbis planorbis	Pulmonata	60	3.5×10^6	12	0	0
Cloeon dipterum	Ephemeroptera	60	3.5×10^6	8	0	2.5 ± 0.8
Plea leachi	Heteroptera	60	3.5×10^6	7	0	6.7 ± 0
Asellus aquaticus	Isopoda	60	3.5×10^6	8	5.0 ± 1.7	4.3 ± 1.0
Daphnia pulex	Cladocera	75	3.5×10^6	6	4.3 ± 2.3	3 ± 1
Eristalis sp.	Diptera	60	3.5×10^6	3	20	5
Chaoborus crystallinus	Diptera	50	$1.76–28 \times 10^6$	7	35 ± 7–87 ± 1	5 ± 5
Chironomus thummi	Diptera	60	4.4×10^6	6	12.5 ± 7.5	11.5 ± 11.5
Psychoda alternata	Diptera	50	$10^3 \times 10^5$	2	72.3 ± 27.2–99.3 ± 0.9[2]	3.0 ± 3.5

[1] Mean of 2 experiments.
[2] Mean of 3 experiments ($P < 0.05$).

counted 24 and 48 h after the beginning of the test. The results were submitted to a log probit analysis to obtain LC_{50} and LC_{90} values.

RESULTS

Larvicidal activity on Culicidae: The larvicidal activity of *C.b.m.* on the mosquito species tested is summarized in Table 1. *Aedes aegypti* was the most susceptible species with 50% of the population killed by 1.7×10^4 spores/ml and 90% killed by 6.9×10^4 spores/ml 48 h after the application of *C.b.m. Culex pipiens* was less sensitive, with 50% of the population killed by 10.6×10^4 spores/ml and 90% killed by 31×10^4 spores/ml. The bioassay with *An. stephensi* showed that this species is even more susceptible towards *C.b.m.* than the 2 mentioned above, with LC_{50} and LC_{90} values of 0.5×10^4 and 1.8×10^4 spores/ml, respectively. As these were the results of one bioassay only, the LC_{50}/LC_{90} values obtained by Thiery et al. (1992b) with the same lyophilized preparation are added for reasons of comparability (see Table 1).

Toxicity to nontarget organisms: Table 2 summarizes the efficacy of *C.b.m.* on the nontarget organisms tests. *Planorbis planorbis, Asellus aquaticus, Daphnia pulex, Plea leachi,* and *Cloeon dipterum* nymphs, as well as the larvae of the dipterans *Eristalis* sp. and *Chironomus thummi s.l.,* did not show any susceptibility. *Clostridium bifermentans* serovar *malaysia* showed some effect, however, on *Chaoborus crystallinus* and *Psychoda alternata* larvae, with both species reacting with increased mortality rates after treatment with *C.b.m.* spore material.

The larvae of *Psychoda alternata* were shown to be very susceptible to *C.b.m.* The results of the bioassays (LC_{50}/LC_{90} values) with this species are summarized in Table 1. After 48 h of larval exposure to *C.b.m.,* 50% of the population was killed by 0.3×10^4 spores/ml and 90% was killed by 1.2×10^4 spores/ml. According to these results *Psychoda alternata* is even more sensitive to *C.b.m.* than *Ae. aegypti* (about 6 times) and *Cx. pipiens* (about 30 times) and at least as sensitive as *An. stephensi.*

Table 3 presents the results of 2 toxicity tests performed with *Chaoborus crystallinus* larvae. The percent mortality in the treated batches is slightly higher than in the control batches from the 3rd day onward. There is, however, no significant deviation between control and treated batches until the 6th day of the test run. Multiple range tests show that on the 8th day the deviations between control batch and batches with 1,600- and 800-fold concentrations are highly significant ($P = 0.001$), the deviation between control and 400-fold concentration is significant ($P = 0.01$), and there are slightly significant differences ($P = 0.05$) between control and 200-/100-fold concentrations. Similar results were achieved with tests using 100- and 500-fold concentrations. In all experiments, *Chaoborus crystallinus* showed a somehow delayed reaction, with significant mortality rates generally not before 4 or 5 days after treatment with *C.b.m.* It was observed that in the course of the test, larvae were more and more weakened, with their movements and reactions increasingly slowing down. Such weakening of *Chaoborus* larvae was never noted in the control batches. To exclude the possibility of the larvae dying due to altered water conditions, several tests were made with autoclaved and thus nontoxic material. These experiments did not show any difference between control batches and batches treated with autoclaved material. Table 1 shows the LC_{50}/LC_{90} values for

Table 3. Efficacy of *Clostridium bifermentans* ser. *malaysia* on larvae of *Chaoborus crystallinus*.

x-fold LC_{90} *Anopheles stephensi*	Dose (spores/ml)	% mortality ± SE[1]				
		Day 1	Day 3	Day 5	Day 6	Day 7
Control	0	0	0	5 ± 5	5 ± 5	5 ± 5
100 ×	1.76×10^6	3 ± 3	13 ± 9	16 ± 10	33 ± 5	35 ± 7
200 ×	3.5×10^6	3 ± 3	11 ± 7	24 ± 10	35 ± 11	45 ± 15
400 ×	7×10^6	1 ± 1	10 ± 8	35 ± 1	53 ± 5	68 ± 4
800 ×	1.4×10^7	1 ± 1	17 ± 15	34 ± 12	60 ± 4	82 ± 8
1,600 ×	2.8×10^7	0	20 ± 14	37 ± 15	64 ± 4	87 ± 1

[1] Mean of 2 experiments ($P < 0.05$). $n = 50$ per concentration and experiment.

Chaoborus crystallinus. On the 7th day after *C.b.m.* application, 50% of the larvae were killed by 5.95×10^6 spores/ml and 90% by 35.5×10^6 spores/ml. A comparison of these results with those gained for the most susceptible mosquito species (LC_{90} [48 h] *An. stephensi*) shows that about 300-fold more bacteria are required to kill 50% of the *Chaoborus crystallinus* larvae and about 2,000-fold more bacteria to kill 90% of the population within 7 days.

DISCUSSION

This study shows the limited host range and thus specificity of *Clostridium bifermentans* serovar *malaysia* towards nematocerous Diptera. Apart from Culicidae larvae, only *Psychoda alternata* and *Chaoborus crystallinus* show some reaction to *C.b.m.* application.

The bioassays with *Ae. aegypti* (Bora-Bora), *An. stephensi*, and *Cx. pipiens* confirm the high susceptibility of mosquito species described before (de Barjac et al. 1990, Thiery et al. 1992b). The most susceptible species was *An. stephensi*, followed by *Ae. aegypti*. *Culex pipiens* was about 5 to 6 times less sensitive than *Ae. aegypti*. These results confirm those observed by other authors (Thiery et al. 1992b). This is true at least for *An. stephensi* and *Cx. pipiens*, which show much the same LC_{50}/LC_{90} values as the strains used by those authors. In contrast, the *Ae. aegypti* strain (Bora-Bora) used in these experiments was more sensitive than the Bora-Bora strain Thiery et al. tested (LC_{50}: 3 times lower and LC_{90}: 9 times lower). The difference in susceptibility might be due to a genetic change in the Bora-Bora strain in different laboratories over a period of time.

The experiments with several nontarget organisms show that, apart from *Psychoda alternata* and *Chaoborus crystallinus*, they are not affected by *C.b.m.*, even at high concentrations. This is true for *Planorbis planorbis* (Pulmonata), *Asellus aquaticus* (Isopoda), *Daphnia pulex* (Cladocera), *Cloeon dipterum* (Ephemeroptera), *Plea leachi* (Heteroptera), and also for the dipterans *Eristalis* sp. and *Chironomus thummi s.l.* Toxicity tests with the latter group of species were of special interest, as the Chironomidae, especially the subfamily Chironominae, are known to be susceptible to *Bacillus thuringiensis* var. *israelensis* (Ali 1981, Schnetter et al. 1981, Morawcsik 1983[4]). A similar susceptibility towards *C.b.m.* could not be observed.

To the contrary, *Psychoda alternata* is highly sensitive towards *C.b.m.*, even at low bacterial concentrations. Indeed, with LC_{90} value (48 h) of 1.2×10^4 spores/ml, *Psychoda alternata* is more susceptible to *C.b.m.* than most of the mosquito species. As the reaction of *Psychoda alternata* is similar to what has been observed with Culicidae larvae, it can be postulated that the death of *Psychoda* larvae is also due to a direct influence of the sporal toxin. With this result, *C.b.m.* shows some similarity to *B.t.i.* as far as the host range is concerned. Both bacteria show (apart from their toxicity to mosquito larvae) toxic effects on Simuliidae and Psychodidae larvae (de Barjac et al. 1981, de Barjac et al. 1990, Becker and Margalit 1993). The susceptibility of the latter group is interesting in view of its close relationship to the phlebotomines, representatives of which are known to be important disease vectors (the leishmaniases) in the tropics.

The effects of *C.b.m.* on *Chaoborus crystallinus* are not easy to interpret. Five to 6 days postinfection, significant mortality rates are observed among the larvae, which (as tests with autoclaved material show) are not caused by altered water conditions (high turbidity, etc.), but are linked with the application of *C.b.m.* spores. However,

[4] Morawcsik, J. 1983. Untersuchungen zur Wirkung von *Bacillus thuringiensis* var. *israelensis* auf aquatische Nontarget-Organismen. Dissertation. Universitat Heidelberg.

as high bacterial concentrations are required to cause elevated mortality rates and as the reaction of the larvae is rather slow, the experiment does not suggest whether the effects on *Chaoborus crystallinus* are due to a very slow toxicity or to spore germination in the larval digestive tract. This study demonstrates the safety of *Clostridium bifermentans* serovar *malaysia* to aquatic nontarget invertebrates.

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