HOST RANGE OF *CLOSTRIDIUM BIFERMENTANS* SEROVAR. *MALAYSIA*, A MOSQUITOCIDAL ANAEROBIC BACTERIUM

I. THIERY, S. HAMON, B. GAVEN[1] AND H. DE BARJAC

Unité des Bactéries Entomopathogènes, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris cedex 15, France

ABSTRACT. *Clostridium bifermentans* serovar. *malaysia* (*C.b.m.*) is toxic to mosquito larvae. In this study, we quantified its toxicity to the mosquitoes, *Aedes aegypti*, *Ae. albopictus*, *Ae. caspius*, *Ae. detritus*, *Anopheles stephensi*, *An. gambiae*, *Culex pipiens* and *Cx. quinquefasciatus*. *Anopheles* larvae are the most susceptible, followed by *Ae. detritus* and *Ae. caspius*, then *Culex* and other *Aedes* larvae. According to mosquito species, the LC_{50} varies from 7×10^3 to 1.3×10^6 cells/ml. Three concentrations (10^7, 10^6 and 10^5 cells/ml) of *C.b.m.*, *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) and *Bacillus sphaericus* were tested on *Ae. aegypti*, *An. stephensi* and *Cx. pipiens* larvae in order to determine the time necessary for each concentration to kill 50 and 90% of the population. Ninety percent of the 3 mosquito populations are killed within 4–15 h by the *C.b.m.* concentrations. Whatever the concentrations, *C.b.m.* kills at least 10 times less rapidly than *B.t.i.* but always quicker than *B. sphaericus*. Bioassays of *C.b.m.* bacterial cells or final whole culture were not toxic to *Musca domestica* and *Drosophila melanogaster* (Diptera) as well as to *Phaedon cochleariae* (Coleoptera) and *Spodoptera littoralis* (Lepidoptera).

INTRODUCTION

A new strain of *Clostridium bifermentans* Weinberg and Séguin serovar. *malaysia* (*C.b.m.*) named CH18 has been found to be toxic to mosquito and blackfly larvae (de Barjac et al. 1990). It is the first time that an anaerobic bacterium demonstrated high toxicity to Diptera. Larvicidal activity of *C.b.m.* is maximal during the sporulation stage at t_8 (8 h after the beginning of the sporulation process) and in part is related to the presence of parasporal inclusion bodies (Charles et al. 1990). After cell lysis, the larvicidal activity is 10 times less toxic; this is due to the inactivation of extracellular proteases excreted in the supernatant (Nicolas et al. 1990). Bacterial cells are demonstrated non-toxic to mammals and goldfish (Thiery et al. 1992); thus *C.b.m.* can be considered as a new candidate for vector control, although the mode of action of its toxic component(s) is still unknown.

The spectrum of action of *C.b.m.* should be given first priority. Thus this investigation reports comparative bioassays on a greater number of mosquito species. Larvae of different pests from Diptera, Coleoptera and Lepidoptera were also tested with *C.b.m.* cells, as well as an important vector of schistosomiasis, *Biomphalaria glabrata* Say.

MATERIALS AND METHODS

Bacterial culture: The *C.b.m.* was grown in a 6 liter capacity Biolaffite fermentor and stopped at t_8 of sporulation (15-h culture), corresponding to the maximal larval toxicity on *Anopheles stephensi* Liston larvae (Charles et al. 1990). Ten milliliters of TYG medium (g/liter; Bio-Trypcase 30.0; yeast-extract 20.0; HCl L-cysteine 0.5, glucose 0.5) pH 7.5, were inoculated from spores of *C.b.m.* stored in a sealed-tube on a filter paper at 4°C. After 72 h incubation at 30°C, bacteria were heat-shocked for 10 min at 80°C and poured into a 200 ml TYG medium preculture. This preculture grew at 34°C in anaerobic conditions for 6 h 30 min until the exponential phase, as checked by phase-contrast microscopy. It was inoculated in the fermentor containing 4.5 liters of TYG medium. Agitation (400 rpm) was performed with a draft tube. Anaerobic conditions were maintained by bubbling N_2 through the medium as described in Nicolas et al. (1990). After 15 h, the culture was harvested. At this stage, all bacterial cells contained spores and parasporal bodies. Half of the culture was directly frozen in aliquots. The second part of the culture was centrifuged in a MSE High Speed 18 centrifuge refrigerated at 4°C. Centrifugation was performed at 8,000 *g* for 40 min. Pellets were washed once in 1 M NaCl and in distilled water, each for 20 min. The pellets were resuspended in distilled water before being frozen in aliquots. Supernatant was also frozen in aliquots at −20°C.

Counting of bacterial cells was performed using plates previously maintained in an anaerobic incubator (95% H_2, 5% CO_2) at 34°C for at least 48 h. Plating was performed under anaerobic conditions and the plates for viable counts were incubated for 24 h. The final whole culture (FWC) contained 2.3×10^9 cells/ml with 100% spores and was used for all the experiments.

Bacillus thuringiensis Berliner var. *israelensis* (*B.t.i.*) strain 1884, *Bacillus thuringiensis* var. *morrisoni* strain "tenebrionis 256–82" and *Ba-*

[1] Entente Interdépartementale de Démoustication du Littoral Méditerranéen, BP 6036, 34030 Montpellier cedex 1, France.

cillus sphaericus Neide strain 2362 cultures, used for comparison in some experiments, were grown in their appropriate respective medium (Kalfon et al. 1983) for 48 h at 30°C on a orbital shaker.

Bioassays on mosquito larvae: Aedes aegypti (Linn.) (Cayenne and Zanzibar strains), *Aedes albopictus* (Skuse) (Taipei and Tananarive strains), *Culex quinquefasciatus* Say and *Anopheles gambiae* Giles were laboratory reared strains. Larvae of *Aedes caspius* Pallas and *Aedes detritus* Haliday were sampled from mosquito breeding sites (brackish marshland with *Juncus maritimus*) near Montpellier, France. Responses of these larvae to *C.b.m.* FWC were compared with those of *Ae. aegypti* Bora-Bora strain, *An. stephensi* ST15 strain and *Culex pipiens* Linn. Montpellier strain, reared in our laboratory.

Twenty-five fourth-instar larvae of *Aedes* and *Culex* sp. and third-instar larvae of *Anopheles* were placed in plastic cups containing 150 ml of the appropriate bacterial suspension. Two cups were prepared per dilution (series of 6 dilutions, range 10^7- 5 × 10^2 cells/ml according to the tested mosquito) and tests were run in triplicate at 25°C. Two cups of 25 larvae in 150 ml of dechlorinated water served as control. *Aedes detritus* and *Ae. caspius* larvae, were bioassayed in dechlorinated water containing 5 g/liter NaCl. Mortality was determined after 48 h larval exposure. The LC_{50} and LC_{90} ($P < 0.05$) values were calculated from a log-probit regression line program using a Macintosh computer.

LT_{50} determination: The LT_{50} was determined for 10^7, 10^6 and 10^5 cells/ml of *C.b.m.* FWC. The same FWC concentrations of *B.t.i.* and *Bacillus sphaericus* were tested. Twenty-five fourth-instar larvae of *Ae. aegypti, An. stephensi* or *Cx. pipiens* were added per plastic cup to 150 ml of the appropriate bacterial suspension. Twenty cups were run for each bacterial concentration. Mortality was recorded every 5 min (for 10 sec per cup) for *B.t.i.* or every 30 min or hour for *B. sphaericus* and *C.b.m.* according to the tested dilution.

Bioassays on Brachycera: Two series of 25 second-instar larvae of *Musca domestica* Linn. (pupae provided by Procida Company, Marseille) were poured onto 25 ml of artificial diet medium in which 2.5 ml *C.b.m.* whole culture had been incorporated (Larget and de Barjac 1981). Tests were run in duplicate. Mortality was recorded at 4, 6 and 16 days.

The *C.b.m.* FWC was also tested on second-instar larvae of *Drosophila melanogaster* Meigen. One hundred μl of *C.b.m.* FWC (1; 1/10) were spread onto the artificial diet in each glass tube (Larget and de Barjac 1981). Two series of 20 second-instar larvae were bioassayed per tube, tests were run in duplicate at 25°C. Mortality was recorded at 4 and 9 days.

Bioassays on Coleoptera: Larvae of *Phaedon cochleariae* Fabricius (Chrysomelidae) were reared on Chinese cabbage Pe-tsai; *C.b.m.* bacterial cells or supernatant (1 and 1/10) were sprayed with a nozzle (Burgerjon 1956) on 2 cabbage leaves of 1 cm^2, this area received 0.0048 ml suspension/cm^2. These leaves were kept in small boxes in which 6 first-instar larvae were placed. Thirty larvae were used per dilution. The bioassay was performed at 20°C. Mortality was recorded at 48 and 96 h. After 48 h, treated leaves were substituted by fresh ones. For a positive control larvae of *P. cochleariae* were also exposed to *B. thuringiensis* strain "tenebrionis 256–82," under the same conditions.

Bioassays on Lepidoptera: Dilutions of *C.b.m.* FWC (1; 1/10) were plated on artificial diet medium previously poured in small petri dishes (5 cm diam) (Kalfon and de Barjac 1985). Twenty neonates of *Spodoptera littoralis* Boisduval (Noctuidae) were placed per petri dish. The test was run in duplicate, two-fold 20 neonates served as control. Mortality was recorded at 3 and 7 days.

Biomphalaria glabrata: The experiments were performed either on small snails (3 mm diam, 1 month-old) or on masses of eggs (10–20 eggs) of *Biomphalaria glabrata* (Mollusca, Planorbidae), an important vector of schistosomiasis. Snails were treated either individually in 2 ml of dechlorinated water, 24 snails per dose, fed on 20 μg of artificial diet or per group of 10 snails in 150 ml of suspension, fed with 200 μg of sterile lettuce, run in duplicate. Whole culture, washed bacterial cells from the supernatant and supernatant were suspended in dechlorinated water to final dilutions of 1/100 and 1/1,000. A mixture of acids (acetic acid 50 mM, propionic acid 5 mM, phenyl propionic acid 10 mM, isovalerianic acid 5 mM and isocaproic acid 20 mM) corresponding to the composition of acids excreted in the supernatant was also tested on snails at 1/100 and 1/1,000. The final pH of the dechlorinated water was within the 6.8–8 range, depending whether the suspensions were made with *C.b.m.* or organic acids.

RESULTS

Larvicidal activity on Culicidae: Larvicidal activity of *C.b.m.* on Culicidae larvae is summarized in Table 1. *Anopheles gambiae, An. stephensi, Ae. caspius* and *Ae. detritus* larvae were the most susceptible with 50% of their population killed by 0.2 to 1.7 × 10^4 cells/ml. The LC_{50s} of *Ae. aegypti* strains were similar, varying from

Table 1. Larvicidal activity of *Clostridium bifermentans malaysia* on Culicidae larvae.

Larvae and strains	LC_{50}[a]	$LC_{90} \times 10^4$
Aedes aegypti Bora-Bora	5.3 (2.9)	62.1 (18.4)
Aedes aegypti Cayenne	7.1 (2.3)	80.5 (27.6)
Aedes aegypti Zanzibar	9.4 (5.5)	142.6 (115)
Aedes albopictus Taîpei	52.9 (27.6)	1,449 (1,311)
Aedes albopictus Tananarive	7.6 (8.7)	230 (253)
Aedes detritus	1.7 (0.2)	3.4 (0.4)
Aedes caspius	1.6 (0.2)	3.4 (0.2)
Anopheles stephensi	1.6 (0.7)	7.6 (3.6)
Anopheles gambiae	0.2 (0.09)	0.9 (0.2)
Culex pipiens	16.5 (3.9)	53.6 (2.3)
Culex quinquefasciatus	12.8 (2.5)	32 (0.7)

[a] Mean of 3 experiments after 48 h larval exposure (SE) ($P < 0.05$), lethal concentrations are expressed in 10^4 cells/ml of *C.b.m.* whole culture.

5.3 to 9.4×10^4 cells/ml. Although the LC_{50} (12.8 and 16.5×10^4 cells/ml) of *Culex* larvae was slightly higher than that of *Ae. aegypti*, *Culex* larvae were more susceptible when comparing the LC_{90}s. *Aedes albopictus* larvae were the less susceptible species with very high LC_{90}s (230 to $1,449 \times 10^4$ cells/ml); however *C.b.m.* is 10 times more toxic to the *Ae. albopictus* Tananarive strain than to the Taipei strain.

The rapidity of toxicity of *C.b.m.* was compared with *B.t.i.* and *B. sphaericus* (Table 2). Considering the LT_{50}, with 10^7 cells/ml, *C.b.m.* killed *Ae. aegypti*, *An. stephensi* or *Cx. pipiens* larvae within 2 to 3 h whereas *B.t.i.* was much quicker (less than 0.18 h). Moreover, at this concentration the 2 lethal times LT_{50} and LT_{90} were 3 to 5 times lower than the *B. sphaericus* ones. When given 10^6 cells/ml of *C.b.m.* FWC, 50% of the larval populations of *Ae. aegypti*, *Cx pipiens* or *An. stephensi* died within 11.7, 9.5 and 6.6 h, respectively. Ninety percent of these populations were killed in less than 25.4 h. The difference of rapidity observed between *C.b.m.*, *B.t.i.* and *B. sphaericus* toxicities decreased with the concentrations. At the lowest concentration (10^5 cells/ml), ca. 1–2 h were sufficient for *B.t.i.* to kill 50–90% of the 3 larval mosquito populations, however 11–20 h were necessary for *C.b.m.* to kill the same percentages of these populations. At this concentration *B. sphaericus* killed within 25 h except for *Ae. aegypti* larvae, which needed 257 h exposure to be killed.

Toxicity upon other insects: Larvae of *M. domestica*, *D. melanogaster* and *P. cochleariae* were not susceptible to *C.b.m.*; the highest mortality was observed with a 1/10 dilution of *C.b.m.* (2.3×10^8 cells/ml) induced 12% mortality in *M. domestica* larvae. No variations of their larval and imaginal growth was noticed after exposure to *C.b.m.*

In coleopteran experiments, we could not spray the FWC as it was too thick to pass through the nozzle, consequently washed bacterial cells and supernatant were tested separately. Whereas 10^9 and 10^8 cells/ml washed bacterial cells of *B. thuringiensis* strain "tenebrionis 256–82" killed 100% and 30% larvae respectively, *C.b.m.* bacterial cells (2.3×10^9 cells/ml) and supernatant culture did not induce any mortality nor slow down the larval growth which was similar to the untreated control.

Neonates of *S. littoralis* were not susceptible even to the pure *C.b.m.* culture. No mortality and no growth abnormality occurred.

Effect of C.b.m. on B. glabrata: Snails either individually or per group were treated with washed bacterial cells, FWC, supernatant and with a mixture of organic acids representing the main acids excreted in the supernatant (Cato et al. 1986) (Table 3). The bacterial cells (10^7 cells/ml) were not toxic to snails, whereas a high mortality, 60 to 93%, at 1/100 dilution was observed with FWC and supernatant in both experiments. These results were confirmed on egg masses. The mixture of acids was also very toxic causing 87.5% mortality, as well as each acid when tested separately on groups of snails induced between 25–75% mortality (data not shown). When a lower dilution (1/1,000) was tested, no mortality was noted.

DISCUSSION

This study shows the specificity of *C.b.m.* cells toward nematocerous Diptera, which appears as selective as *B.t.i.* and *B. sphaericus* toxins. No significant mortality was noticed either on lepidopteran, coleopteran and brachyceran dipteran larvae tested.

In respect to the effect on *B. glabrata* snails, *C.b.m.* culture supernatant showed a real toxicity. However, a similar level of mortality can be obtained with an artificial mixture of 4 organic acids reproducing the main acids excreted by *C.b.m.* cells. So, even if the intervention of some unknown toxin in the supernatant cannot be

Table 2. Comparative LT_{50} and LT_{90} of 3 concentrations of *Clostridium bifermentans malaya* with *B.t.i.* strain 1884 and *B. sphaericus* strain 2362.

	Cells/ml	10^{7a}		10^{6}		10^{5}	
Larvae	Hours	LT_{50}[b]	LT_{90}	LT_{50}	LT_{90}	LT_{50}	LT_{90}
Ae. aegypti	*B.t.i.*	0.18 (0.18–0.19)[c]	0.30 (0.29–0.31)	0.28 (0.26–0.29)	0.80 (0.49–0.51)	1.10 (1.06–1.14)	1.78 (1.72–1.84)
	B. sphaericus	18.9 (18.8–19.1)	24.5 (24.3–24.7)	29.8 (28.8–30.7)	73.5 (71.7–76)	257 (125–525)	809 (241–2,715)
	C.b.m.	3.4 (3.3–3.4)	4.9 (4.9–5.0)	11.7 (10.6–12.6)	25.4 (25.4–26.5)	12.3 (11.9–12.7)	19.9 (19.3–20.5)
An. stephensi	*B.t.i.*	0.09 (0.07–0.12)	0.48 (0.44–0.52)	0.02 (0.01–0.04)	0.28 (0.22–0.35)	1.83 (1.76–1.88)	2.98 (2.93–3.01)
	B. sphaericus	13.6 (13–14.1)	18.8 (18.6–19)	16.9 (16.6–17.2)	21.4 (21.3–21.6)	21.8 (21.6–22.0)	25.3 (22.2–25.5)
	C.b.m.	3.2 (3.2–3.3)	4.4 (4.3–4.4)	6.6 (6.5–6.7)	8.5 (8.4–8.6)	11.8 (11.4–12.1)	14.5 (14.4–14.5)
Cx. pipiens	*B.t.i.*	0.04 (0.018–0.08)	0.16 (0.12–0.22)	0.09 (0.07–0.11)	0.34 (0.30–0.38)	1.12 (1.07–1.16)	1.87 (1.84–1.91)
	B. sphaericus	7.7 (7.6–7.8)	9.4 (9.3–9.4)	10.4 (10.1–10.8)	17.7 (17.7–18.0)	14.4 (14.2–14.7)	18.4 (18.3–18.6)
	C.b.m.	2.5 (2.4–2.5)	3.2 (3.2–3.2)	9.5 (9.3–9.8)	16.5 (16.3–16.8)	11.4 (10.9–11.8)	15.1 (15.0–15.2)

[a] Concentration of FWC (cells/ml).
[b] LT_{50} and LT_{90} are expressed in hours.
[c] 95% confidential limits, $n = 500$.

Table 3. Effect of *Clostridium bifermentans malaysia* on *Biomphalaria glabrata*.

Suspension	Dilution	Dose (cells/ml)	% Mortality[a] Snails (72 h) 1	Snails (72 h) 2	Eggs (96 h)
Bacterial cells	1/100	10^7	5.5 (6.0)	0	2.6 (3.7)
	1/1,000	10^6	nd	0	0
Whole culture	1/100	10^7	84.7 (16.6)[b]	93.3 (6.2)	100
	1/1,000	10^6	0	0	0
Supernatant	1/100		84.0 (1.0)	60.0 (22.0)	100
	1/1,000		0	0	0
Acids mixture[c]	1/100		nd	87.5 (7.0)	nd
	1/1,000		0	0	nd
TYG culture medium	1/100		4.0 (4.0)[b]	2.5 (2.0)	nd
	1/1,000		0	0	nd
Control			5.0 (3.3)	13.3 (12.4)	19.3 (17)

[a] Mean (SE) of 4 experiments on (1) individual snails ($n = 96$) and of 3 experiments on (2) group of 10 snails ($n = 60$) per 150 ml and on egg masses ($n = 60$). nd = Not determined.
[b] Mean of 2 experiments.
[c] Mixture of the following acids: acetic, propionic, isovalerianic and isocaproic (when tested alone on individual snails at 1/100 dilution they gave respectively 60, 75, 75 and 25% mortality after 72 h).

excluded *a priori*, the presence of such organic acids is relevant to the observed toxicity.

Clostridium bifermentans serovar. *malaysia* is shown to be toxic to all the mosquito species tested, within a range ca. 10^3–10^5 cells/ml. The higher toxicity is observed on *Anopheles* species and on *Ae. detritus* and *Ae. caspius* larvae sampled from nature. This bacterium is 10 times less toxic to *Culex* species and all strains of *Ae. aegypti* larvae, but with the slowest action on *Aedes* spp. larvae. *Aedes albopictus* is the less susceptible, especially the Taipei strain.

The toxicity of *C.b.m.* is 10 times higher than most of the *B.t.i.* or *B. sphaericus* toxic strains on *An. stephensi*, but 10 times lower on *Cx. pipiens* larvae. On *Ae. aegypti* larvae, *C.b.m.* is 10 times less toxic than *B.t.i.* whereas it is more active than *B. sphaericus* toxic strains (Thiery and de Barjac 1989).

The larvicidal activity of *C.b.m.* is slightly quicker than *B. sphaericus* toxicity, acting from 3 to 16 h proportionally to the concentrations employed, but is still much slower than *B.t.i.* activity. Although the mode of action of *C.b.m.* toxin(s) is unknown, it does not act in the midgut as *B.t.i.* or *B. sphaericus* toxins (Charles, unpublished data), but its host-range is similar to these latter. Its toxicity due to bacterial cells is limited to nematocerous Diptera, as far as presently known, and is closer to the *B.t.i.* spectrum as it is also toxic to *Simulium* sp. (de Barjac et al. 1990).

Although the genus *Clostridium* Prazmowski contains species pathogenic for mammals, no strain of *C. bifermentans* has been reported pathogenetic to mammals (Cato et al. 1986). The fear of using a member of the genus *Clostridium* is similar to the previous one with *Bacillus thuringiensis* Berliner belonging to the same genus as *Bacillus anthracis* Cohn. Anyway *C.b.m.* is not intended to be used as such for vector control because of constraints of anaerobic production and storage instability. Our aim is the search for new entomopathogenic toxin(s) whose genes could be integrated further into other organisms.

ACKNOWLEDGMENTS

We are indebted to E. Frachon for performing the anaerobe cultures. We thank F. Rodhain and P. Brey (Ecologie des systèmes vectoriels, Institut Pasteur) for supplying eggs of *Ae. albopictus*, *An. gambiae* and *Cx. quinquefasciatus*. This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

REFERENCES CITED

de Barjac, H., M. Sebald, J.-F. Charles, W. H. Cheong and H. L. Lee. 1990. *Clostridium bifermentans* serovar *malaysia*, une nouvelle bactérie anaérobie pathogène des larves de moustiques et de simulies. C. R. Acad. Sci. Paris 310:383–387.

Burgerjon, A. 1956. Pulvérisation et poudrage au laboratoire par des préparations pathogènes insecticides. Ann. Epiphyties 4:675–684.

Cato, E. P., W. L. George and S. M. Finegold. 1986. Genus *Clostridium* Prazmowski 1880, pp. 1141–1157. *In:* P. H. A. Sneath, N. S. Mair, E. Sharpe and J. G. Holt (eds.), Bergey's manual of systematic bacteriology, Vol. 2. Williams and Wilkins, Baltimore.

Charles, J.-F., L. Nicolas, M. Sebald and H. de Barjac. 1990. *Clostridium bifermentans* serovar *malaysia*:

sporulation, biogenesis of inclusion bodies and larvicidal effect on mosquito. Res. Microbiol. 141:721–723.

Kalfon, A. and H. de Barjac. 1985. Screening of the insecticidal activity of *Bacillus thuringiensis* against the Egyptian cotton leaf worm *Spodoptera littoralis*. Entomophaga 30:177–186.

Kalfon, A., I. Larget-Thiery, J.-F. Charles and H. de Barjac. 1983. Growth, sporulation and larvicidal activity of *Bacillus sphaericus*. Eur. J. Appl. Microbiol. Biotechnol. 18:168–173.

Larget, I. and H. de Barjac. 1981. Spécificité et principe actif de *Bacillus thuringiensis* var. *israelensis*. Bull. Soc. Pathol. Exot. 74:216–227.

Nicolas, L., H. Hamon, E. Frachon, M. Sebald and H. de Barjac. 1990. Partial inactivation of the mosquitocidal activity of *Clostridium bifermentans* serovar *malaysia* by extracellular proteinases. Appl. Microbiol. Biotechnol. 34:36–41.

Thiery, I. and H. de Barjac. 1989. Selection of the most potent *Bacillus sphaericus* strains based on activity ratios determined on three mosquito species. Appl. Microbiol. Biotechnol. 31:577–581.

Thiery, I., S. Hamon, V. Cosmao Dumanoir and H. de Barjac. 1992. Vertebrate safety of *Clostridium bifermentans* serovar *malaysia*, a new larvicidal agent for vector control. J. Econ. Entomol. (In press).