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Screening of Brazilian *Bacillus sphaericus* strains for high toxicity against *Culex quinquefasciatus* and *Aedes aegypti*

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Abstract: In this work, 246 *Bacillus sphaericus* strains were evaluated against *Aedes aegypti* and *Culex quinquefasciatus* larvae to select the most effective ones to be used as the basis of a national product. All strains were isolated from different regions of Brazil and they are stored in a *Bacillus* spp. collection at Embrapa Genetic Resources and Biotechnology. The selected strains were characterized by biochemical and molecular methods. Based on selective bioassays, 87 strains were identified as toxic to one or both target species. All of these strains contain genes that encode the 42, 51 kDa proteins that constitute the binary toxin and the 100 kDa Mtx1 toxin. All toxic strains presented a very high LC_{50} against *A. aegypti*, so, a product based on any of these *B. sphaericus* strains would not be recommended for use in programmes to control *A. aegypti*. S201 had highest activity against *C. quinquefasciatus*, presenting the lowest LC_{50} and LC_{90} in bioassays.

Key words: *Aedes aegypti*, *Bacillus sphaericus*, *Culex quinquefasciatus*, bioassay, biological control, mosquito, vector disease

1 Introduction

Bacillus sphaericus Neide is a bacterial species found commonly in soil and aquatic habitats (Davidson, 1985) and characterized by the production of spherical terminal or subterminal spores in the sporangium. Although most strains of *B. sphaericus* are not pathogenic for insects, the mosquitocidal strains are important tools in mosquito control programs. The first pathogenic strains were isolated from *Culiseta incidens* (Dipt.: Culicidae) larvae and were called strains K and Q (Kellen et al., 1965). Currently many toxic strains are known and many studies have been performed on strains 1593 and 2362 isolated respectively in Indonesia (Singer, 1973) and Nigeria (Weiser, 1984). This organism does not infect non-target invertebrates (including bees) or cold-blooded vertebrates and it is also innocuous to mammals in laboratory tests (Davidson, 1985). The World Health Organization (WHO) recommends the utilization of this bacterium in public health programs (World Health Organisation, 1985).

Almost all mosquito species from the genus *Culex* (Dipt.: Culicidae), some of them vectors of filariasis, are susceptible to *B. sphaericus*, as are members of the genera *Anopheles, Psorophora* and *Mansonia*. The activity of this bacterium against mosquitoes from the genera *Aedes* and *Ochlerotatus* (representing species that used to be defined as *Aedes*), is variable. Some mosquito species are very susceptible and others, particularly *Aedes aegypti*, a major vector of dengue and yellow fever, show low sensitivity. *Bacillus sphaericus* is very effective when used in polluted water, making it a good option to control *Culex quinquefasciatus* and other *Culex*, breeding in polluted water in cities located in tropical and sub-tropical areas. The *B. sphaericus* activity is because of a presence of different kinds of protein toxins that differ both in their composition and time of synthesis. The parasporal crystal of *B. sphaericus*, which is produced during the sporulation phase is a binary toxin composed of two components designated as P51 and P42 on the basis of their molecular masses (Baumann et al., 1987), and now termed BinB and BinA, respectively. The different Mtx toxins, which have molecular masses of 100 kDa (Mtx1) and 32 and 36 kDa (Mtx2 and Mtx3) are expressed during the vegetative growth phase, but low levels of production and instability mean that their toxicity is of minor significance, particularly in the spores that are applied in mosquito control programmes (Thanabalu et al., 1991; Chan et al., 1996; Liu et al., 1996).

Bacillus sphaericus is a very promising microorganism and several laboratories around the world are looking for new strains that may be able to produce novel toxins or which may be more adapted to local environmental conditions, in order to have better effect in the field and which could be used in resistance management (Monnerat and Bravo, 2000).

Embrapa Genetic Resources and Biotechnology has a culture collection of entomopathogenic *Bacillus* spp. in which around 300 *B. sphaericus* strains are stored (Monnerat et al., 2001a). The aim of this work was the

characterization of the most toxic *B. sphaericus* strains for the control of *A. aegypti* and *C. quinquefasciatus* among the Embrapa culture collection to identify strains that could be used as a basis for a Brazilian product.

2 Materials and Methods

2.1 *Bacillus sphaericus* strains

A total of 246 *B. sphaericus* strains were used in this work. They are stored at Embrapa's Culture Collection of Entomopathogenic *Bacillus* spp. and were isolated from soil and water samples collected in different regions of Brazil (Monnerat et al., 2001a).

2.2 Preliminary bioassay

All strains were grown in NYSM (a medium composed of nutrient broth, yeast extract, Mncl2, MgCl2 and Ca Cl2) medium (Yousten, 1984) for 48 h at 28°C and 200 r.p.m. and tested against third-instar larvae of *C. quinquefasciatus* and *A. aegypti*. One millilitre of total culture of each strain was added to 200 ml cups in triplicate with 100 ml of distilled water and 25 larvae of *C. quinquefasciatus* or *A. aegypti*. One cup without bacteria was used as the control. Forty-eight hours later, the numbers of dead larvae were evaluated. The strains that killed more than 50% of the larvae were considered pathogenic (Monnerat et al., 2001b).

2.3 Quantified bioassay

2.3.1 Final whole culture

In order to determine the LC_{50}, the quantified bioassay was performed according to the method recommended by WHO (WHO, 1985), several dilutions of the final culture prepared as described above were used. One millilitre of these dilutions was added into 200 ml cups in triplicate, as for the procedure used in selective bioassays. Forty-eight hours later the numbers of dead larvae were recorded and the LC_{50} was calculated by Probit analysis (Finney, 1971). *Bacillus sphaericus* 2362 (SPH-88, from the Pasteur Institute) was used as standard.

2.3.2 Lyophilized culture

The most toxic strains against *C. quinquefasciatus* were also tested as above, except that lyophilized culture, prepared as described previously (WHO, 1985), was added in place of diluted whole cell cultures. These bioassays were repeated three times.

2.4 Analysis of protein profile

The spore-crystal mixtures of the *B. sphaericus* strains were prepared according to Schenkel et al. (1992). The protein composition of the spore-crystal mixtures was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels.

2.5 Analysis of the presence of *B. sphaericus* toxin genes by polymerase chain reaction (PCR)

The method used was described by Otsuki et al. (1997). *Bacillus sphaericus* strains were grown on NYSM agar for 16 h, at 25°C. Cells were resuspended in MilliQ water and frozen at −80°C for 1 h and then transferred to boiling water for 10 min to lyse the cells.

Primers designed for detection of the binary toxin operon, and the individual *bin* genes, BSN1/BSN2 and BS1/BS2 (*binB*), BSN3/BSN4 and BS3/BS4 (*binA*) and 100.1/100.2 (*mtx1*) toxin were used (Otsuki et al., 1997). Fifteen microlitre of supernatant obtained from cell lysates of the *B. sphaericus* strains were transferred to a 200-μl reaction tube (Bio-Products) containing 0.5 μM of each primer, 0.2 mM of each dNTP, 1x *Taq* polymerase buffer, 1.5 mM $MgCl_2$ and 2.5 U of *Taq* DNA polymerase (Gibco BRL, Life Technologies, Grand Island, NY, USA) in a final volume of 50 μl. PCR amplification was performed with a Programmable Thermal Controller (MJ Research model PTC-100, MJ Research, Inc., Waltham, MA, USA). The conditions used for the PCRs were those described by Otsuki et al. (1997). After amplification, a 15-μl sample of the product from each PCR reaction was subjected to electrophoresis in a 2% (w/v) agarose gel in Tris-borate buffer at 100 V for 1 h and stained with ethidium bromide.

2.6 Sequencing of a bin operon

To determine the sequence of the bin genes from a sample strain, the operon was amplified by PCR using the primers BinF (CAATGATAAGGAGATGAAGA) and BinR (CATCTATTAGTTCAAGAATATTG) at an annealing temperature of 60°C. The amplified fragment was cloned into the *Escherichia coli* vector pGEM-T (Promega, Madison, WI USA) for subsequent sequencing using an ABI Prism 3100 capillary sequencer.

3 Results and Discussion

3.1 Toxicities of *B. sphaericus* strains against *C. quinquefasciatus* and *Aedes aegypti* larvae

Single point bioassays were performed on all 246 strains of *B. sphaericus* in the Embrapa collection. Of these strains, 87 strains were considered toxic, killing more than 50% of insect larvae: 69 were toxic only to *C. quinquefasciatus* and 18 to both *C. quinquefasciatus* and *A. aegypti*.

LC_{50} and LC_{90} values, using a final whole culture against the two target species, were subsequently determined only for the 18 strains that presented dual activity (tables 1 and 2).

Against *C. quinquefasciatus*, the LC_{50} values showed a range between 0.38 and 14.5×10^{-6} fcd (final culture dilution), the LC_{90} values showed a range between 1.52 and 72.9×10^{-6} (table 1). The most effective strains were S201 and S162, having respectively LC_{50} of 0.38 and 0.55×10^{-6} fcd and LC_{90} of 1.52 and 2.20×10^{-6} fcd. This result shows that both strains are at least twice as active against this insect than the standard strain *B. sphaericus* 2362 that had an LC_{50} of 2.1×10^{-6} fcd and LC_{90} of 5.4×10^{-6} fcd in our assays (table 1).

Against *A. aegypti*, the LC_{50} values ranged between 0.003 and 0.35 fcd and the LC_{90} between 0.012 and 2.67 fcd (table 2). The most effective strains were S242, S233 and S260, having LC_{50} of 0.003, 0.004 and 0.004 fcd and LC_{90} of 0.012, 0.018 0.019 fcd respectively. Strains 2362 and S201 were the least toxic

Table 1. *Toxicities of* B. sphaericus *strains against C. quinquefasciatus larvae*

Strains	LC_{50} (fiducial limits 95%)	LC_{90} (fiducial limits 95%)
S201	0.38 (0.28–0.52)	1.52 (1.02–2.74)
S162	0.55 (0.41–0.76)	2.20 (1.46–4.18)
2362	2.12 (1.65–2.87)	5.40 (3.80–9.30)
S242	2.20 (1.70–2.98)	5.47 (3.87–9.40)
S200	2.28 (1.77–3.09)	5.56 (3.94–9.43)
S295	2.28 (1.77–3.09)	5.56 (3.94–9.43)
S260	2.47 (1.91–3.35)	5.74 (4.10–9.46)
S516	2.69 (2.06–3.57)	6.73 (4.89–10.6)
S524	2.69 (2.06–3.57)	6.73 (4.89–10.6)
S15	2.86 (2.19–3.77)	7.58 (5.53–11.7)
S16	3.16 (2.39–4.16)	9.19 (6.67–14.3)
S233	3.16 (2.39–4.18)	7.07 (5.25–10.5)
S444	3.16 (2.39–4.18)	7.07 (5.25–10.5)
S662	3.16 (2.39–4.18)	7.07 (5.25–10.5)
S438	3.54 (2.67–4.62)	8.59 (6.46–12.5)
S64	3.85 (2.84–5.01)	8.70 (6.64–12.2)
S558	4.84 (3.62–6.35)	14.6 (10.6–23.3)
S1	13.7 (9.30–27.7)	66.0 (31.1–428)
S131	14.5 (9.73–31.7)	72.9 (32.9–556)

The unit used was final culture dilution (fcd) $\times 10^{-6}$.

Table 2. *Toxicities of* B. sphaericus *strains against A. aegypti larvae*

Strains	LC_{50} (fiducial limits 95%)	LC_{90} (fiducial limits 95%)
S242	0.003 (0.002–0.005)	0.012 (0.008–0.022)
S233	0.004 (0.002–0.006)	0.018 (0.011–0.033)
S260	0.004 (0.002–0.006)	0.019 (0.012–0.035)
S295	0.005 (0.003–0.007)	0.025 (0.015–0.049)
S516	0.005 (0.003–0.007)	0.025 (0.015–0.049)
S558	0.005 (0.003–0.008)	0.029 (0.017–0.059)
S200	0.006 (0.004–0.010)	0.037 (0.021–0.082)
S444	0.006 (0.004–0.010)	0.037 (0.021–0.082)
S662	0.006 (0.004–0.010)	0.037 (0.021–0.082)
S438	0.007 (0.004–0.010)	0.040 (0.023–0.090)
S524	0.007 (0.004–0.010)	0.040 (0.023–0.090)
S131	0.009 (0.006–0.015)	0.061 (0.031–0.168)
S15	0.020 (0.013–0.029)	0.140 (0.080–0.310)
S64	0.024 (0.017–0.035)	0.160 (0.096–0.0351)
S16	0.047 (0.033–0.069)	0.230 (0.141–0.450)
S1	0.044 (0.032–0.065)	0.220 (0.130–0.411)
S162	0.062 (0.043–0.095)	0.034 (0.205–0.740)
S201	0.233 (0.165–0.330)	1.30 (0.811–2.66)
2362	0.355 (0.243–0.541)	2.67 (1.47–7.07)

The unit used was final culture dilution (fcd).

presenting LC_{50} values of 0.35 and 0.24 fcd and LC_{90} of 2.67 and 1.30 fcd (table 2).

To check whether higher apparent toxicities were because of a greater toxicity per cell or a higher cell density in the final cultures, LC_{50} and LC_{90}values were also determined for the four most toxic strains against *C. quinquefasciatus* using a lyophilized final whole culture (table 3). In this case, S201 and S242 were the most toxic strains, showing LC_{50} of 1.24 and 1.35 ng/ml and LC_{90} of 4.52 and 7.16 ng/ml of lyophilized material, respectively whilst strain S162 showed similar toxicities to that of 2362 with values of 2.97 and 4.15 ng/ml (LC_{50}) and 26.8 and 35.1 ng/ml (LC_{90}).

Table 3. *Toxicities of* B. sphaericus *strains against C. quinquefasciatus larvae. Results are expressed in nanogram of lyophilized bacteria/ml*

Strains	LC_{50} (fiducial limits 95%)	LC_{90} (fiducial limits 95%)
S201	1.24 (0.92–1.67)	4.52 (3.12–7.87)
S242	1.35 (0.95–1.90)	7.16 (4.53–14.9)
S162	2.97 (1.92–4.36)	26.8 (16.6–52.2)
2362	4.15 (2.74–6.04)	35.1 (21.7–69.7)

These results confirm *B. sphaericus* S201 and S242 as promising strains and indicates that for strain S201, better growth characteristics as well as higher toxicity per spore may contribute to the higher activity observed.

3.2 Analysis of protein profile by SDS-PAGE

Spore crystal complexes from 87 mosquitocidal *B. sphaericus* strains and the standard strain 2362 were used. All of them presented the same protein profile, showing two major proteins of 51 and 42 kDa (fig. 1). This protein profile is typical of the binary toxin produced by *B. sphaericus* (Baumann et al., 1987).

3.3 Analysis of the presence of *B. sphaericus* toxin genes by polymerase chain reaction

All 87 *B. sphaericus* pathogenic strains produced the expected PCR amplicons of 0.523, 0.720 and 0.700 kb, indicating the presence of genes encoding BinA, BinB and Mtx1, respectively (fig. 2). These results are consistent with the protein profiles for these strains where the presence of 51 and 42 kDa bands, corresponding to the sizes of BinB and BinA respectively, were observed. The presence of 100 kDa

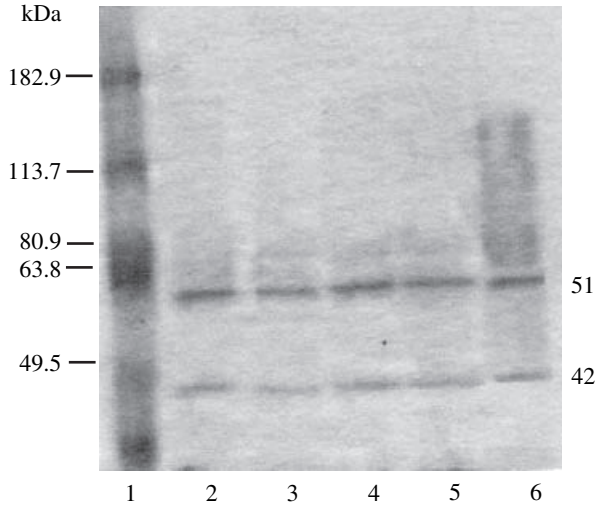

Fig. 1. *Representative SDS-PAGE of spore-crystal from B. sphaericus strains. 1, molecular marker Gibco BRL; 2, 2362; 3, S242; 4, S233; 5, S260 and 6, S295. All other toxic strains exhibited the same profile (not shown)*

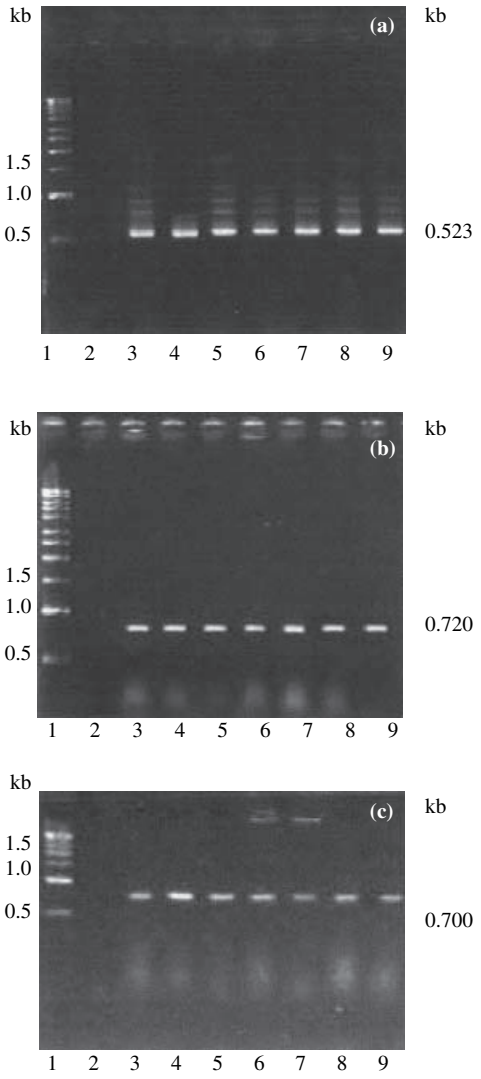

Fig. 2. *Representative agarose gel of PCR products obtained with primers BS1/BS2 (a), BSN3/BSN4 (b) and 100.1/100.2 (c). 1, molecular marker 1 kb Pharmacia; 2, negative control; 3, 2362; 4, S200; 5, S233; 6, S242; 7, S260; 8, S295 and 9, S516. All other toxic strains exhibited the same profile (not shown)*

bands corresponding to the Mtx1 toxin were not observed on SDS-PAGE and would not be expected in spore crystal mixes due the fact that this is a vegetative protein produced in low amounts with low stability that has not been observed previously in spores (Thanabalu et al., 1992; Thanabalu and Porter, 1995).

3.4 Sequencing of a bin operon

To assess whether this toxicity was because of a new variant of the bin operon, the bin genes from S242 were amplified and sequenced. The results showed that the bin operon in this strain was identical to bin type 2 as found in *B. sphaericus* strains such as 2362 (Humphreys and Berry, 1998). This indicates that the greater toxicity to *A. aegypti* may be because of higher levels of toxin production or to the presence of an extra, unidentified toxin active against this species.

The assays performed in this work demonstrated that the toxic *B. sphaericus* strains isolated from different regions of Brazil all appear to be typical of highly toxic strains of this bacterium as they encode Bin toxins, along with the Mtx1 protein although they present a range virulence levels towards the two mosquito species studied. It is also important to emphasize that the LC_{50} obtained against *C. quinquefasciatus* is not indicative of the relative toxicity against *A. aegypti*. Although many strains are more toxic than 2362 against *A. aegypti*, the LC_{50} are still very high when compared with *Bacillus thuringiensis israelensis* (Goldberg and Margalit, 1997) and *B. thuringiensis medellin* (Orduz et al., 1994) so, a product based on any of these *B. sphaericus* strains would not be recommended for use in programmes to control *A. aegypti*. The compilation of the results shows that S201 is the best strain to be used as a basis of a product against *C. quinquefasciatus*, as this strain presented the lowest LC_{50} and LC_{90} in both kinds of bioassays with a significantly better activity against this mosquito.

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