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SCIENTIFIC NOTE

AN ISOLATE OF *BACILLUS CIRCULANS* TOXIC TO MOSQUITO LARVAE

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ABSTRACT. A new strain of *Bacillus circulans* isolated from a larva of *Culex quinquefasciatus* showed larvicidal activity on 3 mosquitoes of medical importance. Compared to *Bacillus sphaericus* strain 2362, this *B. circulans* isolate proved less toxic to *Cx. quinquefasciatus* and *Anopheles gambiae* but was 107 times more toxic to *Aedes aegypti.* Moreover, in comparison to other studies, *B. circulans* was at least as pathogenic as *B. thuringiensis* var. *israelensis* in *Ae. aegypti.* The tests have showed that the toxicity of the bacterial culture of *B. circulans* resulted from its spores and not from the insecticidal effect of chitinases or exotoxins.

KEY WORDS *Bacillus circulans,* spores, chitinases, exotoxins, mosquitoes

The entomopathogenic bacteria most virulent to mosquito larvae were discovered by accident, such as *Bacillus thuringiensis* var. *israelensis* isolated from dead larvae of *Culex pipiens* L. in the Negev desert, Israel (de Barjac 1978a), and *Bacillus sphaericus* strain 2362 from an adult black fly in Nigeria (Weiser 1984). These 2 bacilli have shown such a strong entomopathogenic potency that in both cases pathogenicity was equal to the toxicity induced by chemical insecticides. Hundreds of thousands of liters of *B. thuringiensis* var. *israelensis* have been spread each year since 1982 against black fly larvae by the Onchocerciasis Control Program in West Africa (Hougard and Back 1992). A large-scale evaluation of *B. sphaericus* was conducted in Maroua, Cameroon, in 1991. That study proved that this bacillus can be efficient in the control of the urban nuisance caused by *Culex quinquefasciatus* Say (Barbazan et al. 1997). To date, no new entomopathogenic bacteria is operationally used. That is why, faced with the developing resistance to bacterial toxins, as is already the case for *B. sphaericus* (Rao et al. 1995), the World Health Organization has set up reference centers whose activities focus on the search for, isolation of, and identification of new entomopathogenic agents. One of these centers, located at the Centre Pasteur in Yaounde, Cameroon, directed its investigations to finding entomopathogenic bacteria of the genus *Bacillus* (Hougard 1991). From 1990 to 1992, 1,166 sporogenic bacilli were isolated from 385 samples of mosquito larvae and soils collected in the savanna and forest areas of Cameroon. One bacillus proved pathogenic to mosquito larvae (Darriet 1998). This bacillus, extracted from a living larva of *Cx. quinquefasciatus* collected in N'gaoundere, Cameroon, was identified as *Bacillus circulans* (de Barjac, personal communication) by the entomopathogenic bacteria laboratory of the Institut Pasteur of Paris in 1992.

The virulence of *B. sphaericus* against mosquito larvae comes from parasporal inclusions, but also from the toxicity of the spore itself and its wall (Charles et al. 1996). However, which factor was responsible for the insecticidal activity within the whole culture remained to be determined for *B. circulans.* Indeed, some strains of *B. circulans* were proven to release, in culture media, chitinases that could have insecticidal activity (Wiwat et al. 1999). Moreover, some *Bacillus* sp., particularly *B. thuringiensis* strains, produce nonspecific entomopathogenic exotoxins that are found in the supernatant fluid after culture media have been centrifuged (Hernandez et al. 2001). These exotoxins are excreted by the early vegetative cells, whereas the endotoxins (crystal) appear at the sporulation phase.

In the present study, the pathogenic activity of *B. circulans* was compared to that of *B. sphaericus* strain 2362 to assess the potency of *B. circulans.* The cultures of the 2 bacilli were grown from strips of filter paper impregnated with spores inoculated into MBS culture media (Kalfon et al. 1983). The whole cultures were incubated in a steam room at 30°C for 48 h. The media were then diluted in 10^{-2} to 10^{-6} of whole culture aliquots to allow counting of spores. These aliquots were heat-shocked at 80°C for 10 min to destroy the vegetative cells. Then, 0.1 ml of each aliquot was spread on MBS agar and incubated in a steam room at 30°C for 48 h. These dilutions produced a carpet of very distinct colonies that served as inoculums for 5 new MBS agars. Bioassays were performed to determine the toxicity of whole cultures of *B. sphaericus* and *B. circulans* to the larvae of 3 mosquitoes of medical interest: *Cx. quinquefasciatus* (S. Lab strain), *Anopheles gambiae* Giles (Kisumu strain), and *Aedes aegypti* (L.) (Bora-Bora strain). The bioassays followed a WHO standardized protocol (WHO 1999) and the results were expressed in percentages of larval mortality after 48 h of exposure. Median lethal concentration (LC_{50}) and LC_{95} values were also determined

Table 1. Lethal concentrations 50% (LC_{50}) and 95% (LC_{95}) of *Bacillus circulans* and *Bacillus sphaericus* against 3 mosquito species.

Mosquitoes	LC_{50} (95% CL)[1]	LC_{95} (95% CL)[1]	Regression line	
			Slope	P^2
	Bacillus circulans			
Culex quinquefasciatus	17,947 (11,983–23,878)	771,445 (429,415–1,962,782)	1.007	0.11
Anopheles gambiae	14,447 (13,741–15,020)	20,993 (19,767–22,969)	10.14	0.19
Aedes aegypti	13,739 (12,217–15,024)	45,843 (37,692–62,231)	3.14	0.38
	Bacillus sphaericus			
Culex quinquefasciatus	854 (757–963)	3,389 (2,696–4,632)	2.75	0.07
Anopheles gambiae	2,268 (1,991–2,538)	7,013 (5,872–8,975)	3.36	0.51
Aedes aegypti	1.47×10^6 (1.22×10^6–1.79×10^6)	24.58×10^6 (15.43×10^6–46.67×10^6)	1.34	0.17

[1] Expressed in number of spores per milliliter. CL, confidence limit.
[2] If this value exceeds 0.05, the regression line is representative with 95% probability.

for each bacillus (in spores/ml). To assess the action of chitinase enzymes or exotoxins on mosquito larvae, 2 ml of the whole culture of *B. circulans* was centrifuged at 8,000 rpm for 40 min. The supernatant was collected to test the possible activity of the chitinases or exotoxins released in the medium. The pellet of spores was diluted in 2 ml of sterile distilled water then heat-shocked at 80°C for 10 min to destroy the vegetative forms left. Both the supernatant and pellet were diluted at 10^{-3} and tested on the larval stages of *An. gambiae* and *Ae. aegypti* (the 2 species that proved the most sensitive to this bacillus).

For *B. sphaericus,* an average of 63.6 colonies was harvested on 10^{-6} of whole culture, that is, 636×10^6 spores/ml of whole culture. The *B. circulans* dilution at 10^{-4} yielded an average of 198 colonies, that is, 19.8×10^6 spores/ml. The results presented in Table 1 confirmed the toxicity of *B. sphaericus* to *Cx. quinquefasciatus* and *An. gambiae* but indicated a lesser pathogenicity to *Ae. aegypti.* Compared to *B. sphaericus, B. circulans* proved less toxic to *Cx. quinquefasciatus* and *An. gambiae* but much more toxic to *Ae. aegypti* (107 times on the basis of the LC_{50}). Moreover, de Barjac (1978b) estimated the LC_{50} of *B. thuringiensis* var. *israelensis* in *Ae. aegypti* larvae at 24×10^3 spores/ml. The LC_{50} of *B. circulans* on the same species averages 14×10^3 spores/ml, which ranks *B. circulans* at least as pathogenic as *B. thuringiensis* var. *israelensis* in *Ae. aegypti.* After 48 h of exposure, the pellet of spores induced a 96% mortality rate among the larvae of *An. gambiae* and *Ae. aegypti.* However, no mortality was recorded in the supernatant that could have contained chitinases or exotoxins. Therefore, the supernatant does not seem to be toxic to mosquito larvae, which suggests either that it is devoid of chitinases or exotoxins or that they are not potent enough to act at that dilution.

Because no entomopathogenic strain of *B. circulans* had yet been described, it was considered important to add this one to the list of new agents for biological vector control. Analysis of the toxicity data of *B. circulans* suggests that a new toxin may be present that probably is different from those of *B. sphaericus* and *B. thuringiensis* var. *israelensis.* These results suggest that searches for new entomopathogenic bacteria should be continued, and even intensified.

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