A new black fly isolate of *Bacillus thuringiensis* autoagglutinating strain highly toxic to *Simulium pertinax* (Kollar) (Diptera, Simuliidae) larvae

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Formulations containing the entomopathogenic Bacillus thuringiensis *serovar* israelensis *strain IPS-82 has been widely applied for mosquito control around the world. Strain IPS-82 is highly active against* Aedes aegypti *but less active against other well-known vectors such as* Culex quinquefasciatus *and* Simulium spp. *larvae. Eighteen strains of* B. thuringiensis *were isolated from* Simulium pertinax *larvae naturally occurring in rivers of Southeast Brazil with one demonstrating special toxic effects. Simulated field tests against* S. pertinax *larvae showed that the native Brazilian autoagglutinanting* B. thuringiensis *(LFB-FIOCRUZ 1035) has an LC_{50} at least 25 times lower than the standard IPS-82 strain. The same bacterial preparation was also tested against* Ae. aegypti *larvae in laboratory trials and the LC_{50} values obtained with LFB-FIOCRUZ 1035 were at least three times lower than the one for the IPS 82 strain. The results indicate that this strain is more toxic than the standard* B. thuringiensis *serovar* israelensis *(H14) in the two Dipteran species tested. It is noteworthy that differences between LC_{50} values were more pronounced in* S. pertinax *larvae, the source of the original isolation.*

Key words: entomopathogenic bacteria - biological control - black fly

Bacillus thuringiensis is a Gram-positive, spore-forming bacterium which produces a parasporal body during sporulation. The proteins contained in the parasporal crystal (delta-endotoxins) are specific insect gut toxins which have no significant effect on non-target organisms (Siegel 2001). The mode of action after ingestion involves solubilization of the protein in the alkaline conditions of the insect midgut, followed by activation of the protoxin into toxin through cleavage of specific regions by gut proteases. Then, the so called toxin binds to specific receptors in the brush border membrane vesicles of the midgut columnar cells. The binding is a two-step process, the second part being the insertion of the toxin into the cell membrane leading to pore formation, osmotic imbalance, disruption of the cell membrane and finally, death of the insect (Van Rie et al. 1990, Knowles 1994).

The use of bioinsecticides based on *B. thuringiensis* to control insects from the orders Diptera, Coleoptera, and Lepidoptera and more recently against Hymenoptera, Orthoptera, and Mallophaga, has stimulated the search for other natural pathogens (Schenpf et al. 1998). Strains have been isolated from different sources, such as mud, silt, and soil. However, only a few reports have been published concerning the isolation of entomopathogenic *Bacillus* from insects.

In 1984, Weiser described a highly toxic strain of *B. sphaericus* isolated from an adult of *Simulium damnosum* in Northern Nigeria. This strain, known as *B. sphaericus* 2362, is the one used in commercial bioinsecticides based on *B. sphaericus*. Asimeng and Mutinga (1992) and Chilcott and Wigley (1993) isolated several additional *B. thuringiensis* strains from Diptera larvae.

In Brazil, Rabinovitch et al. (1995), isolated two new *B. thuringiensis* serotypes from commercial peppers. These isolates belong to serotype H-38 and H-39, *B. thuringiensis* serovar *oswaldocruzi* and *B. thuringiensis* serovar *brasiliensis*, respectively.

Between 1995 to 1998, an isolation program was set up to obtain entomopathogenic *Bacillus* from *Simulium* larvae and adults in the Southeast region of Brazil. Eighteen strains of *B. thuringiensis* and one of *B. sphaericus* were isolated (Cavados et al. 2001). Preliminary bioassays were performed and one of the *B. thuringiensis* strains (LFB-FIOCRUZ 1035) showed promising activity against *Aedes aegypti* and *Culex quinquefasciatus* (Cavados et al. 2001).

In the present study, the toxicity of the autoagglutinating strain LFB-FIOCRUZ 1035 was evaluated against two Dipteran species, *S. pertinax* and *Ae. aegypti* larvae, and compared with a standard powder formulation of the reference *B. thuringiensis* serovar *israelensis* strain IPS-82, which is used in commercially available bioinsecticides.

MATERIALS AND METHODS

Bacterial strains - Two strains of *B. thuringiensis* were used, one of them IPS-82 (LFB-FIOCRUZ 584) serotype H-14, isolated from the standard powder prepared by the Pasteur Institute of Paris, and the other LFB-FIOCRUZ 1035, an autoagglutinating strain isolated from *S. pertinax* larvae collected in a natural breeding-site in

Southeastern Brazil (Cavados et al. 2001). Both are deposited as lyophilized material in the Coleção de Culturas do Gênero *Bacillus* e Correlatos - CCGB, of the Departamento de Bacteriologia, Instituto Oswaldo Cruz-IOC-Fiocruz.

Preparation of active bacterial biomass - The Brazilian strain LFB-FIOCRUZ 1035 and the standard IPS-82, were grown in a fermentation medium based on soy flour and inorganic salts (such as $MgSO_4$, $MnSO_4$, $ZnSO_4$, $FeSO_4$, $CaCl_2$) developed in the Laboratório de Fisiologia Bacteriana, Departamento de Bacteriologia, IOC-Fiocruz (Cavados et al. 1998).

Growth started with a pre-inoculum to reduce the duration of the lag phase of bacterial growth. After inoculation in 125 ml Erlenmeyer flasks containing 50 ml of the medium, the flasks were incubated in a New Brunswick Scientific agitator series 25D, at 175 opm and 30°C for 6 h. Subsequently, 3 ml were transferred to 500 ml Erlenmeyer flasks containing 150 ml of the soy flour and inorganic salts medium and incubated as previously described for a further 72 h period.

Once sporulation had reached 95% of free spores and crystals, each culture was centrifuged (6000 g, 10°C), the supernatant removed and the remaining material was kept in an amber container with the pH adjusted to 5.0 with propionic acid and then formulated (Rabinovitch et al. 1998).

Determination of the dry weight - Samples of 0.5 mg biomass were weighed. For each sample three replications were done which were kept for 24 h in a vacuum oven at 70°C under negative pressure of 62 mm Hg. Dried biomasses were transferred to a desiccator and placed under a vacuum for 1 h until they reached room temperature. Dried material was then weighed up to the fourth decimal place, and then, the mean weight of the dry biomass and its moisture content were determined.

Counting viable cells - A sample corresponding to 25 mg (dry weight) of the biomass was transferred to a 50 ml volumetric flask; distilled sterile water was added to a final volume of 50 ml and the solution was homogenized for 5 min. This standard suspension thus contained 0.5 mg/ml. For the quantification of viable spores, 5 ml of this solution was subjected for 12 min to 80°C in a water bath. Immediately after cooling to room temperature, serial dilutions down to $1:10^5$ were prepared in sterilized tubes, 0.1 ml was drawn from the last three dilutions ($1:10^3$, $1:10^4$, $1:10^5$) and spread onto the surface of nutrient agar poured into Petri dishes. Three dishes were used for each dilution and incubation was for 24 h at 33°C. The colonies were counted, the average determined and the number of spores/mg calculated.

Bioassays against Ae. aegypti - The World Health Organization assay protocol (WHO/8CV/IC-GE/8713) was used to evaluate the 50% lethal concentration (LC_{50}) against L_3-L_4 of *Ae. ageypti* (strain Rockfeller). For each bioassay was used 600 larvae and the mean LC_{50} was based at least on three different assays performed at room temperature. Assessment of the biological activity was determined through Log-Probit analysis (Finney 1952).

Bioassays against S. pertinax - The formulations of strains LFB-FIOCRUZ 584 and 1035 were sent to the Laboratório de Simulídeos, Superintendência de Controle de Endemias, São Paulo. Assays against black fly larvae (each one with 600 larvae) were carried out in artificial breeding-sites of *S. pertinax* in the field according to the methodology described by Araújo-Coutinho (1995) and the mean LC_{50} was calculated based on at least three different bioassays. Assessment of the biological activity was determined through Log-Probit analysis (Finney 1952).

RESULTS AND DISCUSSION

The use of biological control as one of the strategies to control insect populations has increased over the past few years with the use of *B. thuringiensis* serovar *israelensis* based insecticides in many vector control programs around the world (Araújo-Coutinho 1995, Hougard 1997, Gray et al. 1999).

From 1995 to 1998 a program to isolate new bacteria was conducted by the Laboratório de Fisiologia Bacteriana in order to find entomopathogenic *Bacillus* naturally occurring in *Simulium* larvae and adults. The larvae were collected from different breeding-sites in the states of Rio de Janeiro and São Paulo. Some of the breeding-sites were treated with *B. thuringiensis* serovar *israelensis* products, and others were used as control areas. Eighteen strains of *B. thuringiensis* and one *B. sphaericus* were isolated (Cavados et al. 2001). One *B. thuringiensis* strain (LFB-FIOCRUZ 1035), an autoagglutinanting strain demonstrated high activity against *Ae. aegypti* larvae in preliminary tests. It is important to emphasize that "autoagglutinanting" strains have eluded H-classification because suspensions of these strains in sterile NaCl 0.85% agglutinated spontaneously in the absence of specific antiserum (Cavados et al. 2001)

The biological activity of the formulated biomass from the autoagglutinating strain (LFB-FIOCRUZ 1035) was then determined and compared with that of the reference, *B. thuringiensis israelensis* IPS 82. The results showed that this new strain was more toxic against *Ae. aegypti* and also against *S. pertinax* than the classic strain used as the active ingredient in commercial insecticides. In the assays of biological activity against *Ae. aegypti* larvae using the strain LFB 1035, the LC_{50} was 0.0014 mg/l (IC 95% = 0.0012-0.0017) and for the standard the LC_{50} was 0.0039 mg/l (IC 95% = 0.0029-0.0053). For *S. pertinax* larvae, the bioassays were performed in simulated field trials and the LC_{50} for the strain 1035 was 0.135 mg/l (IC 95% = 0.049-0.403) and for the strain LFB 584 the LC_{50} was 3.358 mg/l (IC 95% = 2.841-3.970).

Strain 1035 was 2.8 times more effective against *Ae. aegypti* than the IPS-82 strain, and it was 25 times more effective against *S. pertinax* than the standard strain used routinely in programs to control black flies in the Southeast region of Brazil (Araújo-Coutinho 1995).

Finally, we noted that the number of colony-forming units of the LFB 1035 sample was smaller by a factor of ten than that of IPS-82 strain, thus showing that even with a lower concentration of toxic proteins per milligram

of material applied, it was still more toxic. The results of the counting of the number of viable cells/colony formation units per milligram (ufc/mg) showed that strain LFB 1035 had 6.9 x 10^6 cells/mg of biomass, while strain LFB 584 had 2.45 x 10^7 cells/mg.

The results obtained here with this autoagglutinating *B. thuringiensis* strain are in accordance with those of Ragni et al. (1996) and Cavados et al. (1998), which showed that autoagglutinating strains of *B. thuringiensis* can be more effective against certain insects than the strain currently used for biological control of Dipteran vectors.

Additional studies are needed to explain why this strain is more toxic to its natural host, *S. pertinax* than to *Ae. aegypti*, since the results of protein profile analysis (Cavados et al. 2001) showed that this sample has the same proteins as present in the parasporal body of *B. thuringiensis* serovar *israelensis* IPS 82 which does not produce exotoxins. But whatever the underlying cause, it appears to be very promising for future use as a biological control agent.

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