RESEARCH NOTE

A New Strain of *Bacillus thuringiensis* Serovar *israelensis* Very Active against Blackfly Larvae

Leon Rabinovitch/+, Clara de Fátima G Cavados, Jeane Q Chaves, Carlos José PCA Coutinho*, Viviane Zahner, Katia Regina A Silva***, Lucy Seldin*****

Laboratório de Fisiologia Bacteriana, Departamento de Bacteriologia **Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, Av. Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brasil *Superintendência de Controle de Endemias, Secretaria de Estado de Saúde de São Paulo, SP, Brasil ***Instituto de Microbiologia Prof. Paulo de Góes, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

Key words: *Bacillus thuringiensis* - *Aedes aegypti* - *Aedes albopictus* - *Anopheles* - blackfly - mosquito control

Some strains of the Gram-positive spore-forming bacterium *Bacillus thuringiensis* are very toxic to various insect larvae. The insecticidal activity is determined by different cytoplasmic insoluble proteins produced during sporulation which aggregate to form parasporal crystals (E Schnepf et al. 1998 *Microbiol Mol Biol Rev 62*: 775-806). A few strains of *B. thuringiensis* are used as bioinsecticides to control pests and vector insects in the orders Diptera, Lepidoptera and Coleoptera (PAW Martin & RS Travers 1989 *Appl Environ Microbiol 55:* 2437-2442) and more recently new isolates of *B. thuringiensis* have been shown to be active against Hymenoptera, Homoptera, Orthoptera and Mallophaga, as well as nematodes, mites and protozoa (Schnepf et al. *loc. cit.*). Nevertheless, not all *Bacillus* isolates bearing crystals seem to be able to show toxicity against Diptera and/or Lepidoptera (L Rabinovitch et al. 1995 *Mem Inst Oswaldo Cruz 90*: 41-42).

The crystal-protein genes of several *B. thuringiensis* have been characterized and cloned (H Hofte & HR Whiteley 1989 *Microbiol Rev 53*: 242-255). Previously, SS Gill et al. (1992 *Ann Rev Entomol 37*: 615-636) reviewed the classification of the crystal-protein genes related to toxicity including a gene encoding a protein that is both toxic and cytolytic (CytA) and grouped them into 18 distinct gene types, ranging from *cryI* to *cryIV*; the *cryIV* group includes genes encoding toxins for mosquito larvae. JJ Estruch et al. (1996 *Proc Natl Acad Sci USA 93*: 5389-5394) described a *cryV* gene encoding a protein bearing a wide spectrum of activities against Lepidoptera.

Recently, N Crickmore et al. (1998 *Microbiol Mol Biol Revs 62*: 807-813) proposed a revision of the nomenclature for *B. thuringiensis* crystal protein changing from a function-based to a sequence-based nomenclature which allows closely related toxins to be ranked together. In such proposed revision "Roman numerals have been exchanged for Arabic numerals, for instance, CryIA(a) is now Cry1Aa to be better accomodate the large number of expected new proteins" and more than 100 sequenced crystal proteins genes are known (Schnepf et al. *loc. cit*). Serology based on flagellar antigen has been used to characterize *B. thuringiensis* serovars since 1962 (H de Barjac & A Bonnefoi *Entomophaga 23*: 309-319) and most of the strains toxic to mosquito larvae belong to serotype H 14 (*israelensis*).

Since resistance of mosquito larvae to chemical insecticides is increasing (B Lambert & M Peferoen 1992 *BioSciences 42*: 112-122) and field and laboratory resistance has been demonstrated to some isolates of *B. thuringiensis* (J van Rie et al. 1989 *Science 247*: 72-74, WH McGaughey & ME Whallon 1992 *Sciences 258*: 1451-1455, GG Kennedy & ME Whallon 1995 *J Econ Entomol 88*: 454-460), several laboratories have been searching for novel *B. thuringiensis* strains and toxins. This approach has been practiced in the Culture Collection of the Genus *Bacillus* from Fiocruz, Brazil, for the last few years.

The methodology used for isolating the strains was similar to that of RS Travers et al. (1987 *Appl Environ Microbiol 53*: 1263-1266). It consisted basically of the inhibition of the *B. thuringiensis* germination by high concentration of sodium acetate. After isolation, bacterial colonies were mor-

Partially supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico.
+Corresponding author. Fax: +55-21-270.6565. E-mail: leon@ioc.fiocruz.br
Received 25 January 1999
Accepted 10 June 1999

phologically and microscopically examined. Presumptive *B. thuringiensis* strains were selected for further studies. All sporeforming bacteria bearing a parasporal body were screened with a view to find their taxonomic position based on spore and cell morphology, biochemical and physiological characteristics according to the methods and procedures described by D Claus and RCW Berkeley (1986 Bergey's Manual of Systematic Bacteriology, p. 1105-1139. In PHA Sneath, NS Mair, ME Sharpe, J-G Holt (eds), Williams & Wilkins, Baltimore), RE Gordon et al. (1973 USDA Handbook 427, Washington, p. 97-98) and H de Barjac and E Frachon (1990 *Entomophaga 35*: 233-240). Multilocus enzyme electrophoresis screening using 13 loci (V Zahner et al. 1989 *J App Microbiol 67*: 275-282) was studied also. The *B. thuringiensis* serovar *israelensis* derived from the standard powder bacterial strain produced by the Pasteur Institute of Paris (IPS-82) was used as reference for these tests.

Several strains of *B. thuringiensis* were isolated in our laboratory from different sources, such as mud, soils (including mosquito breeding-sites and rhizospheres in selected national forest reserves and parks of Brazil), and commercial native spices. One strain, named LFB-Fiocruz 710 was particularly interesting because of its high toxicity against *Simulium pertinax* larvae. Phenotypic and genetic tests performed in this work will help to better characterize this strain potentially useful for biological control of mosquito and blackfly larvae in Brazil.

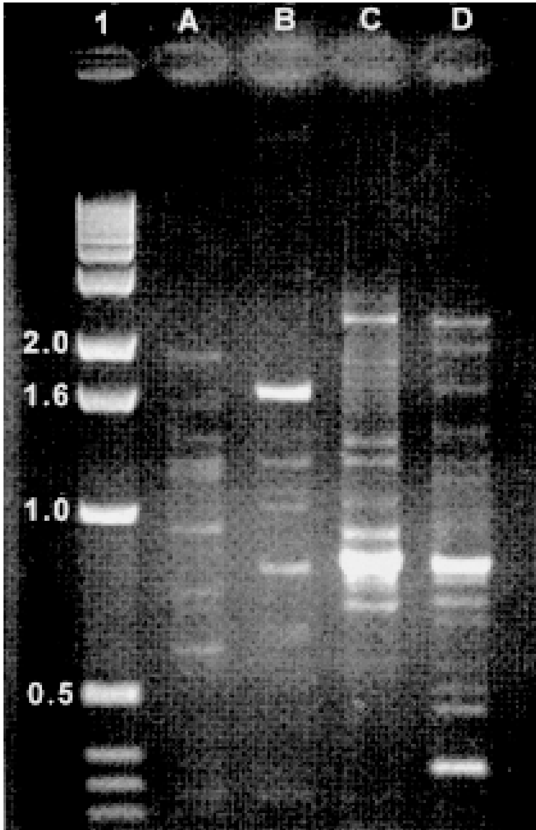
Strain LFB-Fiocruz 710 was isolated from soil of the rhizosphere of a *Ficus doliaria* tree, collected in the National Forest Preserve of Monte Pascoal (Bahia, Brazil) where contamination by commercial microbial inseticides would not be possible. It was identified as *B. thuringiensis* based on the characteristics previously described for the species (Claus & Berkeley *loc. cit.*). Concerning the determination of its serotype, suspensions of strain LFB-Fiocruz 710 agglutinated with H-14 antiserum at a dilution of 1:25,600 (M-M Lecadet, Pasteur Institute of Paris, pers. commun.). When this strain was compared to the reference strain, both strains showed positive results for oxidase, lecithinase, acetyl-methyl-carbinol, reduction of nitrate, serumcoagulase, argynine-dihydrolase, anaerobic growth, esculine hydrolysis and acid from mannose; and negative results for urea hydrolysis, pigment, mannitol and raffinose. When this new strain was tested by using the API system (API50CH), it produced the pattern specific for *B. thuringiensis* (NA Logan & RCW Berkeley 1984 *J Gen Microbiol 130*: 1871-1882), although in three carbohydrates (arbutine, salicine and sucrose) negative results were observed. Different biochemical patterns have been already demonstrated before among *B. thuringiensis* strains belonging to serotype H-14 (de Barjac & Frachon *loc. cit*).

When the isoenzyme profile analysis of strain LFB-Fiocruz 710 was performed according to V Zahner et al. (1994 *Isozyme Bull 27*: 70) by employing agarose gel electrophoresis (H Momen et al. 1987 *J Appl Bacteriol 67*: 275), it was demonstrated that this new strain had the same electrophoretic type as *B. thuringiensis* strain IPS-82 (zymovar 1); both strains belong to electrophoretic type 6. By using sodium-dodecyl sulphate polyacrylamide-gel electrophoresis these strains showed the same electrophoretic standard protein profile (delta-endotoxins).

In order to determine the relationship between strains LFB 710 and IPS-82, their genomic DNAs were amplified using primers corresponding to conserved DNA sequences of rep elements-repetitive extragenic palindromic sequences (J Versalovic et al. 1994 *Meth Mol Cell Biol 5*: 25-40). Different patterns were observed using both primers BOXA1R and REPI/REPII. A common band of 0.8 kb was observed when strains 710 and IPS-82 were amplified using primer BOX (Figure). However, it is clearly shown that strains 710 and IPS-82 are genomically different.

Preliminary toxicity tests were established against different mosquito larval species. The bioassays were conducted using cells grown on Soil Extract Agar (Gordon et al. *loc. cit.*) during 48 hr/30ºC. Two loopfulls of the well sporulated cell growth were homogenized with 1 ml of distilled water and added into plastic cups containing 9 ml of distilled water and ten mosquito larvae per cup, each strain was assayed twice. By this way strain LFB-Fiocruz 710 showed mortality higher than 50% to mosquito larvae (L_3-L_4) of the following species: *Aedes aegypti, Ae. albopictus, Anopheles darlingi, An. deaneorum, An. aquasalis* and *Culex quinquefasciatus*. Strain IPS-82 was used as a positive control, while larvae without inoculation of bacterial cells as a negative control.

The World Health Organization assay protocol (WHO/8CV/IC-GE/8713) was used to evaluate the half lethal concentration (LC_{50}). The LC_{50} against *Ae. albopictus* was 0.03 mg (dry weight basis) per liter after 24 hr while in *An. darlingi* it was 4.8 mg (dry weight basis) per liter. These results are in the same concentration levels of the ones obtained by us with IPS-82 strain. Bioassays against *Ae. aegypti* and *Cx. quinquefasciatus* larvae showed a mortality level higher than 50% by using dilutions of



Amplification patterns of the two *Bacillus thuringiensis* strains used in this study using rep-polyclonal chain reaction 1: 1kb ladder GibcoBRL - A: strain LFB-Fiocruz 710 amplified using primers REP I and II; B: strain IPS-82 amplified using primers REP I and II; C: strain LFB-Fiocruz 710 amplified using primer BOX 1AR and D: strain IPS-82 amplified using primer BOX 1AR.

whole culture as high as 9.7 $x10^{-7}$ and $1.7x10^{-5}$, respectively.

Bioassays using blackfly larvae in field artificial breeding sites was done by employing the methodology described by CJPC Araújo-Coutinho (1995 *Mem Inst Oswaldo Cruz 90:* 131-134) and the mean LC_{50} , based at least on three different assays, each one with five dilutions of wet biomass (LFB 710 with 85.48% and IPS-82 with 87.67% of moisture), obtained for LFB 710 was 2.25 mg/l (95% confidence interval 1.95-2.60) and for the IPS-82 was 3.40 mg/l (95% confidence interval 2.70-4.30), respectively.

According to these bioassays, strain 710 seems to be more efficient bioinsecticide agent against blackflies when compared with those strains of *B. thuringiensis* previously described. Additional studies within this new strain should be encouraged as they could help for further utilization of an alternative bioinsecticide agent against the vector of *Onchocerca volvulus*, the etiological agent of onchocerciasis or river blindness, which according to AJ Shelley et al. (1996 *Bull Nat His Mus Lon 66*: 1-121) and M Maia-Herzog et al. (1999 *Trans R Soc Trop Med Hyg 93*: 1-5) was observed in Brazil.

Acknowledgments: to Professor M-M Lecadet and to the Center of Entomopathogenic Bacteria of the Pasteur Institute-Paris, for providing the H-serotyping. To Dr Cristiano CA Marques, Superintendência de Controle de Endemias, São Paulo, for the bioassays in *Aedes albopictus* larvae and to Rosângela da Costa for secretarial assistance.