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BIOLOGICAL CONTROL

Plasmid Patterns of Efficient and Inefficient Strains of *Bacillus thuringiensis* against *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae)

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Abstract

Bacillus thuringiensis harbors genes encoding Cry proteins found in chromosomes or plasmids of different sizes (4-150 Mb). Although the smaller plasmids are more abundant in *B. thuringiensis*, their specific function is unknown. As for the megaplasmids, their main recognized function is to harbor *cry* genes, although the sequencing of some of these plasmids indicates the occurrence of other important genes. This work used a new protocol for practical and rapid extraction of plasmid DNA in order to characterize the plasmid patterns of Brazilian strains belonging to Embrapa Milho e Sorgo research center *B. thuringiensis* bank. We tried to further assess the relationship of plasmid patterns with strains belonging to the same serovars and strains causing 100% and no mortality to *Spodoptera frugiperda* (J.E. Smith) larvae. It was possible to characterize 59 strains based on the migration of bands in agarose gel. Strains belonging to the same serovars showed different plasmid sizes (from 1,636 bp to 23,200 bp), with the exception of two strains belonging to serovar *galleriae*. The strain T09 *Bt tolworthi* showed a plasmid migration pattern identical to strains belonging to serovar *galleriae*. Plasmid patterns differed for 46 strains, confirming that this is a useful tool to discriminate specific strains. However, it was not possible to associate the plasmid pattern or the occurrence of particular plasmids with the pathogenicity of a given species towards *S. frugiperda* larvae.

Introduction

Spodoptera frugiperda (J.E. Smith) is responsible for substantial losses in maize production. Its control is mainly achieved using chemical insecticides. However, biological control using *Bacillus thuringiensis* Berliner (*Bt*) and the production of genetically modified plants with insect resistance genes are promising alternatives to control this insect (Valicente & Barreto 2003).

Bacillus thuringiensis (*Bt*) is a Gram-positive bacterium that produces protein crystalline inclusions during the stationary phase, called Cry proteins, encoded by different *cry* genes (Yamamoto & Dean 2000). The crystalline inclusions, along with the spores, have a great potential to control a number of insect pests belonging to Lepidoptera, Diptera and Coleoptera. Therefore, they represent a valuable tool for Integrated Pest Management (IPM) (Vidyarthi *et al* 2002, Valicente *et al* 2010). *Bacillus thuringiensis* can be found in different substrates such as soil, water, plant surfaces, dead insects, grain dust, spider webs and stored grains (Glare & O´Callagham 2000, Valicente & Barreto 2003, Miralles & Pérez 2004).

The genes encoding for the Cry proteins are found in chromosomes and mainly plasmids of different sizes (4-150 MDa), not only in different combinations or multiple copies within a plasmid, but also in combinations of these plasmids in different strains of *Bt* (Lereclus *et al* 1993). Two different groups of plasmid patterns can be recognized: those that are ≤30 MDa and those that are ≥30 MDa, called megaplasmids. For practical purposes, each group is divided according to the chromosomal band in agarose gel. Smaller plasmids are below that band, and megaplasmids are above it. Smaller plasmids are generally present in high copy numbers, and megaplasmids are present in low copy numbers (Reyes-Ramirez & Ibarra 2008). Most of the smaller *Bt* plasmids are still referred as cryptic plasmids, since no specific function have been attributed to them. As for the megaplasmids, their main recognized function is harboring *cry* genes (Berry *et al* 2002, Loeza-Lara *et al* 2005, Roh *et al* 2007).

Several techniques for extraction and purification of plasmids have been optimized because of the importance of *cry* genes in host cells and their use as molecular tools (Gitahy *et al* 2005). The most used technique is the extraction using alkaline lysis and purification under gradient ultracentrifugation in cesium chloride (Sambrook *et al* 1989). This was one of the first biochemical method developed for obtaining plasmids of various microorganisms (Gitahy *et al* 2005). Despite several adjustments, this technique is still slow and laborious, with a high level of contamination when ethidium bromide is used. Ramirez & Ibarra (2008) developed a more practical and faster protocol to obtain the plasmid DNA of *Bt*.

The objectives of this study were to characterize the plasmid profile of a group of *Bt* strains efficient and not efficient against larvae of the fall armyworm, *S. frugiperda*, and evaluate the relationship between plasmid profiles of strains belonging to the same subspecies.

Material and Methods

Bacterial strains

Out of a total of 59 *Bt* strains (Table 1), eight were kindly provided by the USDA (United States Department of Agriculture), eight kindly provided by the Institut Pasteur (IP), eight belong to the Embrapa Milho e Sorgo *Bt* bank and identified by the IP, and 35 strains with no subspecies information (Embrapa´s collection). The first twenty-four strains with subspecies identification had been previously tested against *S. frugiperda* (Table 1 in Valicente & Fonseca 2004), as well as the other 36 strains (Table 1 in Valicente & Barreto 2003). These strains are stored in glycerol at -20ºC and -80ºC.

Media preparation

Four *Bt* strains (HD73 *Bt kustaki,* T09 *Bt tolworthi*, 344 *Bt tolworthi* and strain 1644) were used to test two different media for plasmid extraction efficiency and quality. Plasmid extraction was performed according to Reyes-Ramírez & Ibarra (2008), with some modifications. A loopful of each strain was inoculated in 50 ml of Spizizen medium (0.2% NH_4SO_4, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% sodium citrate, 0.02% $MgSO_4.7H_2O$, supplemented with 0.5% glucose, 0.1% casamino acid and 0.01% yeast extract) or LB medium enriched with salts and glucose (0.1% glucose, 0.8% nutrient broth, 0.5% yeast extract, 0.03% $MgSO_4.7H_2O$, 1% tryptone, 0.002% $FeSO_4$, 0.002% $ZnSO_4$, 0.002 % $MnSO_4$, and 0.5% NaCl). Flasks were incubated under constant agitation (250 rpm) for 16h at 28°C.

Plasmid extraction

Optical density of each sample was measured at 600 nm. Samples were centrifuged (Sorval ® Super T21) at 4°C for 15 min at 20,200 *g*. Each pellet was resuspended in 20 ml of cold TES (30 mM Tris base, 5mM EDTA, 50 mM NaCl, pH 8.0) and centrifuged under the same conditions. Pellets were resuspended in 2 ml of lysis buffer (TES buffer containing 20% sucrose, 2 mg/ml lysozyme, and 10 µg/ml RNase) and incubated at 37°C for 90 min. Three milliliters of 10% sodium dodecyl sulfate were added in TES buffer and the suspension was incubated at 65°C for 15 min. Then, 1.5 ml of 3 M sodium acetate (pH 4.8) was added and incubated at -20°C for 30 min. The suspension was centrifuged at 20,200 *g* for 20 min at 4°C. Two volumes of cold pure ethanol were added to the supernatant and incubated overnight at -20°C and centrifuged under the same conditions. Each pellet was dissolved in 100 µl of Tris-EDTA pH 8.0 (10 mM Tris-HCl, 1 mM EDTA), and stored at -20°C until further use.

Electrophoresis

Ten microliters of each sample were applied to a 0.5% agarose gel and ran for approximately 4h at 100 V. TAE buffer was the running buffer (0.001M EDTA pH 8.0, 0.04 M TRIS pH 8.0, 0.02 M acetic acid), and the gel was stained in a solution of ethidium bromide (1 µg/ml) for approximately 15 min, and washed in water for about 30 min. Gel images were recorded in Gel Logic 200 Imaging System.

Results and Discussion

The OD value (Table 2) showed that the LB medium enriched with salts provided a better growth for the four strains with an OD value ranging from 2.12 to 2.18, while the OD ranged from 1.00 to 1.98 in the Spizizen medium.

Table 1 Strains used for plasmid characterization.

N°	Strain identification	Mortality (%)	Origin
	USDA		
1	HD-2 *Bt thuringiensis*	37.8	USA
2	HD-3 *Bt finitimus*	5.2	USA
3	HD-4 *Bt alesti*	6.8	USA
4	HD-7 *Bt dendrolimus*	5.4	USA
5	HD-11 *Bt aizawai*	7.8	USA
6	HD-12 *Bt morrisoni*	28.0	USA
7	HD-29 *Bt galleriare*	12.8	USA
8	HD-73 *Bt kurstaki*	2.7	USA
	Institut Pasteur		
9	T-07 *Bt aizawai*	80.8	France
10	T-09 *Bt tolworthi*	95.8	France
11	T-06 *Bt entomocidus*	9.8	France
12	T-24 *Bt neoleonensis*	17.9	France
13	T-27 *Bt mexicanensis*	17.0	France
14	T-23 *Bt japonensis*	33.5	France
15	T-10 *Bt darmstadiensis*	77.9	France
16	T-16 *Bt indiana*	12.2	France
	Embrapa Milho e Sorgo		
17	344 – *Bt tolworthi*	100	Paraná
18	426 – *Bt tolworthi*	100	Ceará
19	474 – *Bt galleriae*	100	Paraná
20	348B – *Bt alesti*	100	Paraná
21	348L – *Bt galleriae*	100	Paraná
22	460 – *Bt darmstadiensis*	100	Paraná
23	461A – *Bt tolworthi*	100	Paraná
24	462A– *Bt galleriae*	100	Paraná
25	S460	100	Paraná
26	BTLM	100	Goiás
27	566BLR	100	Paraná
28	701A	100	São Paulo
29	701B	100	São Paulo
30	1119C	100	Goiás
31	1124E	100	Goiás
32	1131A	100	Goiás
33	1131C	100	Goiás
34	1132A	100	Goiás
35	1132C	100	Goiás
36	1138G	100	Goiás
37	1145B	100	Goiás
38	1145C	100	Goiás
39	1148F	100	Goiás
40	1132E	100	Goiás
41	1135B	100	Goiás
42	1136B	100	Goiás
43	1139K	100	Goiás
44	1354	100	Minas Gerais
45	1355	100	Minas Gerais
46	1357E	100	Minas Gerais
47	1603B	100	Santa Catarina
48	1641	100	Paraná
49	1644	100	Paraná
50	376B	0	Ceará
51	436	0	Pernambuco
52	257	0	Goiás
53	844K	0	Minas Gerais
54	1097	0	Minas Gerais
55	1096A	0	Minas Gerais
56	1425B	0	Pernambuco
57	1431B	0	Sergipe
58	1540A	0	Pernambuco
59	1530A	0	Paraná

Table 2 Value of optical density for Lurian Bertani enriched with salts and glucose and Spizizen media.

Strains Bt	OD_{600nm}	
	Medium Lurian Bertani Salts	Medium Spizizen
344	2.13	1.93
1644	2.15	1.98
T09	2.18	1.92
HD73	2.12	1.00

The quality of the plasmid profile of the isolates grown in two different media could be observed in agarose gel (Fig 1). Isolates that were grown in LB medium showed thicker and brighter bands that hindered the number of plasmids, but not all isolates produced the same plasmid profile on both culture media. All strains grown in the Spizizen medium resulted in a profile easier to be discriminated, and Strain T09 *Bt tolworthi* revealed a megaplasmid when cultivated in Spizizen medium, but not when cultivated in LB medium. Then, the Spizizen medium was used for plasmid extraction and analysis.

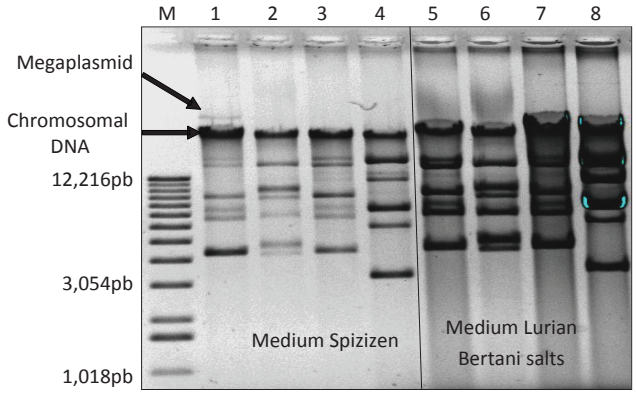

Fig 1 Plasmid patterns in 0.5% agarose gel and product quality provided by Spizizen and LB + salts media. M (1 Kb DNA ladde Invitrogen); 1 and 5 (T09 *Bt tolworthi*); 2 and 6 (344 *Bt tolworthi*); 3 and 7 (1644); 4 and 8 (HD73 *Bt kurstaki*).

Strains showed optical density between 1.00 and 2.39 when evaluated at 600 nm (value proportional to cell density) during the fermentation process. The number of plasmids varied from 0 (zero) to a maximum of 12 plasmids per strain. The megaplasmids (plasmids located above the chromosomal DNA band) were used as a second option for characterization, because migration in the agarose gel during electrophoresis is very limited, and therefore it is more difficult to distinguish the bands. Thus, comparisons focused on the plasmids that migrated below the chromosomal DNA band and megaplasmids were used as second choice in the characterization.

The studies of *Bt* plasmids started in the 70s, then the attention turned to the location of *cry* genes in the plasmids and the ability to transfer plasmids between different strains of *B. thuringiensis* and from *B. thuringiensis* to *B. cereus* (González & Carlton 1980, Kronstad *et al* 1983, Jesen *et al* 1995, Hu *et al* 2004). Studies involving plasmids have been published; however there are few articles that relate the importance of the plasmid profile in the characterization of *Bt* strains.

In general, plasmid patterns were unique to each strain (Figs 2-4). Strains T09 (lane 5), 344 (lane 6) and 426 (lane 7) (Fig 2c), all belonging to the subspecies *Bt tolworthi*, showed distinct plasmid patterns. Strains T09

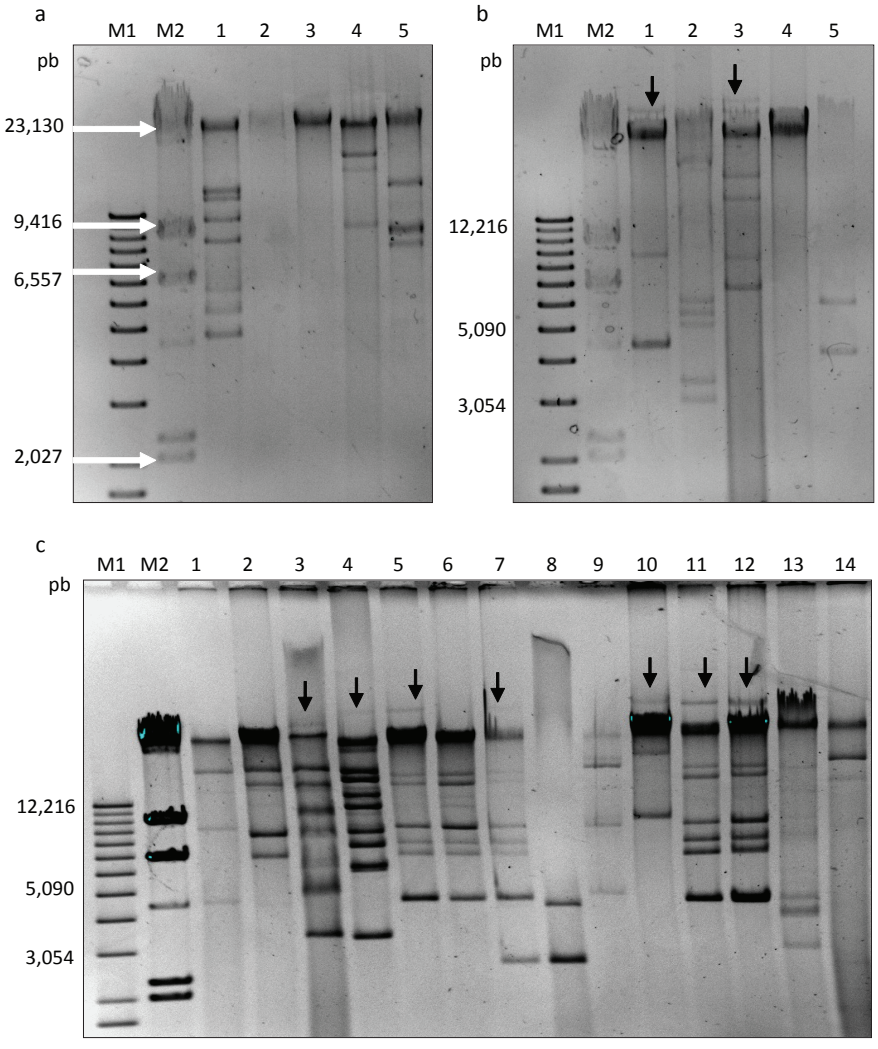

Fig 2 Plasmid patterns of strains of *Bacillus thuringiensis* in 0.5% agarose gel. Strains with subspecies identification. a) Lanes: 1, HD2 (*Bt thuringiensis*); 2, HD3 (*Bt finitimus*); 3, HD7 (*Bt dendrolimus*); 4, HD12 (*Bt morisoni*); 5, HD73 (*Bt kurstaki*). b) Lanes: 1, T06 (*Bt entomocidus*); 2, T24 (*Bt neoleonensis*); 3, T27 (*Bt mexicanensis*); 4, T23 (*Bt japonensis*); 5, T16 (*Bt indiana*). c) Lanes: 1, HD4 (*Bt alesti*); 2, 348B (*Bt alesti*); 3, HD11 (*Bt aizawai*); 4, T07 (*Bt aizawai*); 5, T09 (*Bt tolworthi*); 6, 344 (*Bt tolworthi*); 7, 426 (*Bt tolworthi*); 8, 461A (*Bt tolworthi*); 9, HD29 (*Bt gallerire*); 10, 474 (*Bt galleriae*); 11, 348L (*Bt galleriae*); 12, 462A (*Bt galleriae*); 13, 460 (*Bt darmstadiensis*); 14 T10 (*Bt darmstadiensis*). M1 (1Kb DNA ladder); M2 (λ DNA Hind III marker); (↓) megaplasmids.

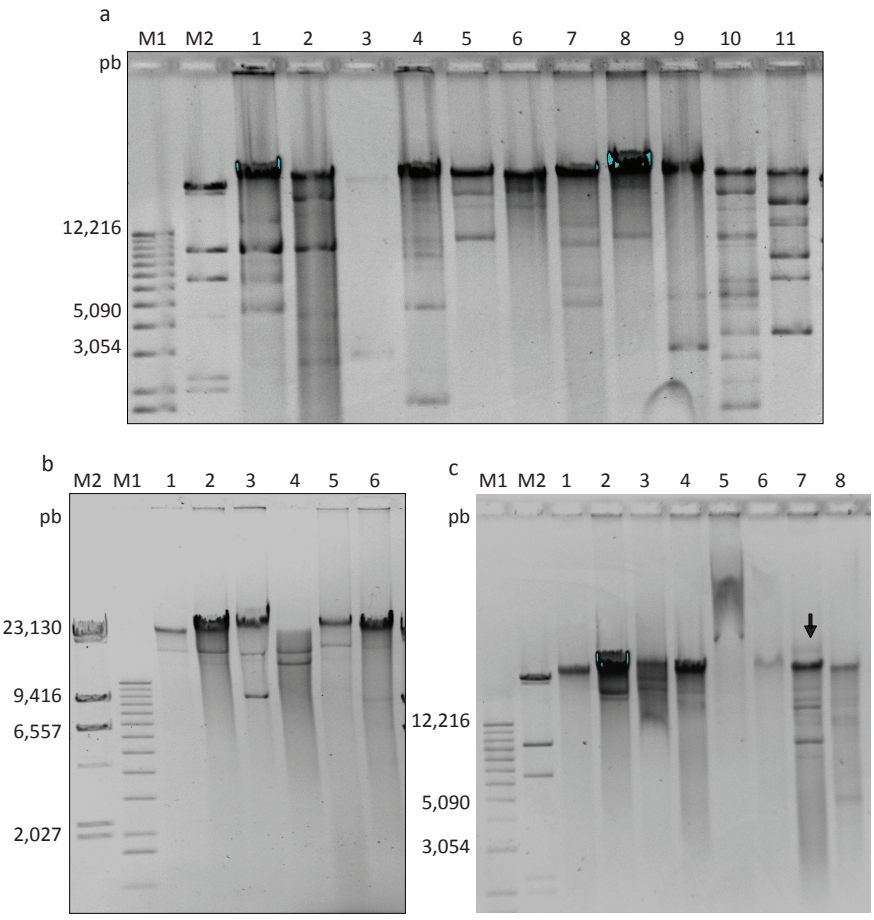

Fig 3 Plasmid patterns of *Bacillus thuringiensis* strains in 0.5% agarose gel. Strains 100% efficient against *Spodoptera frugiperda*. a) Lanes: 1, S460; 2, BTLM; 3, 566BL; 4, 701A; 5, 1124E; 6, 1119C; 7, 1354R; 8, 1135B; 9, 1603; 10, 1641; 11, 1644. b) Lanes: 1, 1131C; 2, 1132A; 3, 1132C; 4, 1138G; 5, 1136B; 6, 1139K. c) Lanes: 1, 701B; 2, 1131A; 3, 1145B; 4, 1145C; 5, 1148F; 6, 1132E; 7, 1355; 8, 1357E. M1: 1Kb DNA ladder; M2: λ DNA Hind III marker, (↓) megaplasmids.

and 426 showed six plasmids between 4,361 bp to 23,000 bp, and could be differentiated from one another due to the presence of a plasmid of approximately 2,500 bp in strain 426. Strain 344 differed from strains T9 and 426 by the presence of two plasmids with about 12,200 bp and 13,200 bp. Strain 461A (lane 8, Fig 2c) also showed two different plasmids of 2,700 pb and 4,200 bp. All four strains belonging to the serovar *tolworthi* showed a very specific and different plasmid pattern for each strain.

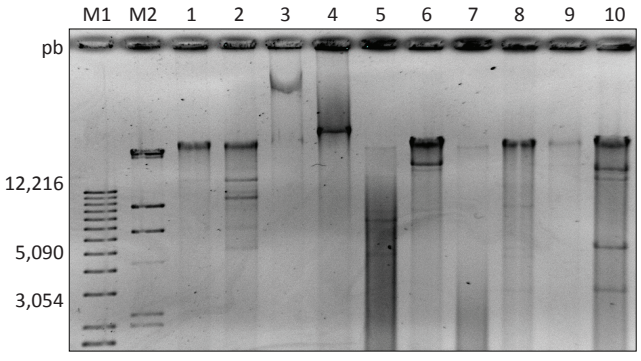

Fig 4 Plasmid patterns of *Bacillus thuringiensis* strains in 0.5% agarose gel. Strains 0% efficient against *Spodoptera frugiperda*. Lanes: 1, 376B; 2, 436; 3, 257; 4, 844K; 5, 1097; 6, 1096A; 7, 1425B; 8, 1431B; 9, 1540A; 10, 1530. M1: 1Kb DNA ladder; M2: λ DNA Hind III marker.

Strains 348L and 462A (lanes 11-12, Fig 2c) belonging to subspecies *galleriae* showed similar band migration with six plasmids between 4,361 to 23,000 bp and one megaplasmid each. However strain HD29 (*Bt galleriae*) (lane 9, Fig 2c) harbors four plasmids showing a different profile from strains 462A and 348L. The strain 474 *Bt galleriae* (lane 10, Fig 2c) also presented a megaplasmid and two distinct small plasmids. Reyes-Ramirez & Ibarra (2008) also reported this difference in migration among strains belonging to the same serovars with strains belonging to subspecie *kenyae*. Likewise, these authors observed pattern diversity in 14 strains belonging to serovars *sotto* and *dendrolimus*.

Serovars T09 *Bt tolworthi* (lane 5, Fig 2c), 348L and 462A *Bt galleriae* (lanes 11-12, Fig 2c) showed similar migration of plasmids, although they belong to different serovars. Although plasmids have the same size, the DNA sequence may be different, and should be considered for future work.

The HD4 and 348B serovars *alesti* (lanes 1-2, Fig 2c), 460 and T10 *darmstadiensis* (lanes 13-14, Fig 2c) and HD11 and T07 *azawai* (lanes 3-4, Fig 2c) showed unique plasmid patterns. Also, the serovars *thuringiensis* (HD2, lane 1, Fig 2a), *morrisoni* (HD12, lane 4, Fig 2a), *kurstaki*

(HD73, lane 5, Fig 2a), *entomocidus* (T06, lane 1, Fig 2b), *neoleonensis* (T24, lane 2, Fig 2b), *mexicanensis* (T27, lane 3, Fig 2b) and *indiana* (T16, lane 5, Fig 2b) represented by only one strain of one subspecies, showed unique patterns. The serovars *finitimus* (HD3, lane 2, Fig 2a), *dendrolimus* (HD7, lane 2, Fig 2a) and *japonensis* (T23, lane 4, Fig 2b) are represented by only one strain and showed only chromosomal DNA.

Similar plasmid migration was found within (348L and 462A Bt *galleriae*) and among (348L, 462A *Bt galleriae* and T9 *Bt tolworthi*) serovars. Despite belonging to different serovars, these strains (348L, 462A and T09) may have evolved from a single common ancestor. The other strains may have evolved from different ancestors and *B. thuringiensis* is able to lose plasmids during evolution, which may explain the differences found in plasmid patterns (Lereclus *et al* 1982, Aptosoglou *et al* 1997).

Among the strains with efficacy of 100% against *S. frugiperda* larvae, strain 1641 showed the largest number of plasmids, a total of 12, ranging between 1,636 bp and 23,000 bp (lane 10, Fig 3a). These strains with 100% efficiency generally showed a great diversity and a unique plasmid profile (Fig 3). Strains 701B and 1132E (lanes 1-6, Fig 3c) showed only chromosomal DNA. However, other strains showed one or more plasmids, and strain 1355 showed only one megaplasmid.

Out of 25 efficient strains against *S. frugiperda*, only one strain contained megaplasmids that leads to the hypothesis that genes responsible for their efficiency are located on the chromosome, because according to Lereclus *et al* (1993), such genes encoding the Cry proteins are found in chromosomes or plasmids of different sizes.

Among the strains collected in Goiás State (lanes 1-5-8, Fig 3a) two of them (1124 and 1135B) showed a similar plasmid profile, with three small plasmids, while the strain BTLM presented four plasmids (Fig 3a). All strains collected in the state of Goiás had small plasmids varying in number from one to three (Fig 3b), while those of the state of Paraná showed from one to 12 small plasmids (lanes 1-3-10-11, Fig 3a) and state of Minas Gerais from four to seven plasmids with distinct profiles (lane 7, Fig 3a; lanes 7-8 Fig 3c), Only one out of the two strains from state of São Paulo presented plasmids (lane 4, Fig 3a; lane 1, Fig 3c), while the only strain from state of Santa Catarina had two plasmids (lane 9, Fig 3a). The similarity between the plasmid profile of strains from state of Goiás reinforces reports of Hongyu *et al* (2000) that the evolution of *B. thuringiensis* may be related to geographic location; however, this was not our major objective.

Five out of the 10 strains (Fig 4 - 376B lane 1, 257 lane 3, 844K lane 4, 1425B lane 7 and 1540A lane 9) belonging to the group that showed no mortality against *S. frugiperda*, had only chromosomal DNA and the other five strains (Fig 4 – 436 lane 2, 1097 lane 5, 1096 lane 6, 1431B lane 8 and 1530 lane 10) showed from one to four small plasmids. Strains not pathogenic to *S. frugiperda* can show mortality towards other insect species or insect orders. For this reason, it was not possible to associate the plasmid pattern or the presence of some plasmids with the pathogenicity towards *S. frugiperda*. However, this fact does not make this technique less important.

All other strains showed a unique plasmid profile with the exception of strains T09, 348L and 462A, and the strains that showed only chromosomal DNA. Strains collected in Goiás state showed a profile similar or even identical among them, and strains collected in Paraná, São Paulo and Minas Gerais showed unique profiles. Reyes-Ramirez & Ibarra (2008) reported that all strains showed unique plasmid patterns with the exception of serovars *israelensis* and *malaysiensis*, what makes it impossible to analyze the results using cluster analysis. Small size plasmids varying between 1,636 bp and 23,000 bp were sorted as it follows: 4% with fragments between 1,636 bp and 3,054 bp, 31% between 3,054 bp and 6,108 bp, 28% between 6,108 bp and 12,216 bp and 37% between 12,216 bp and 23,000 bp.

We detected megaplasmids in 13% of the evaluated strains. In our work we used many strains without identification to subspecies that were collected in different regions of Brazil (Valicente & Barreto 2003). As in Reyes-Ramirez & Ibarra (2008), the comparisons focused only on those plasmids migrating below the chromosomal DNA band as megaplasmids are easily degraded during storage, leading to the loss of the information regarding to each pattern.

In conclusion, the present paper identified similar and unique plasmid migration patterns, and even identical patterns were found within and between serovars, and the method is a valuable tool in intellectual property claims. As also stated by Reyes-Ramirez & Ibarra (2008) the major concerns are the reproducibility and feasibility of the technique used to isolate the plasmid.

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