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Toxic Activity of *Bacillus sphaericus* SSII-1 for Mosquito Larvae

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Using larvae of the mosquito *Culex pipiens* var. *quinquefasciatus* as a bioassay system, we have verified an earlier proposal that pathogenicity of *Bacillus sphaericus* SSII-1 is a toxin-mediated rather than an infectious process. Chloroform or ultraviolet-light treatments that decreased the viable count of SSII-1 cells by 4 or 5 logs did not significantly alter the ability of the bacterial cells to kill larvae. Three lines of evidence indicated that toxic activity was not related to sporulation: (i) cells grown in either a complex or a defined medium were toxic at all ages; (ii) when supplemental Mn^{2+} was excluded from a complex medium, the culture yielded few spores but was of equal toxicity to a culture containing many spores; and (iii) several early blocked oligosporogenous mutants were isolated that had toxic activities comparable to that of the parent. The toxin was shown to be relatively unstable because activity was destroyed by heat and decreased by refrigeration, a freeze-thaw cycle, or two methods of cell breakage. Thin sections of SSII-1 cells did not reveal the presence of any inclusion body that might be related to toxicity.

In 1965, Kellen et al. (5) described the isolation of a strain of *Bacillus sphaericus* that exhibited larvicidal activity against 10 species of mosquitoes. The strain was isolated from a moribund fourth-instar larva of the mosquito *Culiseta incidens*. More recently (7, 8) a new, more pathogenic strain of *B. sphaericus*, SSII-1, was described. Infection of larvae by *B. sphaericus* SSII-1 takes place via the alimentary canal, but histological studies (1) indicated that bacterial invasion of the hemocoel did not occur until after larval death. These observations led Davidson et al. to propose that toxic material was released when the bacterial cells were digested and that bacterial multiplication in the larval gut was not essential to the pathogenesis (1). Evidence that the postulated toxic substance is cell associated rather than an excreted metabolite came from the finding of toxicity in the cell pellet rather than in the supernatant of a centrifuged culture (8).

Before our studies, no growth curve had been published that would allow one to relate toxicity to a phase of the bacterial growth cycle or stage of sporulation. Consequently, we began by investigating this relationship. In addition, this study sought to verify the proposal that larval pathogenesis is toxin mediated, to examine the relative stability of the toxin, and to determine if a parasporal body or special inclusion body was produced by toxic SSII-1 cells.

MATERIALS AND METHODS

Bioassay. Before addition to bioassay cups, the bacterial cells were removed from a volume of growth medium by centrifugation and resuspended in an equal volume of sterile, dechlorinated tap water. Tap water was dechlorinated by passage over a column of activated carbon and contained approximately 50 μg of residual chlorine per liter. For all bioassays, 10 second-instar larvae of *Culex pipiens* var. *quinquefasciatus* (*C. pipiens fatigans*) were suspended in a total volume of 20 ml of dechlorinated tap water in plastic cups. Routinely, three cups (or 30 larvae) were tested per 10-fold dilution of bacterial cells, with 10 cups each containing 10 larvae serving as controls. Larval death was recorded daily. A 0.1-ml amount of 10% (wt/vol) debittered yeast was added to each cup on days 1 and 3 of the 4-day test period. Toxic activity is reported as LC_{50} values, that is, the concentration of bacterial cells that killed 50% of the test insect population in 4 days. For clarity, the LC_{50} is expressed in two forms as follows: (i) in terms of the dilution of cell suspension and (ii) in terms of the number of viable cells that the LC_{50} value represents. The LC_{50} in terms of dilution was calculated by the method of Hoskins (4) after appropriate corrections for control death were made with the Abbots formula (2). For emphasis, the dilutions are reported with positive exponents; thus, an LC_{50} value of 1×10^4 would mean that a dilution of 1 to 10,000 of cell suspension would kill 50% of the larval population. The LC_{50} value in terms of number of viable cells was determined by dividing the number of viable cells by the LC_{50} dilution.

Culture medium and growth conditions. *B. sphaericus* SSII-1 was obtained from Samuel Singer,

Western Illinois University. Bacteria were cultivated in a complex medium (NYSM) containing nutrient broth (Difco) supplemented with 0.05% yeast extract, 5×10^{-5} M $MnCl_2$, 7×10^{-4} M $CaCl_2$, and 1×10^{-3} M $MgCl_2$. A 5-ml amount of broth was inoculated from slants and incubated statically overnight at 33°C. A 5% (vol/vol) inoculum was used to inoculate flasks, which were shaken at 200 rpm in a New Brunswick model G25 shaker at 33°C. To assure an inoculum of young cells, vegetative cells were transferred three times at approximately 2-h intervals (as the turbidity reached an absorbance of 0.06 at 660 nm) before the final flasks were inoculated. For cell cycle experiments, cells of various ages were harvested by centrifugation and resuspended in sterile, dechlorinated tap water to a constant absorbance of 0.4 at 660 nm. This corresponded to 5×10^8 to 7×10^8 cells per ml. The cell suspensions were then diluted into each bioassay cup. The total number of viable cells added to each assay was determined by plating dilutions of the cell suspensions in NYSM agar. The number of heat-stable spores was determined by heating 1 ml of culture for 12 min at 80°C and sonically treating the heated sample for 2 min to declump spores before performing plate counts.

For studies in which cells were grown in a chemically defined medium, bacteria were cultivated in SSM broth (Samuel Singer, personal communication) that contained (per 100 ml): $MnSO_4 \cdot H_2O$, 1 mg; $MgSO_4 \cdot 7H_2O$, 10 mg; $CaCl_2$, 10 mg; ethylenediaminetetraacetic acid, 10 mg; biotin, 0.1 mg; thiamine hydrochloride, 1 mg; nicotinic acid, 1 mg; calcium pantothenate, 1 mg; DL-lysine, 100 mg; DL-isoleucine, 100 mg; DL-valine, 100 mg; DL-methionine, 100 mg; L-glutamic acid, 600 mg; glycerol, 1 ml; K_2HPO_4, 100 mg; and tris(hydroxymethyl)aminomethane, 1.21 g. The pH was adjusted to 7.15 before use.

Ethyl methane sulfonate mutagenesis. Oligosporogenous mutants of SSII-1 were obtained by adding 0.4 ml of ethyl methane sulfonate (Eastman Kodak Co.) to 10 ml of an 8-h shaken culture and incubating the mixture for 30 min at 33°C with shaking at 200 rpm. The cells were sedimented by centrifugation, resuspended in 10 ml of sterile MYSM broth, and grown with shaking at 33°C for 3 h before being plated on NYSM agar. The plates were examined for translucent colonies after 48 h of incubation at 33°C. These colonies were picked, restreaked several times, and grown with shaking in NYSM broth to determine sporulation frequencies.

Chloroform and UV light treatments. To produce chloroform-treated cultures, cells of *B. sphaericus* SSII-1 and ATCC 14577 were grown for 8 h in the NYSM broth without the metal supplement and harvested by centrifugation. After resuspension in sterile, dechlorinated tap water, 5 ml of cells was mixed with 1.2 ml of chloroform and shaken at 200 rpm at 33°C for 5 min. The chloroform and the aqueous layer containing the cells were allowed to separate for 5 min, the aqueous layer was decanted, and the residual chloroform was removed by bubbling air through the broth for 20 min.

To produce ultraviolet (UV)-treated cultures, cells of ethyl methane sulfonate mutant SSII-1-30 were grown for 3 h and harvested as above. A total of 10 ml of cell suspension in dechlorinated tap water was incubated in 100-mm-diameter plastic petri plates with constant stirring under a UV lamp (30,000 ergs/cm^2 per s). After a 1-min exposure, the cells were diluted and added to the bioassay cups. In both the chloroform and UV experiments, bacitracin (100 μg/ml, final concentration) and rifampin (1 μg/ml) were added to inhibit replication of the surviving cells in the assay cups. These levels of each antibiotic had been shown to exceed the minimum inhibitory concentration for *B. sphaericus* SSII-1.

Toxin stability. The effect of storage on toxic activity was investigated by bioassaying cells that had been grown with shaking for 8 h in NYSM broth before and after 2 weeks of refrigeration (4°C) or freezing in sterile, dechlorinated tap water. The effect of two methods of cell breakage on toxic activity was also investigated. The cells were passed once through a cold French pressure cell at 20,000 lb/in^2, and the resultant suspension was assayed for toxic activity. Cells were also broken by sonic treatment on ice (10 1-min treatments) with a model 150 Virsonic cell disrupter operating at a power setting of 60. The probe was cooled in ice between treatments.

Electron microscopy. Eighteen-hour cells of the SSII-1 parent and oligosporogenous mutants were fixed by the method of Kellenberger et al. (6) and embedded in Epon 812. All samples were cut with a diamond knife, and the thin sections were stained with 2% uranyl acetate and 0.4% alkaline lead citrate before examination in a Jeolco 100-B electron microscope operating at 80 kV.

RESULTS AND DISCUSSION

In 1973, Singer (7) published studies of a new isolate of *B. sphaericus*, strain SSII-1, that was reported to be pathogenic for mosquito larvae. The lethal factor was shown to be cell associated rather than being present in the supernatant of a broth culture (8). We have confirmed the above observations of toxic activity of *B. sphaericus* SSII-1. Thus, when SSII-1 was grown in NYSM broth, LC_{50} values of approximately 3×10^5 cells per ml were observed. Larval death was observed as early as 12 h after the addition of the bacterial cells. Filter-sterilized culture supernatants were inactive, as were cells of nontoxic strains ATCC 14577 and ATCC 7054.

Our investigations into the relationship of toxin synthesis and the bacterial growth cycle yielded unexpected results. It has been reported that insecticidal activities of *B. sphaericus* "first appear during the initial stages of sporulation" (8) and that "activity is detectable after growth and vegetative division of the cells have ceased" (7). However, the data shown in Fig. 1 indicate that the lethal factor is associated with cells of all ages, and its synthesis is not a sporulation-related event. As indicated above, the inoculum for these experiments contained cells that had been transferred three times at 2-h intervals to assure a population of vegetative-phase cells.

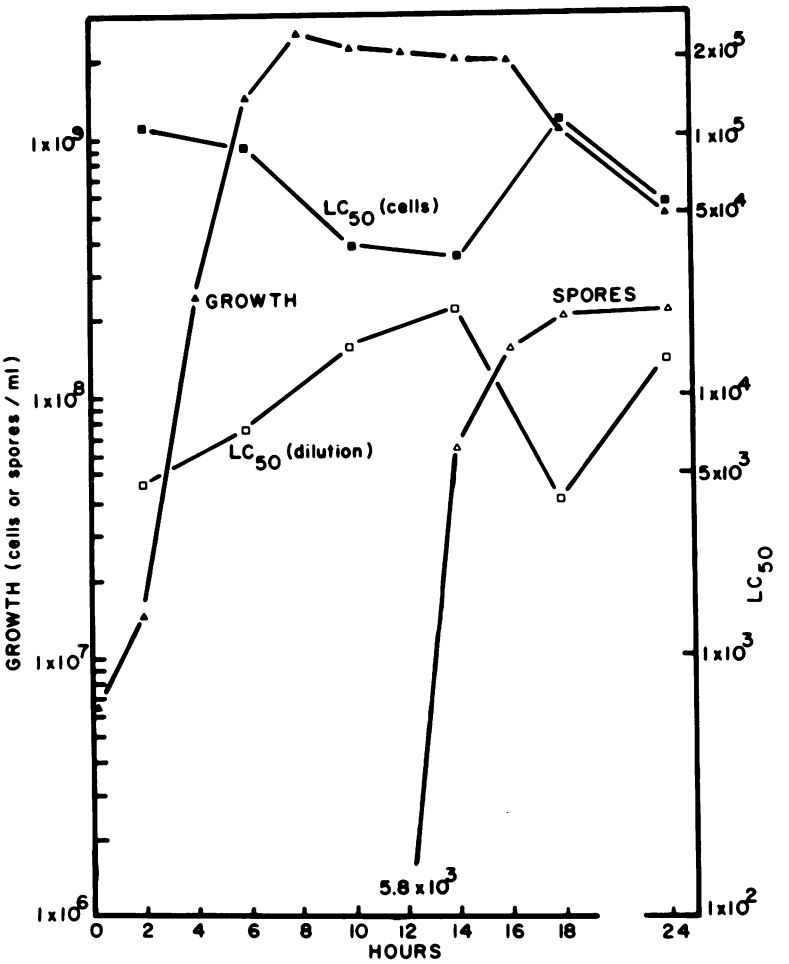

FIG. 1. *Relationship of toxic activity and cell age of SSII-1 cells grown in a complex medium. Symbols: LC_{50} values expressed in terms of dilution of cells (□); LC_{50} values expressed as the number of viable cells present at that dilution (■); growth curve indicating the approximate stage of growth at which the cells were tested for toxic activity (▲); and spore counts indicating the degree of sporulation of the culture when assayed for toxic activity (△).*

Because the genes for sporulation are derepressed at the end of exponential growth (3), the 2- and 6-h cells would not have entered the sporulation sequence, and yet they were about as toxic as older cells. If the SSII-1 toxin is a product or result of the sporulation cycle as has been suggested (7), then one would expect a several-log increase in toxic activity as the vegetative cells enter sporulation. Further, if the lethal factor is associated with sporulating cells only, then the logarithmic increase in activity should occur after the cells have completed exponential growth, i.e., at 6 to 10 h. At this time, the vegetative cells present would have entered the sporulation sequence to produce 2.0×10^8 heat-stable spores per ml by 18 h (as compared with 5.8×10^3 per ml present at 12 h). Although there was a several-log increase in the number of spores between 12 and 18 h (as a result of the conversion of approximately 2.0×10^8 sporulating cells to heat-stable spores), there was not a corresponding several-log increase in toxic activity at 10 h as these cells entered sporulation. Similar results were observed when cells were grown in SSM broth.

Further evidence that toxicity is not related to sporulation came from studies on the exclusion of a metal supplement from the growth medium of SSII-1. Because the Ca^{2+}, Mg^{2+}, and Mn^{2+} present in NYSM broth are required for good sporulation, the exclusion of this supplement from the medium inhibited sporulation

and yielded a population composed largely of vegetative cells and few heat-resistant spores. However, this culture was of equal toxicity to a population of cells containing many more spores (Table 1).

A third line of evidence that toxic activity is not related to sporulation came from studies with oligosporogenous mutants of SSII-1. By using ethyl methane sulfonate as a mutagen, we isolated mutants that sporulated at a very low frequency but that were of comparable toxicity to the parent, which produced many more spores (Table 2). Figure 2 is an electron micrograph of a thin section of ethyl methane sulfonate mutant SSII-1-30 after 18 h of growth. The lack of an axial nuclear filament or forespore membrane suggests that the mutational block is early in the sporulation sequence, probably at stage 0 or I. Examination of many sections of the oligosporogenous mutants indicated that all are blocked at stage 0 or I. Thus, if sporulation is inhibited either nutritionally by excluding the metal supplement or genetically through mutation, the cells are of comparable toxicity to preparations containing many sporulating cells.

It was unknown whether replication of bacterial cells in the bioassay cups occurred before

TABLE 1. *Effect of a metal supplement on toxic activity and sporulation of B. sphaericus SSII-1*[a]

Metal supplement	Viable cells/ml	Spores/ml	LC_{50} in terms of:	
			Dilution	Cells/ml
Mg^{2+}, Ca^{2+}, Mn^{2+}[b]	1.7×10^9	6.5×10^7	3.5×10^3	4.9×10^5
None	1.6×10^9	1.0×10^4	3.2×10^3	5.0×10^5

[a] Cells were grown for 14 h in nutrient broth (Difco) supplemented with 0.05% yeast extract.

[b] Concentrations: 1×10^{-3} M $MgCl_2$, 7×10^{-4} M $CaCl_2$, and 5×10^{-5} M $MnCl_2$.

TABLE 2. *Toxic activity of oligosporogenic mutants of B. sphaericus SSII-1*[a]

Strain	Viable cells/ml[b]	Spores/ml	LC_{50} in terms of:	
			Dilution	Cells/ml
SSII-1	2.9×10^9	5.5×10^7	1.2×10^4	2.5×10^5
SSII-1-a-1	6.2×10^8	1.9×10^3	1.3×10^4	4.6×10^4
SSII-1-17	9.5×10^8	2.5×10^4	5.7×10^3	1.7×10^5
SSII-1-14	5.6×10^8	5.1×10^3	3.0×10^4	1.9×10^4
SSII-1-30	3.0×10^8	6.0×10^1	4.0×10^3	7.5×10^4

[a] Cells were grown with shaking for 16 h in NYSM broth.

[b] Petroff microscopic counts and absorbance measurements indicated that the oligosporogenic mutants grew to about the same total populations as the parental strain. Lower viable counts reflect death of many mutant cells upon failure to sporulate.

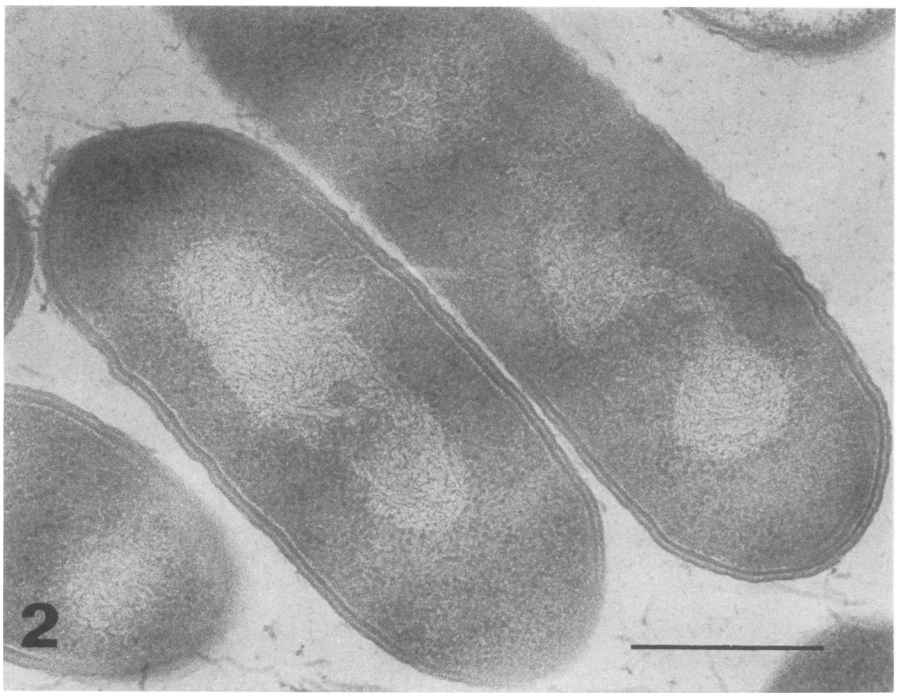

FIG. 2. *Thin section of 18-h cell of oligosporogenous mutant SSII-1-30. Note the lack of forespore membrane and axial nuclear filament. Bar, 0.5 μm.*

the expression of larval death. To determine if replication was related to activity, antibiotic was added to the bioassay cups at levels that would inhibit bacterial replication but that had no effect (data not shown) on normal larval growth and development. Table 3 shows that the LC_{50} values for assays containing bacitracin and rifampin were equivalent to assays without antibiotic additions. These data indicate that replication of the bacteria in the assay system did not occur before the expression of insecticidal activity.

If larval pathogenesis is a toxin-mediated rather than an infectious process, dead but intact bacterial cells should be as lethal to larvae as living cells. To test this, cells of SSII-1 and of the oligosporogenous mutant SSII-1-30 were treated with chloroform and UV radiation, respectively. After these treatments, which destroyed vegetative cells but not spores, the bacterial cells were added to assay cups containing antibiotic to inhibit replication of the surviving bacterial spores. The data (Table 4 and 5) suggest that dead cells are of equal toxicity to viable cells. Thus, chloroform treatment of the culture decreased the viable count by several logs but did not decrease the LC_{50} of the preparation (Table 4). No larval mortality was evidenced in the control cups containing chloroform-treated cells of the nontoxic strain 14577 or chloroform-treated water controls. This indicates that the mortality observed with chloroform-treated SSII-1 was not due to residual chloroform but rather to activity associated with the cells themselves. These data parallel the observations of Singer (8). We have extended this type of experiment to include treatments with UV radiation and obtained similar results (Table 5). Treatment of cells with UV radiation for 1 min lowered the viable count by approximately 5 logs, but it had little effect on the LC_{50}. Further, if the LC_{50} is expressed in terms of the number of viable cells, it apparently took only seven viable cells to kill 50% of the insect population. Because we have previously shown that approximately 5×10^5 cells per ml of the same culture is needed to obtain an LC_{50}, it is obvious that dead cells produced by UV treatment were contributing to insecticidal activity. Because of the presence of antibiotic in the assay, the observed LC_{50} values were not due to growth by the surviving bacterial cells in the cups. These data, combined with the observations of Davidson et al. (1) that bacterial invasion of the larval hemocoel did not occur until after the host was dead, support the suggestion that larval pathogenesis is a toxin-mediated and not an invasive phenomenon.

The toxin of *B. sphaericus* SSII-1 was shown to be heat labile (Table 6). Heat treatment of the culture for 15 min at 80°C eliminated vegetative bacteria as well as toxic activity. Note that, although this preparation contained 1.9×10^7 heat-stable spores per ml, they are apparently nontoxic. This would suggest that the toxin is not enclosed within the spore at a location that would render it heat resistant.

Because it has been reported that the toxin of SSII-1 is relatively unstable (1), studies were undertaken to investigate the effects on activity of various treatments that might be used in

TABLE 3. *Effect of added antibiotic on toxic activity of B. sphaericus SSII-1*

Addition	Viable cells/ml	LC_{50} in terms of:	
		Dilution	Cells/ml
None	3.2×10^9	4.5×10^3	7.0×10^5
Bacitracin and rifampin[a]	3.2×10^9	3.5×10^3	9.1×10^5

[a] Bacitracin was added at a final concentration of 100 μg/ml and rifampin was added at 1 μg/ml to bioassay cups.

TABLE 4. *Effect of chloroform on toxic activity and total viable count of B. sphaericus SSII-1*[a]

Treatment	Viable cells/ml	LC_{50} in terms of dilution
None	2.7×10^9	6.9×10^3
Chloroform	7.4×10^4	3.2×10^3

[a] Cells were grown for 8 h in NYSM broth without the metal supplement. Bacitracin was added at a final concentration of 150 μg/ml and rifampin was added at 1 μg/ml to bioassay cups.

TABLE 5. *Effect of UV radiation on toxic activity and total viable count of B. sphaericus SSII-1-30*[a]

Treatment	Viable cells/ml	LC_{50} in terms of:	
		Dilution	Cells/ml
None	1.45×10^8	2.7×10^2	5.4×10^5
1-min UV	1.31×10^3	1.8×10^2	7.3

[a] Cells were grown for 3 h in NYSM broth without the metal supplement. Bacitracin was added at a final concentration of 100 μg/ml and rifampin was added at 1 μg/ml to bioassay cups.

TABLE 6. *Effect of heat on toxic activity and total viable count of B. sphaericus SSII-1*[a]

Treatment	Viable cells/ml	Spores/ml	LC_{50} in terms of:	
			Dilution	Cells/ml
None	1.8×10^9	2.0×10^7	3.5×10^3	4.9×10^5
80°C, 15 min	1.9×10^7	1.9×10^7	NA[b]	NA

[a] Cells were grown for 14 h in NYSM broth. Bacitracin was added at a final concentration of 100 μg/ml and rifampin was added at 1 μg/ml to bioassay cups.
[b] NA, Not active.

procedures for toxin isolation. Table 7 shows that about 1 log of activity was lost when cells were frozen or refrigerated for 2 weeks. Similarly, when the cells were broken via one pass through a French pressure cell or by sonic treatment, a 1-log drop in activity was observed. It is interesting that the decrease in total viable cells that occurred as a result of the various treatments did not directly parallel the decrease in the LC_{50}, suggesting again the role of a labile toxin in larval pathogenesis.

Figure 3 shows an electron micrograph of a thin section of an 18-h cell of *B. sphaericus* SSII-1 that has sporulated. Sections of cells grown in NYSM or SSM broth were examined. The noteworthy observation is the apparent lack of a toxic inclusion such as the parasporal body of *B. thuringiensis*. This protein crystal is produced at the time of sporulation and is responsible for the toxic activity of *B. thuringiensis* for *Lepidoptera* larvae.

TABLE 7. *Effect of various treatments on toxic activity of B. sphaericus SSII-1*[a]

Treatment	Viable cells/ml	LC_{50} in terms of dilution
Untreated	2.8×10^9	6.9×10^3
Cold storage	2.1×10^7	4.3×10^2
Freeze-thaw	4.6×10^8	3.2×10^2
1 Pass French pressure cell	1.6×10^7	3.0×10^2
Sonic disruption	4.4×10^8	3.1×10^2

[a] Cells were grown for 8 h in NYSM broth without the metal supplement.

In summary, the pathogenicity of *B. sphaericus* SSII-1 for mosquito larvae appears to be a toxin-mediated rather than an invasive phenomenon. Toxic activity is associated with cells of all ages and is apparently not a sporulation-related event in strain SSII-1. Some preliminary evidence indicates that this may not be true in all toxic strains. Filter-sterilized culture supernatants are inactive, indicating that the cells themselves contain the lethal factor. The toxin is relatively unstable in strain SSII-1, as activity is destroyed by heat and decreased by refrigeration, a freeze-thaw cycle, or two methods of cell breakage. Unlike *B. thuringiensis*, the toxin is apparently not associated with a toxic inclusion body.

We are presently examining the structural components of the cell to determine the location of the toxic substance. Comparative ultrastructural studies and biochemical analyses of toxic and nontoxic strains may point to differences between the two. A combination of structural information and bioassay results should allow us

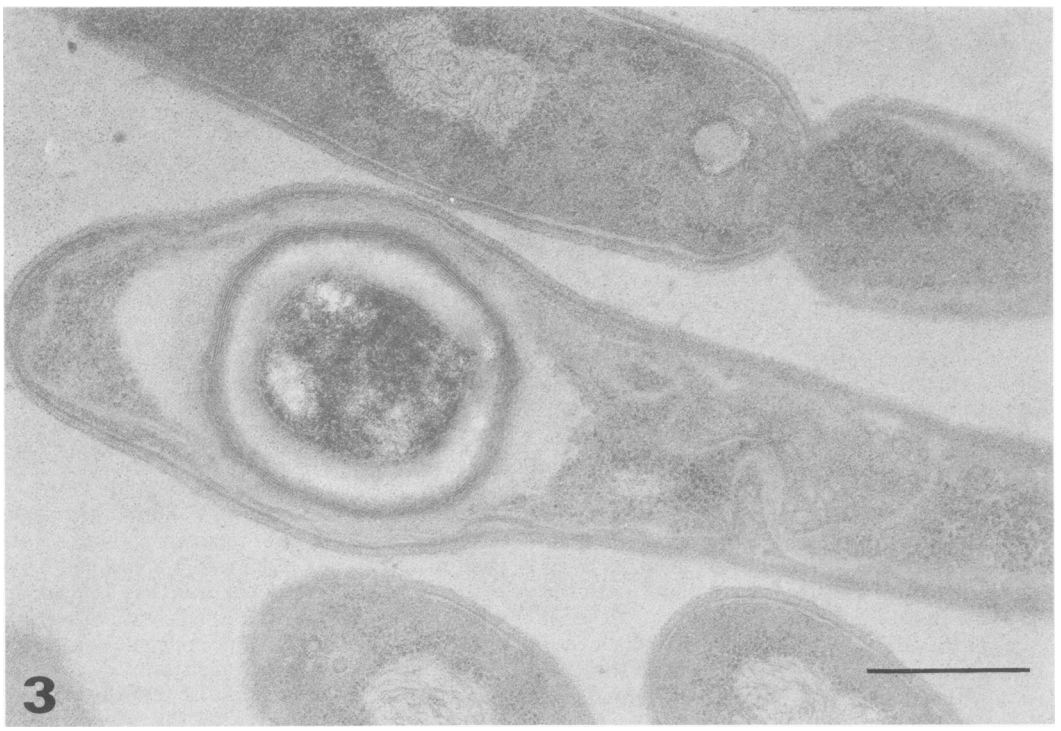

FIG. 3. *Electron micrograph of a thin section of an 18-h cell of SSII-1 that has sporulated. Note the lack of a parasporal body. Bar, 0.5 μm.*

to locate the site of toxin in or on the cell and facilitate fractionation and purification of the toxic substance.

ACKNOWLEDGMENT

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