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A NOVEL INSECTICIDAL SEROTYPE OF *CLOSTRIDIUM BIFERMENTANS*

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ABSTRACT. A novel *Clostridium bifermentans* strain toxic to mosquito larvae on ingestion was isolated from a soil sample collected from secondary forest floor. This strain was designated as serovar *paraiba* (*C. b. paraiba*) according to its specific H antigen. *Clostridium bifermentans paraiba* is most toxic to *Anopheles maculatus* Theobald larvae (LC_{50} = 0.038 mg/liter), whereas toxicity to *Aedes aegypti* (Linn.) (LC_{50} = 0.74 mg/liter) and *Culex quinquefasciatus* Say (LC_{50} = 0.11 mg/liter) larvae was 20 and 3 times lower, respectively. The toxicity to *An. maculatus* larvae is as high as that of *Bacillus thuringiensis* serovar *israelensis. C. b. paraiba* was also found to exhibit significant *per os* insecticidal activity toward adult *Musca domestica* (Linn.).

Aerobic bacteria, viz. *Bacillus thuringiensis* Berliner and *Bacillus sphaericus* Neide, are increasingly being used in the control of dipteran larvae. No obligatory anaerobe toxic to insects was reported until 1990, when, during a nationwide screening program for indigenous microbial agents, a strain of *Clostridium bifermentans* Weinberg and Seguin was isolated from a coastal mangrove swamp soil sample (Lee and Seleena 1990). This *C. bifermentans* has a specific H-antigen and was subsequently designated *C. bifermentans* serovar *malaysia* (*C. b. malaysia*) (de Barjac et al. 1990).

The continuous search for microbial agents has resulted in the isolation of another Gram-positive anaerobic rod-shaped bacterium with a subterminal spore (designated B13) with mosquitocidal activity from a soil sample collected from secondary forest floor. Through a series of biochemical tests, it was identified as *C. bifermentans.* The biochemical characters of this isolate were as follows: fermentation of fructose, glucose, maltose, mannose, ribose, sorbitol; no fermentation of arabinose, cellulobiose, inositol, lactose, mannitol, melibiose, raffinose, rhamnose, salicin, sucrose, trehalose, xylose; no hydrolysis of esculin and starch; hydrolysis of gelatin; production of indole; and no reduction of nitrate. The cellular fatty acid analysis using a phase-gas chromatography, performed at the Pasteur Institute, Paris (WHO Collaborating Center for Entomopathogenic Bacillus), confirmed that B13 is a *C. bifermentans* strain.

The flagellar (H) antigenic structure for strain B13 was serotyped at the Pasteur Institute, Paris, following the protocol described by Thiery and Frachon (1997). The H agglutinating structure of B13, compared by cross reactions with 17 other known *C. bifermentans* serovars, including *C. b. malaysia,* demonstrated that it has a specific H antigen as deduced from the values of agglutinating titers observed with the procedure of de Barjac (1981). The new serovar is designated *Clostridium bifermentans* serovar *paraiba* (*C. b. paraiba*).

The larvicidal activity of this new serovar was determined by conducting bioassays using lyophilized powder of the lysed bacteria according to the WHO protocol (de Barjac and Larget-Thiery 1984). Forty-eight-hour whole cultures of *C. b. paraiba* grown on blood agar plates were scraped with a sterile scalpel and freeze dried. A stock solution was prepared by suspending 50 mg of the lyophilized powder in 10 ml distilled water and was used for further test dilutions. Bioassays were conducted in 200-ml plastic disposable cups containing 150 ml of each test solution. Twenty-five 3rd- to 4th-instar larvae of 3 species of laboratory-bred mosquitoes, *Anopheles maculatus* Theobald, *Culex quinquefasciatus* Say, and *Aedes aegypti* (Linn.), were used for bioassays. Two cups were prepared per concentration (series of 10 concentrations, range 0.0005–2.0 mg/liter according to the tested mosquito), and tests were run in triplicate on different days at 28°C. Two cups of 25 larvae in 150 ml distilled water served as control. All test and control larvae were fed during the assay. The mortality of the larvae was determined after 24 and 48 h continuous exposure. There was no mortality in the control larvae. A probit analysis program (Raymond 1985) was used to analyze the data. Bioassay showed that the *An. maculatus* larvae are the most susceptible, with *Ae. aegypti* being the least susceptible (Table 1). For comparison, the other anaerobic larvicidal bacterium, *C. b. malaysia,* was produced in the same manner as *C. b. paraiba* and bioassayed against the larvae of the 3 species. The trend of susceptibility and the potency of *C. b. paraiba* are very different from those of *C. b. malaysia. Clostridium bifermentans paraiba* is about 10-fold less toxic to *An. maculatus,* but it is toxic to *Cx. quinquefasciatus,* unlike *C. b. malaysia,* which is inactive toward this mosquito under this culture condition. *Clostridium bifermentans malay-*

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Table 1. Larval toxicity of mosquitocidal *Clostridium bifermentans.*

	LC_{50} (mg of dry powder/liter) (95% confidence limit)			
	Clostridium bifermentans serovar *malaysia*		*Clostridium bifermentans* serovar *paraiba*	
Mosquito species	24 h	48 h	24 h	48 h
Anopheles maculatus	0.0026 (0.0018–0.0036)	0.0009 (0.0004–0.0013)	0.038 (0.031–0.047)	0.014 (0.011–0.018)
Aedes aegypti	0.40 (0.28–0.59)	0.12 (0.08–0.16)	0.74 (0.50–1.42)	0.12 (0.019–0.17)
Culex quinquesfasciatus	>3.0	>3.0	0.11 (0.053–0.23)	0.04 (0.018–0.086)

sia has been shown to exhibit some toxicity to *Cx. quinquefasciatus* larvae when grown in TYG liquid medium under anaerobic conditions (Thiery et al. 1992).

As *C. b. paraiba* is most toxic to *An. maculatus* larvae, its potency was also compared to IPS82, the international potency standard for *B. thuringiensis* serovar *israelensis* against *An. maculatus* larvae. Lyophilized powder of IPS82 grown on blood agar plates in similar conditions as *C. b. paraiba* was used for bioassay. Bioassay was done in the same manner as for *C. b. paraiba. Clostridium bifermentans paraiba* was half as toxic to *An. maculatus* as IPS82 (LC_{50} = 0.019 mg/liter).

The spectrum of the *C. b. paraiba* insecticidal toxin activity was also determined by feeding laboratory-bred, less-than-7-day-old adult houseflies, *Musca domestica* Linn., and adult cockroaches, *Blattella germanica* Linn., that had been starved overnight with *C. b. paraiba. Clostridium bifermentans paraiba* cultured in brain heart infusion (BHI) broth was used. A 24-h culture and a 48-h culture (the whole culture, bacterial pellet, and supernatant) were fed to the insects. No mortality was observed in adult *B. germanica,* which were fed with the 24- and 48-h *C. b. paraiba* cultures. This indicates that *C. b. paraiba* does not produce any blatticidal toxic metabolites, unlike *C. b. malaysia,* which was shown to release toxin(s) against adult *B. germanica* in a 24-h BHI culture (Seleena and Lee 1995).

Mortality of 95–100% was observed in adult flies after 72 h continuous feeding with the 48-h *C. b. paraiba* final whole culture and the supernatant (Table 2). Based on these preliminary data, further work to determine the toxicity of *C. b. paraiba* to *M. domestica* adult flies was carried out using the supernatant of a 48-h *C. b. paraiba* culture in BHI. Acetone (1 vol.) was used for overnight precipitation of the muscacidal toxins at 4°C. The precipitate was air-dried and weighed. Various amounts of the precipitate (series of 8 concentrations, range 0.025–0.5 g/ml) were added to the sucrose solution (10%) and fed to 15 adult flies that had been starved overnight in a cage measuring 15 × 15 × 15 cm. The mortality was observed after 72 h of continuous feeding. The results indicated that 0.17 g/ml of the dried acetone precipitate was required to cause 50% mortality in the test population. This study in part shows that the bacterial cells of *C. b. paraiba* are not toxic to *M. domestica.* However, the toxicity could be attributed to the extracellular metabolic by-products that are released into the supernatant by the fermenting *C. b. paraiba* cells.

Table 2. Effect of a 48-h *Clostridium bifermentans* serovar *paraiba* culture in brain heart infusion (BHI) broth on adult *Musca domestica* flies.

C. b. paraiba culture in BHI broth	Mortality after 72 h exposure (%) (mean ± SE)[1]
Whole culture	95.8 ± 5.8
Supernatant	100.0 ± 0
Bacterial pellet	17.5 ± 11.32

[1] Mean of 3 experiments on groups of 15 flies per experiment.

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