Identification of Entomopathogenic *Bacillus* Isolated from *Simulium* (Diptera, Simuliidae) Larvae and Adults

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Entomopathogenic bacteria isolated from Simulium *larvae and adults from breeding sites in the states of São Paulo and Rio de Janeiro, Brazil, were identified as 18 strains of* Bacillus thuringiensis *and one of* B. sphaericus. *Most of these strains were serotyped according to their flagellar antigens. However, nine of the* B. thuringiensis *samples, could not be serotyped and were designated as "autoagglutinating"; they were also shown to be toxic in preliminary tests against* Aedes aegypti *larvae. Additionally,* B. sphaericus *was also shown to be toxic towards* Culex quinquefasciatus *larvae.*

Key words: biological control - *Simulium* - *Bacillus thuringiensis* - *Bacillus sphaericus* - black fly - Brazil

Simuliids are hematophagus insects naturally occurring in waterways throughout Brazil. Although some species have been involved in the transmission of human onchocerciasis in other regions (Gerais & Ribeiro 1986, Maia-Herzog et al. 1999) the main importance of these insects in the southeast of Brazil is socio-economic. The frequent and intensive attacks by simuliids on the transitory population in this area can reduce tourism in summer months, a fact that could negatively impact the economies of affected municipalities. To decrease the presence of these insects in these areas, a full-scale programme, based on *Bacillus thuringiensis* serovar *israelensis*, has been carried out in the north littoral zone of the State of São Paulo since 1990 (Araújo-Coutinho 1995).

Recent findings of *B. thuringiensis* serovar *oswaldocruzi* and *B. thuringiensis* serovar *braziliensis* from unusual habitats (Rabinovitch et al. 1995) encourage the continued search for other endemic entomopathogens. For that purpose, *Simulium* larvae and adults from the north littoral zone of São Paulo and some other rivers in the State of Rio de Janeiro were evaluated for the presence of entomopathogenic bacteria.

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MATERIALS AND METHODS

Collection of simuliids - Black fly larvae and adults were collected from breeding sites in Pau d'Alho river, State of São Paulo. This river was divided into two regions, one downstream of *B. thuringiensis* serovar *israelensis* applications and another, upstream. Another collection point was the Soberbo river, Guapimirim, State of Rio de Janeiro (Fonseca et al. 1998). This river has never been exposed to commercial formulations based on *B. thuringiensis* serovar *israelensis.* Only larvae were collected in this waterway. The third point where larvae were collected was in Rio das Pedras, Mangaratiba, State of Rio de Janeiro.

Isolation of Bacillus - To eliminate external contamination, the insects were sterilized following the methodology described by Alves (1986) with a slight modification; "Superbonder" glue (cyanoacrilate ester) was used to close both oral and anal cavities. This modification was adopted because *Simulium* larvae are very small and the dental floss used in the original technique was not applicable. After this step, the original method was performed passing the larvae through three solutions, first in 70% alcohol for 2 sec, second in 5% sodium hypoclorite for 3 min and finally in sterile 10% sodium thiossulfate for 5 min. The specimens were then washed three times in sterile distilled water.

Two different methods were used to isolate *B. thuringiensis* samples. In both, *Simulium* larvae were transferred aseptically into a sterile mortar and macerated with a sterile pestle. In the first method, the macerate was placed in tubes containing distilled water (10 ml). The suspension was heated (65ºC) for 12 min, and immediately diluted ($1x10^{-2}$

to $1x10^{-4}$). Dilutions were plated on nutrient agar and plates were incubated (33ºC ± 2ºC) for 24 h. The second isolation procedure was that described by Travers et al. (1987) using the same recommended temperature for heating the larvae. The main difference between the two methods is that the second can select the spores of entomopathogenic bacteria such as *B. sphaericus* and *B. thuringiensis*. This selection occurs because sodium acetate added into the medium (0.5 M to give a final concentration of 0.25 M) is able to inhibit spore germination of entomopathogenic bacteria. On the other hand, the first method is more applicable when the amount of material is not large.

Identification of the samples - Bacteria were scraped off from the agar surface and saline water suspensions (NaCl 0.85%) were observed under light microscope (1,000x) to confirm the presence of parasporal crystals, a typical characteristic of *B. thuringiensis*. Biochemical, cytological and physiological characteristics according to Bergey's Manual of Determinative Bacteriology (Sneath 1986), de Barjac and Frachon (1990) and Thiery and Frachon (1997) were also assayed for these samples to confirm the classification of *B. thuringiensis* (data not shown). A strain of *B. sphaericus* was also identified using the same methodology described for *B. thuringiensis*. *B. thuringiensis* and *B. sphaericus* isolates were sent to Unité des Bactéries Entomopathogènes, Institute Pasteur, Paris, where they were submitted to serovar identification based on the flagellar antigens.

Protein profiles of the protoxins - The protein profiles of crystal components from the *B. thuringiensis* strains were determined by SDS-PAGE analysis, as described by Laemmli (1970), using 12% acrylamide separating gels. Samples (5-15 µg) of washed spore-crystal mixtures, prepared as described by Thomas and Ellar (1983), were placed in 4X concentrated sample buffer and heated at 100ºC for 10 min, as previously described by Lecadet et al. (1992) and loaded onto the gel immediately before electrophoresis.

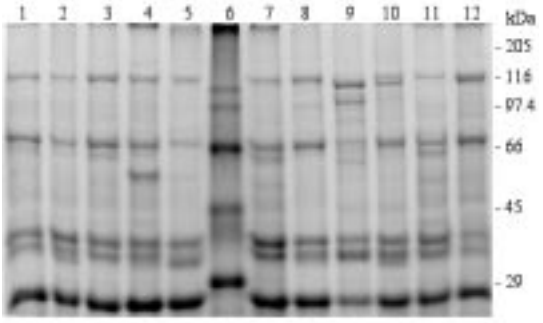
Bioassays for toxicity - To evaluate toxicity against *Aedes aegypti* or *Culex quinquefasciatus* larvae, *Bacillus* strains were grown on Nutrient Agar plates for 48 h (33ºC ± 2ºC). Bacteria were scraped off from the surface of the culture medium and suspended in 0.85% NaCl solution at an optical density of 0.1 at 600 nm. One ml aliquots of the suspensions were added to 50 ml of water (free of chlorine) containing 15 larvae (L_3-L_4) and kept at 25ºC for 24 h for *Ae. aegypti* and 48 h for *Cx. quinquefasciatus*. Strains 2362 (*B. sphaericus*) and IPS-82 (*B. thuringiensis* serovar *israelensis*) were used as reference strains. All strains were tested in duplicate and two containers without added bacteria were maintained as a negative control. The average number of dead larvae was recorded and strains characterized as very toxic (100% mortality) or non-toxic (0% mortality) (Oliveira et al. 1998).

RESULTS

Isolation - Eighteen strains of *B. thuringiensis* were obtained from *Simulium* sp. collected in different rivers of the states of Rio de Janeiro and São Paulo. Seventeen were isolated from larvae of *Simulium* sp. and a single strain from an adult fly. Only one *B. sphaericus* strain was isolated, also from larvae collected in the Soberbo river (Table).

Serotype - Among the strains obtained, only two were shown to belong to serotype H-14, *B. thuringiensis* serovar *israelensis*. Nine "autoagglutinating" strains and seven *B. thuringiensis* serovar *oswaldocruzi*, H-39, were also identified.

Protein profiles of the protoxins - The protein profiles of crystal components were determined by SDS-PAGE analysis, useful for characterizing δ-endotoxin families. The results of SDS-PAGE analysis referring to crystal proteins of the "autoagglutinating" strains are shown in the Figure. Two of the strains (LFB-FIOCRUZ 1038 and 1043) showed a different protein profile when compared to *B. thuringiensis* serovar *israelensis* strain IPS-82. These profiles had multiple components (lanes 4 and 9) ranging between 55 kDa and 100 kDa. Strains LFB-FIOCRUZ 1035, 1036, 1037, 1039, 1041, 1042 and 1044 presented the same protein profiles of the reference strain IPS-82.



SDS-PAGE analysis of parasporal crystals from "autoagglutinating" strains of *Bacillus thuringiensis*. Experiment was performed as described in Material and Methods; lane 1: strain LFB-FIOCRUZ 1035; lane 2: LFB-FIOCRUZ 1036; lane 3: LFB-FIOCRUZ 1037; lane 4: LFB-FIOCRUZ 1038; lane 5: LFB-FIOCRUZ 1039; lane 6: Molecular Weight; lane 7: LFB-FIOCRUZ 1041; lane 8: LFB-FIOCRUZ 1042; lane 9: LFB-FIOCRUZ 1043; lane 10: LFB-FIOCRUZ 1044; lane 11: LFB-FIOCRUZ 1075 (*B. thuringiensis* serovar *israelensis*); lane 12: LFB-FIOCRUZ 584 (strain IPS 82 of *B. thuringiensis* serovar *israelensis*)

Toxicity - The two *B. thuringiensis* serovar *israelensis*, the "autoagglutinating" strains and the strain of *B. sphaericus* were shown to have toxicity (100%) in preliminary bioassays against *Ae. aegypti* and *Cx. quinquefasciatus* larvae, respectively. The strains belonging to serotype H-39 did not exhibit any toxicity (Table). It must be emphasized that these tests were qualitative.

DISCUSSION

The procedure of sealing with "Superbonder" glue was very useful for the purpose of isolation and also highly applicable to *Simulium* larvae.

The easiest method for bacterial isolation described in this study, which employed serial decimal dilutions in water could be useful in cases where the amount of material is limited. These findings were in accordance with Chilcott and Wigley (1993) who established that the most important factor in programme for the isolation of entomopathogenic bacteria was not the nature of the culture medium employed but rather the source of the material. Previously Asimeng and Mutinga (1992) isolated *B. thuringiensis* from mosquito breeding-sites in Kenya, but they did not sample *Simulium* larvae.

It was not possible to establish any relation between the locality of isolation and the serotype determined. Bacteria pertaining to *B. thuringiensis* serovar *israelensis* and *B. thuringiensis* serovar *oswaldocruzi* groups were found inside *Simulium* from different rivers.

Interestingly, the number of "autoagglutinating" strains of *B. thuringiensis* found in black flies from Caraguatatuba, in areas that were not treated and those areas treated with *B. thuringiensis* serovar *israelensis*, was higher than the number of isolated H-14 strains. Cavados et al. (1998) identified one "autoagglutinating" Brazilian strain which was as toxic as *B. thuringiensis* serovar *israelensis* applied in the region against *Ae. aegypti* larvae, but which was more toxic than this strain when the bioassays were performed against *Chrysomya megacephala* (Diptera, Calliphoridae) and it is necessary to say that this strain does not produce β-exotoxin. Rabinovitch et al. (1999) tested one Brazilian strain, classified as a *B. thuringiensis* serovar *israelensis*, against *S. pertinax*, *Cx. quinquefasciatus* and *Ae. aegypti*. The Brazilian sample had the same toxicity against *Ae. aegypti* and *Cx. quinquefasciatus*, but was

TABLE

Strains of *Bacillus thuringiensis* isolated from *Simulium* sp. larvae and adults collected from different rivers of Southeast Brazil and results of preliminary bioassays against *Aedes aegypti* larvae

Strain (LFB-FIOCRUZ)	Location	Serotype	Toxicity
1030	Caraguatatuba – Treated	H39	-
1031	Caraguatatuba – Treated	H39	-
1032	Caraguatatuba – Treated	H39	-
1033	Caraguatatuba – Treated	H39	-
1034	Caraguatatuba – Treated	H39	-
1035	Caraguatatuba – Control	Autoagglutinating	+
1036	Caraguatatuba – Treated	Autoagglutinating	+
1037	Caraguatatuba – Treated	Autoagglutinating	+
1038	Caraguatatuba – Treated	Autoagglutinating	+
1039	Caraguatatuba – Control	Autoagglutinating	+
1040	Caraguatatuba – Treated	H14	+
1041	Caraguatatuba – Treated	Autoagglutinating	+
1042	Caraguatatuba – Treated	Autoagglutinating	+
1043	Caraguatatuba – Treated	Autoagglutinating	+
1044	Caraguatatuba – Treated	Autoagglutinating	+
1045	Caraguatatuba – Adult flies	H39	-
1063	Guapimirim – Control	H39	-
1075	Mangaratiba – Control	H14	+

LFB-FIOCRUZ: Laboratório de Fisiologia Bacteriana, number from Coleção de Culturas do Gênero *Bacillus* from Fundação Oswaldo Cruz, Rio de Janeiro, Brazil; Caraguatatuba: Pau d'Alho river localized in Caraguatatuba, State of São Paulo, Brazil; Guapimirim: Soberbo river localized in Guapimirim, State of Rio de Janeiro, Brazil; Mangaratiba: Mangaratiba river localized in Mangaratiba, State of Rio de Janeiro, Brazil; autoagglutinating: sample of *B. thuringiensis* designated as "autoagglutinating" because they could not be serotyped by serological tests in Institute Pasteur, Paris; (+) : very toxic (100% mortality) in laboratory bioassays against *Aedes aegypti* larvae (L_3-L_4); (-): not toxic in laboratory bioassays against *Ae. aegypti* larvae (L_3-L_4); Treated: region treated with formulations containing a suspension of *B. thuringiensis* serovar *israelensis*; Control: region without any report of *B. thuringiensis* serovar *israelensis* application

more toxic to *S. pertinax* than the *B. thuringiensis* serovar *israelensis* (IPS-82) strain. The "autoagglutinating" strains have eluded H-classification. For unknown reasons, suspensions of these strains agglutinated spontaneously in the absence of specific antiserum (Lecadet et al. 1999). It is interesting that seven of the "autoagglutinating" strains isolated had the same protein profile of the *B. thuringiensis* serovar *israelensis* strain IPS-82, the same results described by Ragni et al. (1996). All of them were very toxic to *Ae. aegypti* larvae and they will be submitted to quantitative tests in order to determine the LC_{50}. Strains LFB-FIOCRUZ 1038 and 1043 presented a different protein profile when compared with IPS82, despite the fact that both were very toxic for *Ae. aegypti* larvae. It will be necessary to run the LC_{50} bioassays in order to determine whether or not they are more toxic than the other ones.

The isolation of serotype H-39 from *Simulium* larvae is also a novel finding, because since the original isolation from commercial peppers, this serotype has appeared only in soil samples. The range of target insects of this serotype has not yet been determined. The strain LFB-FIOCRUZ 1040 may actually be the *B. thuringiensis* serovar *israelensis*, which has been applied in the Caraguatatuba region, where these larvae were collected. The isolation of *B. sphaericus* from *Simulium* sp. has been reported previously. Weiser (1984) isolated the strain 2362, from an adult of *S. damnosum*. Strain 2362 is the strain used widely for mosquito control, however, it is extremely interesting that *B. sphaericus* does not demonstrate any toxicity towards *Simulium* sp. larvae.

This was the first report of the isolation of entomopathogenic *Bacillus* from *Simulium* sp. larvae collected in Brazil.

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