

Insecticidal Activity of *Bacillus laterosporus*

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The *Bacillus laterosporus* strains 921 and 615 were shown to have toxicity for larvae of the mosquitoes *Aedes aegypti*, *Anopheles stephensi*, and *Culex pipiens*. The larvicidal activity of *B. laterosporus* was associated with spores and crystalline inclusions. Purified *B. laterosporus* 615 crystals were highly toxic for *Aedes aegypti* and *Anopheles stephensi*.

The toxicity of the entomopathogenic strains of *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus* against mosquitoes is associated with protein crystal production (2, 3, 7, 8). Entomopathogenic activity of *Bacillus laterosporus*, which was proposed to be moved to the genus *Brevibacillus* in a recent taxonomic study (10), has also been reported. The insecticidal activity of the described *B. laterosporus* strains, however, is 100 times less than that of *B. thuringiensis*. Moreover, these *B. laterosporus* strains do not produce crystalline inclusions (6, 9). We have demonstrated the ability of two strains of *B. laterosporus*, 16-92 and LAT 006, to produce crystalline inclusions (11, 12). This observation raises the possibility that crystal-forming strains of *B. laterosporus* may possess a high mosquitocidal activity similar to the activity of *B. thuringiensis* and *B. sphaericus*.

To examine this question, we conducted bioassays with sporulated cells of these two crystal-forming *B. laterosporus* strains. The first strain, *B. laterosporus* 16-92, was isolated from dead insects (13) and was characterized as *B. laterosporus* in accordance with the results of morphological and phenotypical diagnostic tests (14). The second strain, *B. laterosporus* LAT 006, was obtained from the IEBG Collection of the Unité des Bactéries Entomopathogènes (Institut Pasteur, Paris, France). Both strains were grown in Nickerson broth supplemented with D-glucose (1%), methionine (10 µg/ml), and thiamine (10 µg/ml) (pH 7.0) at 30°C with rotary shaking and plated on Nickerson agar. Single colonies were grown in Nickerson broth and selected according to mosquitocidal activity, sporulating capacity, and the ability to produce crystals. For both *B. laterosporus* strains, approximately 200 colonies were screened (data not shown). The colonies with the highest mosquitocidal activity and sporulating capacity and the ability to produce crystals were isolated from strains 16-92 and LAT 006 and designated strains 921 and 615, respectively. They were characterized as *B. laterosporus* in accordance with the results of morphological and phenotypical diagnostic tests (14) and used throughout this study.

The mosquitocidal activities of sporulated cells from *B. laterosporus* 921 or 615 for the second-instar larvae of *Anopheles stephensi* (Liston), *Aedes aegypti* (L.), and *Culex pipiens* (L.) were determined. All tests were carried out at 26°C. For the growth of the test larvae, two meatballs of cat biscuits were

placed in glass cups with dechlorinated tap water. Twenty-five early L2 larvae of mosquitoes were incubated in 100-ml glass cups containing 50 ml of the appropriate dilution of sporulated cells of *B. laterosporus* 921 or 615. Six concentrations of tested samples with serial twofold dilutions were used. Two or four cups of each concentration of the test samples and of the control sample, which contained 50 ml of deionized water only, were used. Larva mortality was determined after 48 h. Each bioassay was performed four or five times with larvae from different batches. The 50% lethal concentrations (LC₅₀s) were determined by probit analysis.

The assessment of the toxicities of *B. laterosporus* 921 and 615 for mosquito larvae was done with cells harvested after 72 h of cultivation in liquid medium. By that time the bacterial culture had been in the last stage of sporulation and contained predominantly spores and crystalline inclusions. The levels of spore formation of *B. laterosporus* 921 and 615 were approximately 60 and 95%, respectively. The mosquitocidal activities of the *B. laterosporus* strains are shown in Table 1.

It is evident from Table 1 that *B. laterosporus* 921 and 615 were more toxic for two species of mosquito, *Aedes aegypti* and *Anopheles stephensi*, and were less toxic for *C. pipiens*. The strains had larvicidal activities similar to those demonstrated by many *B. thuringiensis* strains for *Aedes aegypti* and *Anopheles stephensi*. Depending on the strain and the target species, LC₅₀s of *B. thuringiensis* strains vary from 10⁻³ to 10⁻⁶ (in final whole culture [FWC] dilution), corresponding to 10⁵ to 10² spores/ml (3). The toxic activities of *B. laterosporus* 921 and 615 for *C. pipiens* larvae were considerably less than those of most highly active *B. sphaericus* strains for *Culex* spp. (LC₅₀s for fourth-instar larvae of *C. pipiens* are <10⁻⁶ FWC dilution [2, 4]).

It is known that the mosquitocidal activities of some *B. thuringiensis* and *B. sphaericus* strains are caused by soluble toxins produced in culture medium, similar to Mtx toxins of *B. sphaericus* or Vip3A toxins of *B. thuringiensis* (2, 5). We proposed that *B. laterosporus* might produce similar soluble toxins. According to this proposition, we compared the toxicities of FWCs, supernatant fractions (SFs), and pellet fractions (PFs) of *B. laterosporus* 921 and 615. SFs and PFs were obtained by centrifugation at 20,000 × g for 1 h at 4°C from 72-h Nickerson broth-sporulated cultures of *B. laterosporus* 921 and 615. PFs containing the sporulated cells of strain 921 or 615 were washed three times with distilled water by 15 min of centrifugation at 20,000 × g and finally were suspended in the same volume of distilled water. All of the samples (FWC, SF, and PF) were used in bioassays (Table 2).

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TABLE 1. Toxic activities of *B. laterosporus* 921 and 615 for larvae of mosquitoes^a

Strain	Spores/ml (10 ⁷)	LC ₅₀ ^b for:		
		<i>Aedes aegypti</i>	<i>Anopheles stephensi</i>	<i>Culex pipiens</i>
921	4.0	5.6 × 10 ⁻³ (0.4 × 10 ⁻³ –11.9 × 10 ⁻³)	7.7 × 10 ⁻³ (6.9 × 10 ⁻³ –8.4 × 10 ⁻³)	5.6 × 10 ⁻² (4.6 × 10 ⁻² –13.5 × 10 ⁻²)
615	2.4	5.3 × 10 ⁻³ (4.0 × 10 ⁻³ –6.5 × 10 ⁻³)	0.11 × 10 ⁻³ (0.05 × 10 ⁻³ –0.2 × 10 ⁻³)	4.6 × 10 ⁻² (3.3 × 10 ⁻² –10.4 × 10 ⁻²)

^a Strains were grown in Nickerson broth at 30°C for 72 h with shaking.

^b LC₅₀s are presented as FWC dilutions. The data are averages of four or five assays, with 95% confidence limits, as determined by probit analysis, in parentheses.

As shown in Table 2, the toxic activities of SFs of *B. laterosporus* 921 and 615 for mosquitoes were approximately 10 and 100 times less, respectively, than those of FWCs and PFs. Using light and electron microscopy, we could not detect any spores in the SFs. These results clearly demonstrated that the mosquitocidal activities of *B. laterosporus* 921 and 615 were associated with the sporulated cells rather than with soluble factors present in the culture medium during sporulation. Moreover, the low level of mosquitocidal activity of *B. laterosporus* SF (Table 2), which fails to demonstrate the presence of spores, may reflect the activity of some other soluble factors, e.g., small soluble crystals, rather than the activity of soluble toxins.

The entomopathogenic activities of *B. laterosporus* 921 and 615 may be due to production of crystalline inclusions similar to those of *B. thuringiensis* and *B. sphaericus*. To clarify this question, we analyzed the mosquitocidal activity of purified crystals of *B. laterosporus* 615.

The results of light and electron microscope analysis have demonstrated the capacity of *B. laterosporus* 615 to increase the production of crystalline inclusions when it is grown on Nickerson agar instead of Nickerson broth (data not shown). Therefore, the agar medium was used for the production of crystals of *B. laterosporus* 615. Cells of *B. laterosporus* 615 were grown overnight at 30°C in Nickerson broth. Thereafter, the overnight culture was spread on 1.8% agar surface plates with Nickerson medium supplemented with D-glucose (1%), methionine (10 μg/ml), and thiamine (10 μg/ml). The plates were incubated at 30°C for 5 to 6 days. The sporulated cells and crystalline inclusions of *B. laterosporus* 615 were washed out from the agar surface plates by using a small volume of distilled water. The suspensions obtained from each plate were pooled and incubated with 0.02% Triton X-100 for 1 h at 18 to 20°C and then overnight at 5°C. This pretreated suspension of *B. laterosporus* 615 was sonicated on ice for two 30-s intervals with

30-s pauses at a relative output of 40 kHz with a VCD16-850 sonic disintegrator. The crystalline inclusions of *B. laterosporus* 615 were purified from spores and cell debris by centrifugation in a nonlinear density gradient of 20, 30, 40, and 50% sodium bromide at 150,000 × g for 2 h at 4°C. The resulting fractions were washed three times, suspended in distilled water, and stored at –20°C. Electron microscopy analysis of the obtained fractions for the presence of spores and crystals was also done. According to the electron microscopy examination, the fraction corresponding to 30% sodium bromide was spore free and contained square crystals without clear corners (Fig. 1). For solubilization of the protein crystals, they were treated with 0.2 M NaOH for 1.5 h at 37°C. The protein concentration in the samples was then measured by the Bradford method (1).

The purified crystal fraction was analyzed for toxicity against larvae of the mosquito species *Aedes aegypti* and *Anopheles stephensi*. The purified crystal fraction was toxic for the larvae of *Aedes aegypti* as well as for the larvae of *Anopheles stephensi*. We found no difference in the toxicities for each of these two species of mosquito: the LC₅₀ for *Aedes aegypti* was 3.0 ng/ml with 95% confidence limits of from 1.2 to 4.1 ng/ml, and that for *Anopheles stephensi* was 5.0 ng/ml with 95% confidence limits of from 3.9 to 11.4 ng/ml. It is known that purified, intact parasporal crystals of *B. thuringiensis* subsp. *israelensis* have an LC₅₀ for *Aedes aegypti* in the range of <4 to 20 ng/ml, while the toxicity for *Anopheles stephensi* is less (7). Thus, the insecticidal activity of crystalline inclusions of *B. laterosporus* 615 against

TABLE 2. Toxic activities of culture medium fractions of *B. laterosporus* 921 and 615 for *Anopheles stephensi* mosquito larvae^a

Strain	Sample	Spores/ml (10 ⁷)	LC ₅₀ ^b
921	FWC	1.0	2.2 × 10 ⁻³ (0.8 × 10 ⁻³ –7.9 × 10 ⁻³)
	SF	ND ^c	4.0 × 10 ⁻² (1.1 × 10 ⁻² –10 × 10 ⁻²)
	PF	9.8	2.4 × 10 ⁻³ (0.7 × 10 ⁻³ –7.1 × 10 ⁻³)
615	FWC	2.2	2.2 × 10 ⁻⁴ (1.8 × 10 ⁻⁴ –3.2 × 10 ⁻⁴)
	SF	ND	1.0 × 10 ⁻² (0.7 × 10 ⁻² –2.3 × 10 ⁻²)
	PF	2.1	2.2 × 10 ⁻⁴ (1.1 × 10 ⁻⁴ –7.2 × 10 ⁻⁴)

^a Both strains were grown in Nickerson broth for 72 h.

^b LC₅₀s are presented as FWC dilutions, SF dilutions, and PF dilutions. The data are averages of four or five experiments, with 95% confidence limits, as determined by probit analysis, in parentheses.

^c ND, not detectable (spores were not detectable by light and electron microscopy).

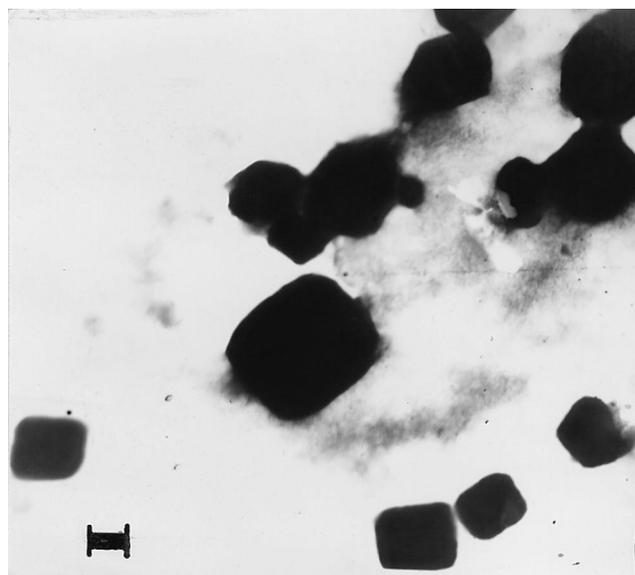


FIG. 1. Pure crystals of *B. laterosporus* 615. Flat square crystals lacking clear corners are predominant. Negative staining was with 1% uranyl acetate. Bar, 100 nm.

larvae of *Aedes aegypti* and *Anopheles stephensi* was similar to the activity of the crystalline inclusions of the highly toxic entomopathogenic strains of *B. thuringiensis* subsp. *israelensis*.

As a result of our studies, it was shown that crystal-forming strains of *B. laterosporus*, i.e., 921 and 615, possessed an insecticidal activity against the second-instar larvae of the mosquitoes *Aedes aegypti*, *Anopheles stephensi*, and *C. pipiens*. The toxic activity of *B. laterosporus* 615 for larvae of the species *Aedes aegypti* and *Anopheles stephensi* was associated with the protein crystals produced during sporulation of *B. laterosporus* 615. The level of toxicity of crystalline inclusions was similar to that of crystalline inclusions of *B. thuringiensis*. The high entomopathogenic activity of *B. laterosporus* 615 indicates that this strain may be a potential candidate for mosquito control.

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