Characterization and Biological Activity of a Brazilian Isolate of *Bacillus sphaericus* (Neide) Highly Toxic to Mosquito Larvae

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Primary powders of Bacillus sphaericus *strain S2 isolated from soil samples in Brazil, and strain 2362 were produced in a 14 liter fermentor. Growth patterns and sporulation observed in three trials with strains S2 and 2362 in the fermentor were similar. Second-instar larvae of* Culex quinquefasciatus, Anopheles albimanus, Anopheles quadrimaculatus, *and* Aedes aegypti *exposed for 48 hr to strain S2 responded with* LC_{50} *values of 0.25, 5.95, 12.28 and 140.0 ppb of lyophilized primary powder, respectively. Under the same conditions, strain 2362 resulted in* LC_{50} *values of 0.39, 7.16, 16.93 and 307.0 ppb of lyophilized primary powder, respectively, in those mosquito larvae. Statistical analysis of the bioassay data did not show significant differences among* LC_{50} *values observed in* B. sphaericus *strains S2 and 2362, at the 0.05 level.*

Toxins of strains S2 and 2362 were extracted at pH 12 with NaOH. Electrophoresis of the extracts in polyacrylamide gel under denaturing conditions revealed the 51 and 42 kDa toxins in both S2 and 2362 B. sphaericus *strains. The presence of the 42 kDa peptide in the extracts was confirmed by Western blot and Elisa, with anti-42 kDa IgG previously prepared from strain 2362.*

Key words: *Bacillus sphaericus* - mosquitoes - biological control - larvicide

Bacillus sphaericus has become an alternative agent for microbial control of mosquitoes since the isolation of highly larvicidal strains of this bacteria. Strain 2362, isolated from *Simulium* in Nigeria (Weiser 1984), is not toxic to black flies, but it is regarded as the most promising isolate for field use against mosquitoes (WHO 1985). Indeed, several authors have demonstrated the efficacy of strain 2362 against field populations of mosquitoes from the genera *Culex*, *Anopheles*, *Psorophora*, and some members of the genus *Aedes*. *Ae. aegypti* and *Ae. albopictus* are insensitive to *B. sphaericus* (Mulla et al. 1986, 1987, 1988a, b, Lacey et al. 1986, 1988, Nicolas et al. 1987, Berry et al. 1987, Arredondo-Jiménez et al. 1990, Karch et al. 1990). Abbott Laboratories has recently formulated a commercial product (Vectolex) of *B. sphaericus* 2362. Generally, *B. sphaericus* strains with high larvicidal activity have been isolated from dead insects (e.g., Weiser 1984, Lysenko et al. 1985, de Barjac et al. 1988, Gharib et al. 1989). However, five isolates of *B. sphaericus* from soil samples in Israel have been reported to belong to phage group three and were found to be as toxic as strain 2362 to *Culex* sp. larvae (Brownbridge & Margalit 1987). During a screening for entomopathogenic bacteria in soil samples carried out at CENARGEN/EMBRAPA in Brazil, several *B. sphaericus* isolates were obtained. One of these isolates (S2) showed high larvicidal activity in bioassays with *Cx. quinquefasciatus* larvae (Schenkel et al. 1988). In this work, the larvicidal activity of the Brazilian isolate of *B. sphaericus* has been compared with strain 2362 in larvae of the mosquitoes *Cx. quinquefasciatus*, *An. albimanus*, *An. quadrimaculatus*, and *Ae. aegypti*.

MATERIALS AND METHODS

Bacterial isolates - *B. sphaericus* S2 was isolated from a soil sample in Brazil (Schenkel et al. 1988). The procedures outlined in the WHO protocol (1985) for selective isolation of insecticidal *B. sphaericus* from soil samples were employed. *B. sphaericus* 2362 was provided by Dr Howard Dulmage (USDA-Brownsville, Texas). Both strains were preserved at 4-8°C as lyophilized cultures grown in NYSM medium (Myers & Yousten 1978). The Brazilian strain was characterized by Dr Allan Yousten as belonging to phage type three, which is the phage group of strain 2362 and other strains highly toxic to mosquito larvae.

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Growth in fermentor - A loopfull of lyophilized culture of each strain was seeded into 250 ml Erlenmeyer flasks with 50 ml of MBS medium (Kalfon et al. 1983) which contains per liter tryptone 10 g, yeast extract 2 g, $MgSO_4$ 0.3 g, $CaCl_2$ 0.2 g, $Fe_2(SO_4)_3$ 0.02 g, $MnSO_4$ 0.02 g and $ZnSO_4$ 0.02 g. Cultures were incubated in G25 New Brunswick rotary shakers at 30°C, 250 rpm. At late exponential phase, 100 ml of culture was inoculated into a New Brunswick 14 liter fermentor with 10 liters of MBS broth (1% v/v). Batch fermentation was carried out at 30°C, 600 rpm with air flow (1.0 liter/liter of broth/min) for 24 hr. Growth was monitored by reading of absorbance at 660 nm. Samples taken every 2 hr were filtered onto 0.45 µm Millipore membranes, dried in an oven at 80°C for 24 hr and used for dry weight determination. Although the pH of the broth was not controlled, the pH of the samples was recorded. Foam production during growth in the fermentor was reduced by the addition of silicone based antifoam (Sigma) previously diluted at 10% v/v. The cultures were maintained in the fermentor for an additional 24 hr, without aeration and agitation. The final broth was centrifuged in a continuous flow system (Sorvall) at 10,000 g with a flow rate of approximately 30 ml/min. The resulting creamy pellets were freeze dried. Spore counts were made by plating serial dilutions from 10 mg/ml suspensions of the dried cultures (primary powder). Before plating in solid MBS medium the samples were heated 15 min at 80°C and sonicated 1 min to unclump heat resistant spores. Three primary powders were produced for each strain (S2 and 2362).

Bioassay conditions - The protocol suggested in the WHO report (1985) was used with slight modifications to compare the toxicity of the primary powders against different mosquito hosts. The mosquito species *Ae. aegypti*, *An. albimanus*, *An. quadrimaculatus*, and *Cx. quinquefasciatus* used in the bioassays were obtained from lab reared colonies at the USDA, Gainesville. One percent (w/v) stock suspensions of primary powder in deionized water were diluted to obtain seven concentrations with five replicates in each bioassay. Twenty second-instar larvae were placed per each 100 ml disposable plastic cups filled with the test spore suspension, and five cups were left untreated as controls. A small amount of powdered dog chow was added to each cup. As an alternative, bioassays with *Ae. aegypti* were run in 24 well plates, with 10 first instar larvae per well, three replicates for each concentration tested, to minimize the consumption of primary powder. Bioassays of the first primary powder of each strain were replicated three times. The remaining batches were bioassayed only once for each mosquito species. Mortality was scored after 48 hr of incubation at room temperature (25°C) and the results were submitted to log-probit computer analysis (POLO-PC).

Toxin studies - Spore suspensions (10 mg/ml) were incubated in 50 mM NaOH, pH 12, for 2 hr with the alkaline extraction procedure previously described (Davidson 1983). After centrifuging for 15 min at 10,000 g at 8°C, the supernatant was filter sterilized and kept under refrigeration. Toxin was precipitated by the addition of sodium acetate buffer pH 4.0, and the pellets obtained after centrifugation (10,000 g, 20 min) were suspended in 0.1 M Tris. BCA reagent from Pierce Laboratories was used for protein determination in the alkali extracts. The toxin titer of each batch was estimated by ELISA (Engvall & Perlman 1971) with antisera against the 42 kDa toxin from *B. sphaericus* 2362 provided by Dr Elizabeth Davidson. Solubilized proteins were visualized in SDS-PAGE, electrotransferred onto nitrocellulose membrane by semi-dry system, and exposed to the antisera previously mentioned (dilution 1/1000).

RESULTS

The use of MBS medium to grow *B. sphaericus* S2 and 2362 in this study confirms previous observations about the efficacy of such a complex medium to produce larvicidal strains of *B. sphaericus* with high toxin yields (Kalfon et al. 1983). It was noticed, however, that a rather high level of foam was generated during exponential growth phase, and in late sporulation stage (lysis of sporangium).

The growth curves of the *B. sphaericus* strains 2362 and S2 in the 14 liter fermentor, showed similar profiles (Fig. 1). The data of dry weight on time collected from the fermentation runs was analyzed using paired t-test. The average dry weight observed for each strain (S2 1 vs S2 2, p=0.4049; 2362 1 vs 2362 2, p=0.1681), as well as the averages between strains, do not differ significantly at the 0.05 level (Avg S2 vs Avg 2362, p=0.1033). Both strains reached stationary growth phase at 12-14 hr, and completed sporulation at 24 hr, with many of the sporangia lysed, liberating free spores with attached parasporal bodies. In all of the fermentation runs change in pH showed a similar pattern, rising in the beginning of the log phase (around 4 hr), to reach about 8.5-8.6 at the end of growth (Fig. 1). Cell counts using a Petroff-Hauser counting chamber resulted in more than 10^9 cells per ml at 24 hr in all trials (data not shown). Spore counts of the primary powders presented in Table I indicate that strains S2 and 2362 sporulate at the same levels in MBS medium. The results of the ELISA of the alkali extracts of the cell preparations reflect the units of OD_{405}/ mg of primary

powder (Table I). Table I also presents the results of the bioassays of individual fermentation that runs against the most susceptible species *Cx. quinquefasciatus* and *An. albimanus*. A poor correlation between the 42 kDa toxin titer measured by ELISA and the larvicidal potency of the primary powders has been observed. The variation among the LC data (50 and 90%) of the primary powders is not statistically significant at the 0.05 level. Paired and independent t-test conducted with the bioassay data (LCs) showed that S2 and 2362 means are not different at the 0.05 level (*Cx. quinquefasciatus* p=2.54, and *An. albimanus* p=1.54).

Sets of bioassay data obtained for individual primary powders were combined per strain. The larvicidal activity along with the mosquito species tested is summarized in Table II. In all the hosts the LC_{50} and LC_{90} of strain S2 is lower than those observed for strain 2362. However, the difference

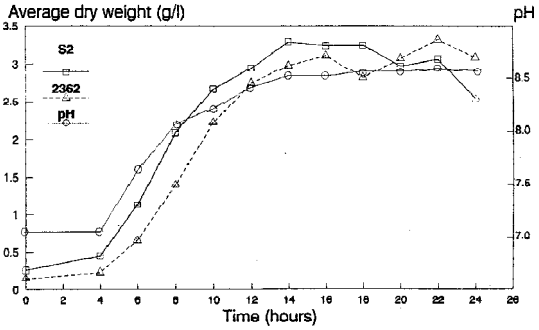

Fig. 1: growth pattern of *Bacillus sphaericus* strains S2 and 2362 in 14 l fermentor. Average dry weight from three fermentation runs was used to plot the growth curves.

is not statistically significant. The 95% confidence limits around the LC_{50} and LC_{90} overlap. The slopes in all cases are similar, except in *Cx. quinquefasciatus*. In this species the slope of the

TABLE I

Spore production and toxicity of the *Bacillus sphaericus* primary powders

Primary powder	Spores/mg[a] (units/mg)	Toxin titer[b]	LC_{50}[c] (95% confidence limits)	
			Culex quinquefasciatus	*Anopheles albimanus*
2362 1	4.1×10^8 ±5.3	11.3	0.42(0.36-0.47)	7.5(5.50-10.0)
2362 2	2.2×10^9 ±1.3	10.0	0.34(0.30-0.39)	6.6(5.10-8.20)
2362 3	1.0×10^9 ±0.9	13.7	0.17(0.15-0.19)	3.9(3.40-4.40)
S2 1	1.2×10^8 ±0.8	19.0	0.29(0.25-0.35)	3.0(2.00-4.10)
S2 2	1.1×10^9 ±8.5	7.7	0.13(0.11-0.15)	14.9(11.7-19.4)
S2 3	2.3×10^9 ±0.8	10.7	0.27(0.24-0.29)	2.9(2.40-3.50)

a: mean of three replicates; *b*: units of $O.D._{405}$/mg of protein determined by Enzyme-Linked Immunosorbant Assay. Values were adjusted to toxin units/mg of primary powder; *c*: values expressed in ppb (µg/l).

TABLE II

Larvicidal activity of *Bacillus sphaericus* strains 2362 and S2 tested in four mosquito hosts. Lethal concentrations expressed in parts per billion result from bioassay data combined from three primary powders of each strain

Host[a]/*B. sphaericus* strain	LC_{50}	LC_{90}	Slope	SD
Culex quinquefasciatus				
2362	0.39 (0.24-0.61)	2.15 (1.22-6.34)	1.74	±0.05
S2	0.25 (0.19-0.31)	1.00 (0.75-1.52)	2.12	±0.06
Anopheles albimanus				
2362	7.16 (5.86-8.63)	38.00 (28.6-55.0)	1.76	±0.04
S2	5.95 (4.66-7.49)	31.20 (22.5-49.2)	1.78	±0.04
Anopheles quadrimaculatus				
2362	16.93 (11.5-24.4)	88.33 (57.0-160.8)	1.78	±0.05
S2	12.28 (8.67-16.9)	62.69 (43.1-101.6)	1.81	±0.08
Aedes aegypti				
2362	307.00 (200-450)	1580.00 (987-3,328)	1.80	±0.16
S2	140.00 (062-243)	830.00 (453-3,055)	1.65	±0.18

a: second instar larvae were used in the bioassays

Brazilian strain is steeper (2.12) than that observed in strain 2362 (1.74). A comparison of the biological performance of the two strains is provided on the basis of the mortality plotted against log dose in Fig. 2. As it is demonstrated graphically the pattern of toxicity is very similar in all mosquito species tested.

The 51 and 42 kDa toxic proteins reported elsewhere (Baumann et al. 1988, Broadwell et al. 1990), were visualized in SDS-PAGE of the alkali extracts from all primary powders. Elution of proteins from slab polyacrylamide gels onto nitrocellulose membranes, and further exposure to the 42 kDa antiserum, confirmed the presence of that peptide in crude extracts from both S2 or 2362 strains (Fig. 3).

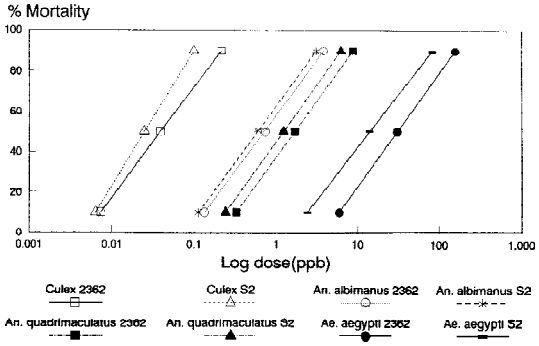

Fig. 2: larvicidal activity of *Bacillus sphaericus*.

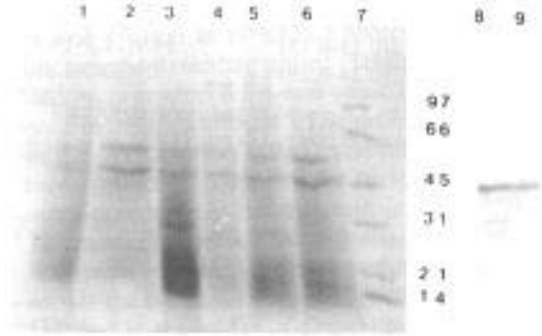

Fig. 3: gel electrophoresis (SDS-PAGE) of crude toxin extracts from *Bacillus sphaericus* strains S2 and 2362 in 5-20% gradient (lanes 1-7). Lanes 1-3: S2 extracts; lane 7: molecular weight standards in kilodaltons; lanes 8-9: western immunoblots of extracts from strains S2 and 2362 developed with anti-42 kDa antisera.

DISCUSSION

As shown by the fermentor results, MBS medium supports a good yield for the *B. sphaericus* strains S2 and 2362 without significant differences in the growth profile. Larvicidal strains of *B. sphaericus* are regarded as a homogeneous group in physiological characters (de Barjac et al. 1980, Krych et al. 1980, Priest et al. 1988). Based on these observations, and the fact that strains S2 and 2362 belong to the same phage type group, marked differences in the growth pattern are not very likely. The growth and spore production of strain 2362 observed in this work are in agreement with the results of Yousten and Wallis (1987) who employed a peptone based broth (NYSM). In four trials using a one liter New Brunswick fermentor, with the same aeration and without pH control, Yousten and Wallis obtained spore counts of 6.0-8.6 x 10^8 spores/ml of final broth of *B. sphaericus* 2362. This study reports spore counts between 1.2 x 10^8 to 2.3 x 10^9/mg (dry weight) in the primary powder of *B. sphaericus* S2, and 4.1 x 10^8-2.2 x 10^9 in *B. sphaericus* 2362 (Table I). During fermentation a significant amount of foam was produced which resulted in the incorporation of varying amounts of anti-foam which remains as a component of the primary powders. The variable amount of anti-foam used during the fermentation runs may explain in part the variation in the calculated number of viable spores per mg of primary powders. Other factors that may account for variation in the spore number of individual preparations are the recovery process (continuous flow centrifugation, lyophilization), the standardization of the inoculum (initial cell count), and the standardization of the growth conditions (aeration, agitation). The larvicidal potency in the cultures produced by Yousten and Wallis (1987) was measured in comparison with the International Standard RB-80 (Pasteur Institute, France), and expressed in toxic units per milliliter of broth. The way the larvicidal toxicity is presented in this paper does not allow comparison of results. However, it is relevant for this work that Yousten and Wallis (1987) report a potency range in *Cx. quinquefasciatus* from 7728-14144 toxin units/mg dry weight for the four trials mentioned above, which represents a variation of up to 100% among batches of *B. sphaericus* 2362 in NYSM broth. As shown in Table I, a similar variation in the potency in terms of LC_{50} and LC_{90} of the primary powders of *B. sphaericus* S2 and 2362 was observed.

The high levels of toxicity of the strains S2 and 2362 in *Cx. quinquefasciatus* were followed by lower levels in *An. albimanus* and *An. quadrimaculatus* (20-40 fold higher LC_{90}'s), with the lowest larvicidal activity observed in *Ae. aegypti* (Table II). Several authors have observed this larvicidal profile for *B. sphaericus* 2362 and other strains (Lacey & Singer 1982, Mulla et al. 1986, Ali & Nayar 1986, Wraight et al. 1987). As reviewed by Lacey and Undeen (1986), *B. sphaericus* spore/crystal preparations kill *Ae. aegypti* larvae at dosages 100-1000 fold greater

than that required for *Cx. quinquefasciatus*. An explanation for this pattern has been proposed by Davidson (1989), based upon the affinity that FITC-labeled toxin binds to midgut of larvae of these mosquito species. It was shown that the toxin does not bind to the midgut of *Ae. aegypti* larvae, and binds with low affinity to *Anopheles* sp. larvae. De Barjac et al. (1988) report LC_{50}'s at dilutions of 7.2-8.5 x 10^{-4} of final whole cultures of *B. sphaericus* 1593 and 2362 respectively, in fourth instar larvae of *Ae. aegypti*. In second instar larvae there are reports of LC_{50}=189 ppm with strain SSII-1 (Wraight et al. 1987), LC_{50}=57.2 ppm with strain 1593 (Mulla et al. 1983), and LC_{50}>50 ppm (third instar larvae) with strains 1593 and 2362 (Ali & Nayar 1986). The results found in this work fall in a range well below previous reports. Indeed, even for *Cx. quinquefasciatus*, a similar high level of larvicidal activity has been reported by Myers and Yousten (1981), who found an LC_{50}=0.37 ppb with pure spores (washed free of culture residues) of strain 1593 in second instar larvae of *Cx. quinquefasciatus*. Yousten (1984) reports LC_{50}'s of 0.04-0.07 ppb with pure spores of strain 2362 in second instar *Cx. quinquefasciatus*. Mulla et al. (1986) tested a primary powder of *B. sphaericus* 2362 produced by Abbott Laboratories (ABG-6184) in lab-reared fourth instar *Cx. quinquefasciatus* larvae, and found an LC_{90}=8.0 ppb. The lowest values of LC_{50} obtained with anopheline mosquitoes are reported by Lacey and Singer (1982). These authors observed LC_{50} values of 18.7-52.7 ppb in second instar *An. albimanus* and *An. quadrimaculatus*, respectively, with *B. sphaericus* strain 2013-4 from Romania. The overall comparison indicates that the preparations of *B. sphaericus* S2 and 2362 obtained in this work are among the most potent, in terms of larvicidal activity for the mosquito species tested.

The use of ELISA with anti-42 kDa antisera failed to provide a good correlation of larvicidal potency with titer of 42 kDa toxin in individual preparations. One reason for this can be the absence of the anti-51 kDa toxin IgG in the antisera used for the ELISA titration. The binary action of the 42 and 51 kDa peptides in the process of toxicity of *B. sphaericus* has been established (Broadwell et al. 1990, Davidson et al. 1990). The role of a third 100 KDa toxin previously characterized (Davidson 1983) which may be influencing the results obtained in this research has been established elsewhere (Thanabalu et al. 1991).

As revealed by the polyacrylamide gel of the toxin extracts (Fig. 3), *B. sphaericus* S2 shows a protein profile typical of the highly larvicidal strains. Previous research has demonstrated the similar toxin profile among the *B. sphaericus* toxin producer strains (Baumann et al. 1985, De Barjac et al. 1988). Schenkel et al. (1992), demonstrated that S2 and three other *B. sphaericus* isolates from Brazilian soils, belong to serotype H5 and produce the toxins found in 2362 strain. It was also demonstrated that these strains were different from 2362 and from each other, based on gas chromatography analysis of fatty acids.

Data generated in laboratory bioassays suggest a trend in strain S2 towards a toxicity higher than that observed in strain 2362 against the mosquito species tested. Additional research should provide valuable information with regard to the field performance of the new isolate.

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