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ULTRASTRUCTURAL AND ENTOMOTOXIC ASPECTS OF *BACILLUS SPHAERICUS* STRAINS ISOLATED FROM BRAZILIAN SOILS

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ABSTRACT

Using transmission electron microscopy we compared the cellular structures (membranes and cytoplasmic inclusions) of three *Bacillus sphaericus* strains isolated from Brazilian soils. The native strains LFB-FIOCRUZ 664 and 666 are described for the first time and were found to possess parasporal bodies with a similar structure to those described for the strains 1593 and 2362 used as standard strains in this study. The strain LFB-FIOCRUZ 152 differed from the other two by the absence of parasporal bodies and crystalline inclusions. Toxicity tests on *Culex quinquefasciatus* larvae showed that the native crystalline strains present a toxicity similar to that of the standard strains 1593 and 2362. On the other hand, the isolate LFB-FIOCRUZ 152 did not show any toxicity under the experimental conditions.

KEY WORDS: *Culex quinquefasciatus, Bacillus sphaericus,* cellular ultrastructure, parasporal body, entomopathogenicity.

INTRODUCTION

Bacillus sphaericus is gaining more attention in the field of biological insecticides (Singer, 1980; Obeta and Okafor, 1983; Davidson et al., 1984; Yap et al., 1988). The species shows a larvicidal effect on mosquito larvae of the genus *Culex* and *Anopheles,* due to the presence of binary toxins of 51 and 42 kDa (Baumann et al., 1991) and 100 kDa (Thanabalu et al., 1991), and other properties which are relevant for its use in the control of insect vectors: *B. sphaericus* spores and toxins possess a prolonged residual activity in the environment, especially in polluted water (Mulla et al., 1984), and the recycling of the microorganism in the environment by multiplication in dead mosquito larvae was shown to occur during several months (Charles and Nicolas, 1986; Nicolas etal., 1987).

In Brazil, due to the high incidence of diseases transmitted by insect vectors, like malaria (Cowley et al., 1992) and filariosis (Regis et al., 1995), the above mentioned properties led to the search for autochtonous *B. sphaericus* strains which are more active and better adapted to local environmental conditions (Vilarinhos et al., 1992; Schenkel et al., 1992,1993). However, no data are available on the cytomorphological characterization of these new isolates.

Our laboratory isolates and characterizes routinely *B. sphaericus* strains (Guaycurus et al., 1992; Silva et al., 1995; Chaves et al., 1996), and this study aims at the cytomorphological characterization by transmission electron microscopy of three autochtonous isolates, two of which are toxic to *Cx. quinquefasciatus* larvae.

MATERIALS AND METHODS

Bacterial strains

Three bacterial strains isolated from Brazilian soil were employed in this study: LFB-FIOCRUZ 152 (phage-group 2, serotype H2a,2b); LFB-FIOCRUZ 664 (phage-group 4, serotype H5a,5b); and LFB-FIOCRUZ 666 (phage-group 3, serotype 5a,5b). The strains are part of the Culture Collection of the Genus *Bacillus* (C.C.G.B.) of the Laboratory of Bacterial Physiology at the Oswaldo Cruz Institute. The isolates 1593 and 2362 (phage group 3, serotype H5a,5b) were used as reference strains.

The purity of the 5 *B. sphaericus* strains was verified by Petri dish cultivation in NYSM solid medium with 1.5% agar (Myers and Yousten, 1980). The isolates were shown to be positive for the following biochemical tests: hydrolysis of gelatin, casein and urea; deamination of phenylalanine; growth in nutrient broth containing 7% of NaCl and lysozyme (100 units/mL). Test results were negative for citrate utilization; growth in nutrient broth containing 10% NaCl; and growth on anaerobic agar. The strains were examined by phase contrast microscopy (magnification 2,000×) for the presence of terminal spherical spores, causing a deformation of the sporangium.

B. sphaericus cultivation and sporulation

All samples were grown in 500 mL Erlenmeyer flasks using 100 mL of NYSM medium. The flasks were agitated at 175 rpm at 30°C for 48 h. Under these conditions we obtained 10^7–10^8 spores/mL and a sufficient number of sporangiae encompassing the parasporal bodies.

Toxicity tests on *Cx. quinquefasciatus* larvae

The bioassays were carried out according to the protocol recommended by the World Health Organization (WHO, 1985) with minor modifications. Biomasses of the five strains used in this study were grown in solid NYSM at 30°C for 72 h. The cells were resuspended and homogenized in 10 mL sterile distilled water. 1 mL aliquots of these stock suspensions were used for the determination of the average dry weight. The suspensions were serially diluted, using a dilution factor of 10^{-1} for LFB-FIOCRUZ 152 and 10^{-2} for the other strains. Susceptibility of *Cx. quinquefasciatus* 4th instar larvae (L_4) was determined by employing aliquots of these dilutions, varying between 1000 µL and 1700 µL, or 3 µL and 120 µL, depending on the strain under investigation. Lethal concentrations for 50% (LC_{50}) of the larvae were determined by linear regression analysis in log-probit scale for each strain.

Preparation of *B. sphaericus* cells for transmission electron microscopy (TEM) studies

60 mL-aliquots of the cell cultures were centrifuged and the bacteria fixed in 2.5% glutaraldehyde at 4°C for 2 h, and subsequently in 1% OsO_4 at 4°C for 1 h. The samples were slowly dehydrated in acetone and embedded with Epon 812 resin. After polymerization at 60°C during 72 h, ultrathin sections were obtained using a Reicher OM-U3 ultramicrotome. The sections

were stained with 5% uranyl acetate for 20 minutes and 0.4% lead citrate for 5 minutes, and subsequently observed and micrographed in a Zeiss EM 10C transmission electron microscope, operated at 80 kV.

RESULTS AND DISCUSSION

In this study the *B. sphaericus* strains 2362 and 1593 were used as reference for toxicity and cellular ultrastructure. The strains LFB-FIOCRUZ 664 and 666 possess parasporal bodies, as evidenced by phase contrast microscopy. On the other hand, using the same technique this structure could not be observed in the strain LFB-FIOCRUZ 152.

The results of the toxicity tests on L_4 larvae of *Cx. quinquefasciatus* are shown in Table 1. According to Baumann et al. (1991), highly toxic *B. sphaericus* strains such as strains 1593 and 2362 present LC_{50} values of 30 µL/L or less, whereas low-toxic strains present LC_{50} values of 5,000 µL/L or higher. According to this classification, the Brazilian strains LFB-FIOCRUZ 664 and 666 belong to the high toxicity group. The isolate LFB-FIOCRUZ 152 has no larvicidal effect since biomass concentrations varying from 1,000 to 4,300 µL/L did not kill *Cx. quinquefasciatus* larvae.

The analysis of ultrathin sections obtained from the native strains LFB-FIOCRUZ 664 and 666 confirmed the existence of parasporal bodies with a parallelepipedal shape and internal longitudinal and transversal striations (Fig. 1). These bodies are contained in an envelope and localized next to the spore. Both are wrapped in the exospore (Fig. 1). These characteristics are analogous to those described by Yousten and Davidson (1982) and De Barjac and Charles (1983) for strain 2297. Parasporal bodies with the shape, internal configuration and localization similar to those described above are considered to be the storage site of the major part of the pro-toxin synthesized by *B. sphaericus* strains (Payne and Davidson, 1984).

Small triangular or oval bodies without internal striations, which are localized above or below the spore between the folds and membranes of the exospore were also observed (Fig. 2). These inclusions were first described in strain 1593 by Davidson (1981) and later have been reported for other toxic strains (Davidson and Myers, 1981; Kalfon et al., 1984). Studies showed that these bodies are not digested in the mosquito midgut, and therefore most likely are not the sites of *B. sphaericus* toxin localization. In addition, their existence has also been described for atoxic strains (Davidson, 1981; Davidson and Myers, 1981).The results of the

TABLE I

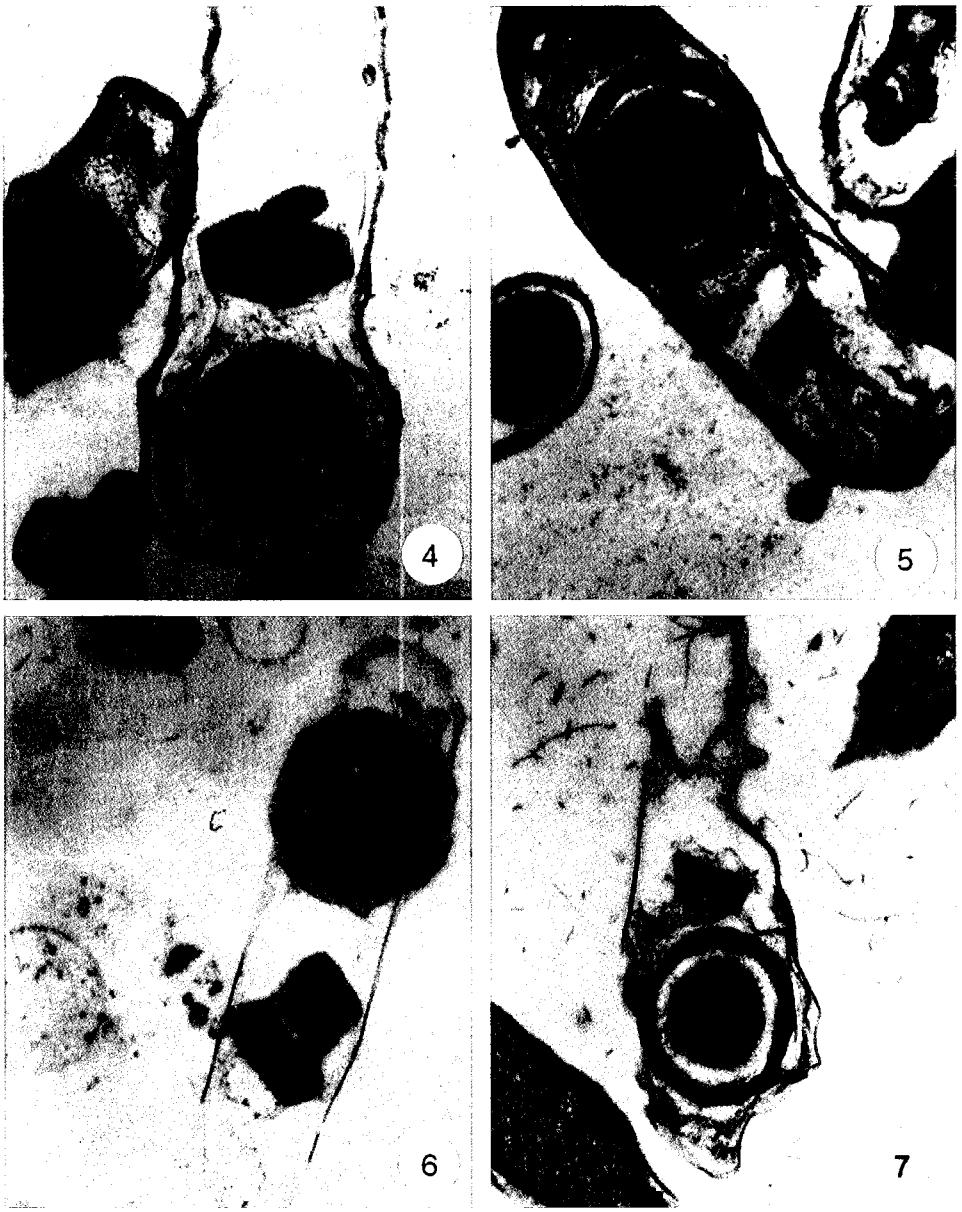
Lethal concentrations of bacterial suspensions from different *Bacillus sphaericus* strains, obtained after growth in NYSM and tested on L_4 larvae of *Culex quinquefasciatus*

Strain	LC_{50} (µg/L)*
1593	4.07 ± 1.51
2362	3.87 ± 1.27
LFB-FIOCRUZ 393	9.14 ± 0.69
LFB-FIOCRUZ 395	6.49 ± 1.58
LFB-FIOCRUZ 65	nd

nd = not detected within the concentration range of 1000–4300 µg/L.

*The numbers express average results of two experiments with their standard deviations.

Figs. 1–3. 1. Spore of strain LFB-FIOCRUZ 666 with parasporal body (157,000×). EX = exosporium; EV = envelope; CE = spore coats; MI = inner menbrane spore; PP = primordial cell wall; * = internal striations of parasporal body. 2. Spore of strain LFB-FIOCRUZ 666 with small inclusions (84,000×). IN = inclusions; EX = exosporium. 3. Sporangium of strain LFB-FIOCRUZ 152 without parasporal body or small inclusions (69,000×). MC = plasmic membrane; PC = cell wall.



Figs. 4–7. See text for details. 4. Sporangium of strain LFB-FIOCRUZ 666 (62,000×). 5. Sporangium of strain LFB-FIOCRUZ 666 (62,000×). 6. Sporangium of strain LFB-FIOCRUZ 666 (60,000×). 7. Sporangium of strain LFB-FIOCRUZ 666 (44,000×).

ultrastructural analysis of the spore-parasporal body complex of the strains 2362 and 1593 are similar to those reported in the literature for both strains (Davidson, 1981; Payne and Davidson, 1984; Charles and Nicolas, 1986; Karch and Charles, 1987). In strain LFB-FIOCRUZ 152, no parasporal body or other inclusions were observed (Fig. 3).

The cellular structures composing the spores of all strains analyzed in this study show the presence of the internal spore membrane, the nucleoid, cortex and spore coats with 4 to 7 lamellae (Fig. 1). These findings are in agreement with those reported by Holt et al. (1975) for the atoxic strain 9602.

The ultrathin sections obtained for the reference strains and the native crystallogenic strains show a division of the parasporal bodies, as evidenced in Fig. 8 for strain 2362, and in Figs. 5 and 7 for strain 666. These findings have not yet been reported in the literature for *B. sphaericus*, although Zelazny et al. (1994) described a putative dislocation of parts forming one of the parasporal bodies in five *B. thuringiensis* isolates. However, although some *B. thuringiensis* strains are as entomotoxic as *B. sphaericus*, several differences exist between the two species. Some subspecies of *B. thuringiensis* synthesize 2 or 3 parasporal bodies per cell (Samasanti et al., 1986; Yousten et al., 1992), while the parasporal body producing *B. sphaericus* strains synthesize only one per cell (Yousten and Davidson, 1982; Kalfon et al., 1984). However, De Barjac et al. (1988) confirmed the presence of 2 or more parasporal bodies in the naturally occurring *B. sphaericus* strain IAB 59.

The "fractured" parasporal body observed in Figs. 5, 7 and 8 could alternatively be explained by the presence of two separate parasporal bodies with a perfect morphological fit. This would oppose the evidence presented in Figs. 4 and 6, where separate parasporal bodies are not morphologically complementary. In addition, the number of *B. sphaericus* cells containing "fractured" parasporal bodies is very small if compared to the number of cells containing only a single body. However, we cannot rule out the possibility that the *B. sphaericus* strains under

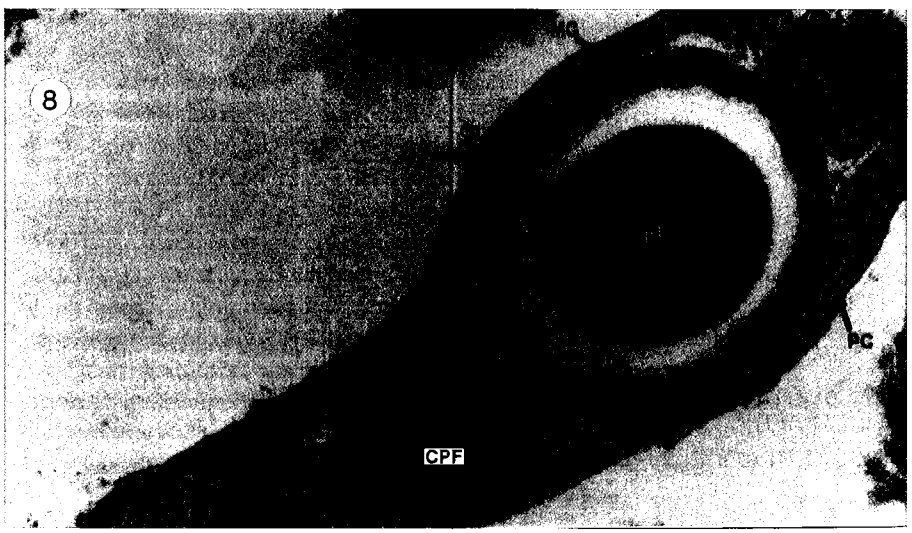


Fig. 8. Sporangium of strain 2362 (105,000×). N = nucleoid; CE = spore coats; CTX = cortex; CT = T layer; PC = cell wall;. MC = plasmic membrane; CPF = "fractured" parasporal body.

investigation are capable of synthesizing a second parasporal body, as can be naturally observed in *B. thuringiensis*.

In the present study we showed by electron microscopy that the three native Brazilian *B. sphaericus* strains under investigation are morphologically related to the reference strains used and possess parasporal bodies, thus confirming their larvicidal effect, although strain LFB-FIOCRUZ 152 is not crystallogenic.

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