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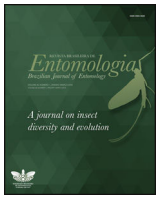

Biological Control and Crop Protection

Isolation and molecular characterization of *Bacillus thuringiensis* found in soils of the Cerrado region of Brazil, and their toxicity to *Aedes aegypti* larvae

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ABSTRACT

This study investigated the potential of *Bacillus thuringiensis* isolates obtained in the Cerrado region of the Brazilian state of Maranhão for the biological control of *Aedes aegypti* larvae. The isolates were obtained from soil samples and the identification of the *B. thuringiensis* colonies was based on morphological characteristics. Bioassays were run to assess the pathogenicity and toxicity of the different strains of the *B. thuringiensis* against third-instar larvae of *A. aegypti*. Protein profiles were obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Polymerase chain reaction assays were used to detect the toxin genes found in the bacterial isolates. Overall, 12 (4.0%) of the 300 isolates obtained from 45 soil samples were found to present larvicidal activity, with the BtMA-104, BtMA-401 and BtMA-560 isolates causing 100% of mortality. The BtMA-401 isolate was the most virulent, with the lowest median lethal concentration (LC_{50}) (0.004×10^7 spores/mL), followed by the *Bacillus thuringiensis* var. *israelensis* standard (0.32×10^7 spores/mL). The protein profiles of BtMA-25 and BtMA-401 isolates indicated the presence of molecular mass consistent with the presence of the proteins Cry4Aa, Cry11Aa and Cyt1, similar to the profile of *Bacillus thuringiensis* var. *israelensis* IPS-82. Surprisingly, however, none of the *cry* and *cyt* genes analyzed were amplified in the isolate BtMA-401. The results of the present study revealed the larvicidal potential of *B. thuringiensis* isolates found in the soils of the Cerrado region from Maranhão, although further research will be necessary to better elucidate and describe other genes associated with the production of insecticidal toxins in these isolates.

Introduction

Aedes (*Stegomyia*) *aegypti* (Linnaeus 1762) is the principal vector in the global resurgence of epidemic dengue, and also transmits a number of other arboviruses that affect human populations around the world, including Zika and Chikungunya, which have been introduced recently into Brazil (Donalisio and Freitas, 2015; Gubler, 1998a, 1998b; Honório et al., 2015; Lima-Camara, 2016; Vasconcelos, 2015).

Dengue fever is a major public health concern in Brazil and many other countries, and is the second most prominent arbovirosis in terms of the total number of persons infected, worldwide (Gubler et al., 2001; MS, 2015; Vasconcelos, 2015; WHO, 2013).

This vector is usually controlled using chemical larvicides containing active ingredients such as organochlorines, organophosphates, carbamates and pyrethroids (Camargo et al., 1998; Carvalho et al., 2004; Oliveira, 1998; Quimbayo et al., 2014; Rebêlo et al., 1999).

These insecticides are toxic to humans and the environment, eliminate natural enemies, and when used indiscriminately, may select for resistance in the target mosquito populations (Braga and Valle, 2007; Vilarinhos et al., 1998). Given these disadvantages, there is a clear need for the investigation of more effective and ecologically secure methods for the control of these vectors.

In recent decades, there has been a gradual reduction in the use of chemical pesticides as the development of biological control

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agents has intensified (Alves, 1998; Guo et al., 2015; Pamplona et al., 2004; Polanczyk et al., 2003). In particular, entomopathogenic bacterial spores have enormous potential for the biological control of insects (Palma et al., 2014; Peralta and Palma, 2017; Schnepf et al., 1998). The inclusion of these spores as control agents enables them to resist adverse environmental conditions and facilitates large-scale industrial production (El-Bendary, 2006; Habib and Andrade, 1998; Polanczyk and Alves, 2003).

While hundreds of bacterial strains may affect insects, only a few can be used effectively for the biological control of the vectors that cause tropical diseases. One especially important species is *Bacillus thuringiensis* (Bt) Berliner 1911, which is known to have entomopathogenic properties that are effective against insects of the orders Lepidoptera, Coleoptera, Diptera, among others (Cavados et al., 2001; De Maagd et al., 2003; Van Frankenhuyzen, 2009, 2013). Given this, Bt has enormous potential for the control of agricultural pests and vectors of human diseases.

B. thuringiensis is an active component in many commercial biopesticides (Rosas-Garcia, 2009; Sanahuja et al., 2011; Sanchis, 2011). The effectiveness of this species is due to its ability to produce protein crystals during sporulation that contain insecticidal toxins known as δ-endotoxins (Cry and Cyt) (Bravo et al., 2011; Glare and O'Callaghan, 2000).

The mode of action of δ-endotoxins Cry involves sequential interactions with several insect midgut proteins that facilitate the formation of an oligomeric structure and induce its insertion into the membrane, forming a pore that involves recognition and subsequent binding of the toxin to membrane receptors that kills midgut cells (Bravo et al., 2005, 2007; Likitvivatanavong et al., 2011; Pardo-López et al., 2012; Vachon et al., 2012).

The δ-endotoxins Cyt, besides being toxic to some orders of insects (Federici and Bauer, 1998), act aiding in the insertion of Cry toxins into the intestinal epithelium of mosquitoes, synergizing the insecticidal activity of Cry proteins of mosquitocidal Bt strains (Cantón et al., 2011; Pardo-López et al., 2009; Pérez et al., 2005, 2007).

The different Cry and Cyt proteins that were developed as biological agents are the result of continuous effort in searching for toxins that present appropriate properties for controlling insects of agricultural importance and human disease vectors (Campanini et al., 2012; Pigott and Ellar, 2007). The identification of new Bt strains with insecticidal properties distinct from those already known is thus a research priority in many regions of the world.

The Cerrado biome has a wealth of endemic species. In Maranhão, the Cerrado is characterized by a diversity of ecosystems, due to the proximity of two major Brazilian biomes, the Amazon and the Caatinga, its considerable climatic variation, and extensive hydrographic network (CONAMA, 2015).

In this context, the present study investigated the potential of the new Bt isolates found in the region's soils as agents for the biological control of *A. aegypti*. The search for new isolates, especially in biologically diverse environment provided, is essential for the production of new combinations of insecticidal toxins, derived from the *cry* and *cyt* genes, for the control of the larvae of mosquito vectors.

Materials and methods

Isolation and identification of Bacillus thuringiensis

To investigate Bt strains, soil samples were collected from 17 municipalities in the Cerrado region of Maranhão. The collection points were georeferenced using a GPS (Global Positioning System). The samples were collected from depths of up to 10 cm using a wooden spatula and stored in sterile 50 mL Falcon tubes. The samples were then taken to the Medical Entomology Laboratory (LABEM) at the *Centro de Estudos Superiores de Caxias of Universidade Estadual do Maranhão* (CESC/UEMA), where they were processed and analyzed for the identification and isolation of Bt strains. Such isolation was based on the procedure described by Polanczyk (2004), which is a modification of the protocol published by the World Health Organization (WHO, 1985).

One gram of the soil of each sample was first mixed with 10 mL of a salt solution (0.006 mM $FeSO_4{\cdot}7H_2O$; 0.01 mM $CaCO_3{\cdot}7H_2O$; 0.08 mM $MgSO_4{\cdot}7H_2O$; 0.07 mM $MnSO_4{\cdot}7H_2O$; 0.006 mM $ZnSO_4{\cdot}7H_2O$). Serial dilutions were conducted in 1% saline buffer. One milliliter of this solution was then homogenized, incubated at 80 °C for 12 min and then placed on ice for 5 min to eliminate vegetative cells. A 100 μL aliquot of this solution was transferred to a Petri dish containing nutrient agar and spread out using a Drigalski spatula. The dishes were then inverted and stored to promote bacterial grow for 48 h in an incubator at 28 °C.

After incubation, bacterial colonies were selected based on the morphological characteristics typical of Bt, such as lack of pigmentation, wavy edges and a circular form (WHO, 1985). Colonies with typical Bt characteristics were inoculated in a nutrient broth containing penicillin G (100 mg/L), which served as the selective medium, and then placed in a rotating incubator for approximately 48 h at 28 °C and 180 rpm. The colonies that grew in the medium with the antibiotic were observed using a phase contrast microscope (100× magnification) to confirm the presence of parasporal material (protein crystals).

The isolates containing crystals were identified as Bt, and were deposited in the Entomopathogenic Bacilli Bank of Maranhão (BBENMA) at LABEM (CESC/UEMA). The isolates were labeled using the standard BBENMA nomenclature, being identified as BtMA (Bt for *B. thuringiensis* and MA for Maranhão), followed by the identification number of the isolation.

Susceptibility bioassays

A total of 300 isolates were selected for the susceptibility bioassays against the third-instar larvae of *A. aegypti*. These larvae were obtained from a colony maintained at LABEM (CESC/UEMA) under controlled conditions, at a mean temperature of 26 ± 2 °C, relative humidity of 85%, and 12 h photophase (Consoli and Loureço de Oliveira, 1994).

Suspensions of bacilli, grown in nutrient agar at 28 °C in bacterial growth incubation for five days until sporulation was observed, were prepared for each isolate. All the bacterial content was then transferred using a platinum loop to Falcon tubes containing 10 mL of autoclaved distilled water. Three replicates of each isolate were prepared in plastic cups containing 10 mL of drinking water, 10 third-instar larvae of *A. aegypti* and 1 mL of the suspension of bacilli. For each bioassay, a replicate with no bacteria was prepared as the negative control. After 24 h and 48 h of the bacilli suspension addition larval mortality was verified by counting living and dead larvae. The larvae that did not move when touched with a sterile stick were considered dead (Dulmage et al., 1990). All isolates presenting mortality higher than 50% were selected to estimate the average lethal concentration (LC_{50}) and to the proteins and genes characterization.

Bioassays to estimate the average lethal concentration (LC_{50})

Suspensions of spores/crystals of the isolates of Bt, previously selected in susceptibility bioassays, including the standard *B. thuringiensis* var. *israelensis* IPS-82 (Bti) strain, were prepared. These isolates were cultured in nutrient agar on Petri dishes, incubated for five days at 28 °C to permit full sporulation

and the release of the crystals. The bacterial content was then transferred using a platinum loop to Falcon tubes containing 10 mL of autoclaved distilled water and 0.01% of Triton® X-100 (spreader-sticker). This suspension was homogenized and used to prepare three suspensions by serial dilution of 10^{-1}, 10^{-2}, and 10^{-3}. The 10^{-2} suspension was counted using a Neubauer hemocytometer according to the method described by Alves and Moraes (1998), in order to standardize a concentration of 1×10^8 spores/mL. From this concentration, 13 different concentrations were prepared by serial dilution for each isolate, ranging from 4.57×10^3 spores/mL to 6.67×10^7 spores/mL. For each concentration five plastic cup were prepared, each containing 20 third-instar larvae of *A. aegypti* in a final volume of 100 mL. For the negative control, a plastic cup was prepared following the same procedure, but without bacteria, and the positive control contained the standard strain (Bti). The experiment was conducted in three repetitions. Mortality was estimated after 24 h and 48 h of the application of the bacterial suspension. The determination of larval mortality was done in the same way the susceptibility bioassays. The LC_{50} was estimated by a Probit analysis run in the POLO PLUS (LeOra Software, 2003) program, based on the data on the larvae mortality (Finney, 1981; Haddad, 1998).

Protein characterization of Bacillus thuringiensis *isolates*

The protein profile of the Bt isolates was obtained by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE 12%) (Laemmli, 1970). Bti IPS-82 standard was used as positive control.

The samples were obtained by growing the isolates in nutrient agar, maintained for five days in a bacteriological growth oven at 28 °C. The proteins were extracted by the protocol of Lecadet et al. (1991) and stored in a protease inhibitor solution at −20 °C. The samples were prepared using 25 μL of the spore/crystal complex, to which 25 μL of sample buffer (0.5 M Tris–HCl pH 6.8, 25% glycerol, 1.0% blue of bromophenol, 10% SDS and 1% β-mercaptoethanol) was added. This mixture was then boiled at 100 °C for 10 min.

An aliquot of 40 μL was extracted from each sample and run in a 12% polyacrylamide gel together with a standard Broad Range Protein Molecular marker (Promega) as a reference for the determination of the molecular weight of the proteins. The electrophoresis was run in a vertical system (Kasvi) filled with 1x run buffer (25 mM Tris-base, 35 mM SDS and 1.92 mM glycine) and charged at 150 V for 2:30 h.

After the run, the gel was stained in *Comassie Brilliant Blue* solution (50% methanol, 10% acetic acid and 0.1% *Comassie Brilliant Blue* R-250) for 1 h at room temperature, and then discolored in a 4:1 methanol:acetic acid solution for 24 h, until visualization of the protein bands corresponding to the toxins. The gel was digitized and analyzed for the presence of proteins of interest, that is, those with insecticidal potential, based on the published data.

Molecular characterization by PCR

The PCR technique was used to detect the presence of the larvicidal (for dipterous) *cry4Aa*, *cry4Ba*, *cry10Aa*, *cry11Aa*, *cry11Ba*, *cyt1Aa*, *cyt1Ab* and *cyt2Aa* genes in the Bt isolates that caused higher mortality in larvae of *A. aegypti* (Table 1). The InstaGene Matrix kit (Bio-Rad) was used to extract the genomic DNA, following the manufacturer's instructions.

The PCR assays were run in a final volume of 25 μL, containing 1× buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 μM of each primer, 1 U *Taq* DNA polymerase, and 2.0 μL of the DNA template. The standard Bti was used as a positive control, and for the negative control, the DNA was replaced by ultrapure water. The genes were amplified in a *Gencycler-G96G* thermocycler (Biosystems). Initial denaturation was 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C for denaturation, 30 s at 50–54 °C for annealing, and 1 min at 72 °C for polymerization, with a final extension of 7 min at 72 °C.

Following amplification, 5 μL of the PCR product was mixed with 3 μL of blue/orange *Loading Dye* (Promega) and run in a 1% agarose gel containing ethidium bromide charged at 90 V, in a TBE 1X (Tris/Borate/EDTA) solution at a basic pH. A 1 kb DNA Ladder (Promega) was used as a marker of molecular weight. The amplification products were visualized and photographed under UV light (L-PIX EX *Loccus* photodocumentator system).

Results

Isolation of Bacillus thuringiensis

Forty-five soil samples from 17 municipalities of the Cerrado region of Maranhão were analyzed and 1225 bacterial colonies were obtained, of which, 383 were identified as Bt, corresponding to 31.26% of the total. The highest number of Bt isolates obtained was recorded for sample 118, collected in the municipality of Paraibano, which contained 32 bacterial colonies, of which, 28 (87.5%) were identified as Bt. The next richest sample was the 120 (85.71% Bt) from São João dos Patos, followed by sample 130 from Balsas, with 83.33% of the bacterial colonies corresponding to Bt (Table 2).

Table 1
Primers used in the PCR to amplify *cry* and *cyt* genes of *Bacillus thuringiensis* that presented toxic activity against *Aedes aegypti*, showing the primer sequences, the size of the target fragment, and the annealing temperature.

Gene (primer)	Nucleotide sequence (5′-3′)	Fragment size (bp)	Annealing temperature (°C)
cry4Aa[a]	5′-GGGTATGGCACTCAACCCCACTT 3′-GCGTGACATACCCATTTCCAGGTCC	777	50
cry4Ba[a]	5′-GAGAACACACCTAATCAACCAAT 3′-GCGTGACATACCCATTTCCAGGTCC	347	52
cry10Aa[a]	5′-ATTGTTGGAGTTAGTGCAGG 3′-AATACTTTGGATGTGTCTTGAG	995	50
cry11Aa[a]	5′-CCGAACCTACTATTGCGCCA 3′-CTCCCTGCTAGGATTCCGTC	470	50
cry11Ba[a]	5′-TACAGGATGGATAGGGAATGG 3′-TAATACTGCCATCTGTTGCTTG	608	52
cyt1Aa[a]	5′-AACTCAAACGAATAACCAAG 3′-TGTTCCTTTACTGCTGATAC	300	53
cyt1Ab[a]	5′-AAGCAAGGGTTATTACATTACG 3′-CCAATACTAAGATCAGAGGG	698	54
cyt2Aa[a]	5′-GCATTAGGAAGACCATTTG 3′-AAGGCTAAGAGTTGATATCG	361	53

[a] Primers designed by Costa et al. (2010).

Table 2
Index of *Bacillus thuringiensis* obtained from soil samples collected in different municipalities of the Cerrado region of Maranhão.

Sample	Municipality	Number of bacterial colonies (BC)	Number of *B. thuringiensis* colonies (Bt index = *n*(Bt/BC)*100)
1	Caxias	5	0 (0%)
2	Caxias	44	0 (0%)
3	Caxias	13	2 (15.38%)
4	Caxias	4	1 (25.0%)
5	Caxias	21	3 (14.28%)
6	Caxias	19	0 (0%)
7	Caxias	22	0 (0%)
8	Caxias	19	0 (0%)
9	Caxias	9	0 (0%)
10	Caxias	20	1 (5%)
11	Caxias	21	6 (28.57%)
12	Aldeias Altas	203	7 (3.44%)
13	Aldeias Altas	93	9 (9.67%)
52	Arari	44	20 (45.45%)
53	Arari	41	8 (19.51%)
54	Santa Luzia	3	1 (33.33%)
55	Santa Luzia	12	2 (16.66%)
66	Bacabal	52	11 (21.15%)
67	Bacabal	84	43 (51.19%)
68	Alto Alegre do MA	27	6 (22.22%)
69	Alto Alegre do MA	5	2 (40.0%)
73	Parnarama	31	6 (19.35%)
115	Colinas	33	25 (75.75%)
116	Colinas	28	10 (35.71%)
117	Colinas	30	16 (53.33%)
118	Paraibano	32	28 (87.5%)
119	Paraibano	24	17 (70.83%)
120	São João dos Patos	28	24 (85.71%)
121	São João dos Patos	20	14 (70.0%)
122	São João dos Patos	13	9 (69.23%)
123	Barão de Grajaú	7	5 (71.42%)
124	Barão de Grajaú	22	14 (63.63%)
125	Pastos Bons	8	2 (25.0%)
126	Benedito Leite	33	13 (39.39%)
127	Benedito Leite	27	17 (62.96%)
128	São Raimundo das Mangabeiras	20	5 (25.0%)
129	São Raimundo das Mangabeiras	13	3 (20.07%)
130	Balsas	24	20 (83.33%)
131	Balsas	8	4 (50.0%)
132	Riachão	10	3 (30.0%)
133	Riachão	22	14 (63.63%)
134	Carolina	6	2 (33.33%)
135	Carolina	7	1 (14.28%)
136	Carolina	9	6 (66.66%)
137	Carolina	9	3 (33.33%)
Total		1225	383 (31.26%)

Table 3
Genes detected in the *Bacillus thuringiensis* isolates from the Cerrado region of Maranhão and the mortality rates of *Aedes aegypti* larvae, following the selective bioassays.

Isolate	Gene								Mortality (%)	
	cry					*cyt*			24 h	48 h
	cry4Aa	*cry4Ba*	*cry10Aa*	*cry11Aa*	*cry11Ba*	*cyt1Aa*	*cyt1Ab*	*cyt2Aa*		
BtMA-25	–	–	–	+	+	+	–	–	53.3	86.6
BtMA-64	–	–	–	–	–	+	–	–	6.6	76.6
BtMA-104	–	–	–	–	–	+	–	–	100	100
BtMA-131	–	–	–	–	–	–	–	–	20.0	76.6
BtMA-194	–	–	–	–	–	–	–	+	13.3	66.6
BtMA-251	–	–	–	–	–	–	+	–	6.6	93.3
BtMA-401	–	–	–	–	–	–	–	–	100	100
BtMA-410	–	–	–	+	–	–	+	+	0	96.6
BtMA-413	–	–	–	+	–	–	–	+	0	93.3
BtMA-450	–	–	–	–	–	+	–	+	73.0	96.6
BtMA-451	–	–	–	–	–	+	–	+	0	86.6
BtMA-560	–	–	–	+	–	–	–	–	100	100

+, amplified the gene; –, did not amplify the gene.

Table 4
Average lethal concentration (LC_{50}) of *Bacillus thuringiensis* isolates for *Aedes aegypti* larvae, 48 h after the application of the bacteria.

Isolate	*N*	Inclination±SD	LC_{50}	(CI 95%)	χ^2 (DF)
Bti	180	4.857 ± 0.457[a]	0.32×10^7	$(0.26–0.40) \times 10^7$	9.9448 (4)
BtMA-25	180	2.593 ± 0.192[a]	0.73×10^8	$(0.54 \times 10^8–0.12 \times 10^9)$	26.795 (6)
BtMA-104	180	1.511 ± 0.106[a]	0.84×10^8	$(0.45 \times 10^8–0.36 \times 10^9)$	39.194 (5)
BtMA-401	180	4.460 ± 0.276[a]	0.004×10^7	$(0.0034–0.0047) \times 10^7$	2.5322 (2)
BtMA-560	180	3.073 ± 0.181[a]	0.65×10^8	$(0.46 \times 10^8–0.12 \times 10^9)$	41.57 (4)

N, total number of insects/doses tested; SD, standard deviation; CI, confidence interval; χ^2, Chi-square; DF, degree of freedom.
[a] Significant ($p \leq 0.05$).

Selection of Bacillus thuringiensis

Overall, 12 (4.0%) of the 300 Bt isolates tested caused mortality in the *A. aegypti* larvae, and only three (BtMA-104, BtMA-401 and BtMA-560) of these occasioned 100% mortality in 24 h. After 48 h, BtMA-251, BtMA-410, BtMA-413, BtMA-450 isolates caused mortality of over 90%, while BtMA-25 and BtMA-451 provided over 80% mortality, BtMA-64 and BtMA-131 occasioned 76.6% mortality, and isolate BtMA 194 caused 66.6% mortality (Table 3).

Average lethal concentration (LC_{50})

The 12 isolates that caused over 50% mortality in the *A. aegypti* larvae were suitable for a linear regression analysis, with significant ($p \leq 0.05$) *t* values (>1.96). However, three of these (BtMA-25, BtMA-104 and BtMA-560) were not suitable for Probit analysis due to the fact that their observed χ^2 were higher than the expected value (Table 4).

The BtMA-401 isolate was the most virulent, with the lowest LC_{50} value (0.004×10^7 spores/mL), followed by the standard bacterium, Bti (0.32×10^7 spores/mL). All other isolates tested returned higher LC_{50} values than the standard bacterium (Table 4).

Proteins present in the Bacillus thuringiensis *isolates*

The isolates BtMA-25 and BtMA-401 presented proteins with a molecular mass between 25 and 150 kDa (Fig. 1), similar to those found in Bti IPS-82. Protein with a molecular weight of approximately 30 kDa was observed in the BtMA-131 isolate. Well-defined bands of molecular mass in the 100–150 kDa range were obtained from the isolates BtMA-64 and BtMA-194, although few proteins were obtained from the isolates BtMA-104, BtMA-251, BtMA-410 and BtMA-450.

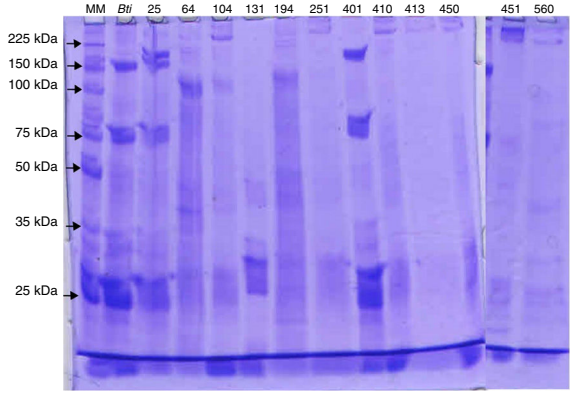

Fig. 1. SDS-PAGE protein profiles of the *Bacillus thuringiensis* isolates most toxic to *Aedes aegypti* larvae. MM, molecular weight marker (kDa); Bti, *Bacillus thuringiensis* var. *israelensis*; 25–560, *Bacillus thuringiensis* isolates.

Molecular characterization of Bacillus thuringiensis *toxin genes by PCR*

One or more Dipteran-specific *cry* and *cyt* genes were visualized in at least 10 of the 12 Bt isolates pathogenic to the *A. aegypti* larvae, when analyzed by PCR. The BtMA-25 and BtMA-410 isolates amplified the highest number of genes, and the *cyt* genes were more frequent, with *cyt1Aa* and *cyt2Aa* being the predominants (Table 3).

Discussion

This study searched for native Bt isolates from the Cerrado biome of Maranhão that can potentially be used for the development of biocontrol tools to help fight mosquito-borne diseases. The soils of the Cerrado biome are poor in nutrients, relatively acidic, and contain large amounts of aluminum. They are often deep red or red-yellowish in color, porous, and permeable (EMBRAPA, 2015). Even so, these soils were shown to have considerable potential as a substrate for Bt (Mourão, 2013; Valicente and Barreto, 2003), with a total 368 Bt colonies isolated from 45 samples.

Soil is the principal natural reservoir of Bt spores and is currently the preferred substrate for the isolation of *Bacillus* species (El-kersh et al., 2016; Hossain et al., 1997; Meadows, 1993; Polanczyk and Alves, 2003; Silva et al., 2012; Soares-da-Silva et al., 2015). Several Bt strains with significantly high larvicidal efficacy against mosquitoes have been isolated from soil samples (Campanini et al., 2012; El-kersh et al., 2016; Soares-da-Silva et al., 2015).

In the present study, the proportion of Bt isolates that caused mortality in the *A. aegypti* larvae was only 4%, although this rate was higher than that recorded in many other insecticidal trials involving mosquito larvae (Dias et al., 2002; Praça et al., 2004; Ootani et al., 2011; Pereira et al., 2013). In general, bacterial strains with mosquitocidal action tend to be rare in comparison their effect against other orders, such as the Lepidoptera (Polanczyk et al., 2004; Silva et al., 2012; Silva-Werneck et al., 2000) and Coleoptera (Martins et al., 2003; Silva, 2008).

The low frequency of isolates with potential for the control of mosquito populations (Costa et al., 2010; Dias et al., 2002; Silva et al., 2002) may be related to the smaller number of described toxins known to affect this group of insects. By contrast, approximately 95 active toxins have already been cataloged for the control of lepidopterans and coleopterans (Van Frankenhuyzen, 2009, 2013).

While only a small number of the isolates analyzed in the present study were toxic to *A. aegypti* larvae, the BtMA-401 strain was more virulent (LC_{50} of 0.004×10^7 spores/mL) than the standard Bti strain (LC_{50} of 0.32×10^7 spores/mL). This isolate thus appears to have enormous potential for the control of mosquito populations, although it will be necessary to identify its active components, given that the molecular analyses using specific primers did not detect any of the expected *cry* and *cyt* genes.

Soares-da-Silva et al. (2015) and Costa et al. (2010) also identified Bt isolates from Brazilian soils with potential as biological agents for the control of *A. aegypti*. Costa et al. (2010) obtained five isolates that were more effective than the standard Bti strain

after 3 h of exposure. These new strains presented LC_{50} values of between 0.01×10^5 and 0.03×10^5 spores/mL, in comparison with a LC_{50} of 0.04×10^5 spores/mL for Bti under the same experimental conditions. It is important to note, however, that these more effective isolates are almost invariably very rare. Praça et al. (2004) and Dias et al. (2002), for example, did not identify any isolate more toxic than the standard strain.

The profiles of isolates BtMA-25 and BtMA-401 indicated the presence of proteins of molecular mass similar to Cry4Aa, Cry11Aa and Cyt1, proteins found in Bti. The BtMA-131 isolate also contained a protein with a molecular weight of approximately 30 kDa, similar to that of the Cyt2 protein. The Cyt1 and Cyt2 classes identified in the present study have a molecular mass of 27–30 kDa, and are known to act synergistically with Cry toxins, which increases the efficiency of an isolate for the control of mosquito populations (Ben-Dov, 2014; Bravo et al., 2007; Crickmore et al., 1998; Jouzani et al., 2008; Praça et al., 2007).

At least one of the genes *cry11Aa*, *cry11Ba*, *cyt1Aa*, *cyt1Ab*, and *cyt2Aa*, which are all known to be toxic to *A. aegypti* larvae, were identified in 83.3% of the isolates analyzed in the present study. Mourão (2013) found these genes in 46.9% of the mosquitocidal strains tested from the Cerrado region. The high frequency of cytolytic toxins found in the Bt isolates from the Cerrado region of Maranhão, which were toxic to *A. aegypti*, reinforces their importance for the control of mosquito populations. The results of the present study are consistent with those of Costa et al. (2010), who recorded the *cyt* gene in 24 of the 45 samples amplified using *cyt* primers. However, Costa et al. (2014) obtained positive results for the *cyt* gene in only 6 (1.2%) of the 500 Bt isolates tested.

It is important to note that, in the present study, two of the larvicidal isolates did not contain the target genes. This emphasizes the need for a broader molecular characterization to investigate additional genes known to be associated with the larvicidal properties of this bacterium for the control of *A. aegypti* larvae. At the present time, approximately 53 toxins have been cataloged, including 15 Cry and five Cyt known to be toxic to *A. aegypti* larvae (Crickmore, 2016; Van Frankenhuyzen, 2009). Soares-da-Silva et al. (2015) obtained similar results to those of the present study in their analysis of six active isolates from the Amazon region, with none of the studied genes being amplified in five (83.3%) of the samples.

Another important finding is that three (30%) of the 10 isolates that were amplified were positive for both *cry* and *cyt* genes. These isolates were relatively effective, which may be related to the association of two classes of toxin, given that their synergistic interaction is known to increase the toxicity of these strains. Praça et al. (2004) concluded that the toxicity of some isolates for the target insects may be the result of the synergistic interaction between the mosquitocidal Bt toxins themselves, or the interaction of these toxins with the bacterial spores.

The *cry11Aa*, *cry11Ba*, and *cyt1Aa* genes were all amplified in the BtMA-25 isolate. Orduz et al. (1998) found that *cry11Ba* is highly toxic to mosquito larvae, regardless of the expression of other genes. Costa et al. (2010) detected *cry11Aa* in all isolates that showed high mortality to *A. aegypti* larvae. Moreover, the toxicity assays suggested the interaction between Cry11Aa and at least one of the Cyt proteins. Hence, the toxicity of BtMA-25 isolate may be consequence of a synergistic effect between Cry11Aa and Cyt1Aa toxins, leading to an efficient alternative to control *A. aegypti*.

The results of the present study have confirmed the larvicidal potential of Bt isolates from the Brazilian Cerrado region. In particular, the BtMA-401 isolate presented better results against *A. aegypti* in the bioassays than the standard Bti test strain.

The Bti strain, despite being widely used to control mosquito larvae and registered in several commercial products for *A. aegypti* control (Harwood et al., 2015; Lopes et al., 2010; Monnerat et al., 2012; Ritchie et al., 2010; Zequi et al., 2011), should be evaluated closely, given the probability of a decrease in the susceptibility of some *A. aegypti* populations to this bacterium. This implies that the constant use of Bti may eventually provoke the emergence of resistant populations (Ben-Dov, 2014; Paris et al., 2010; Tetreau et al., 2012, 2013). In addition, most Bti-based products are imported in Brazil, which results in a considerable increase in the retail price, which means that cheaper chemical insecticides may often be preferred by the users (Angelo et al., 2010; Lopes et al., 2010; Pereira et al., 2013; Silva et al., 2011).

In this context, the search for Bt strains with a greater genetic variability than the Bti, adapted to a diversity of environmental conditions and with high specificity, is of great importance for the development of an effective integrated vector management program. Because these isolates may represent novel genetic resources that can be used to develop new technologies, these studies may result in the development of new microbial insecticides for the control of pest species. However, further research is needed to identify and describe the genes associated with the production of insecticidal toxins in some isolates, which were not detected in the present study.

Conflicts of interest

The authors declare no conflicts of interest

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