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Evidence of *Bacillus thuringiensis* Intra-Serovar Diversity Revealed by *Bacillus cereus* Group-Specific Repetitive Extragenic Palindromic Sequence-Based PCR Genomic Fingerprinting

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Key Words

Argentinean isolates · *Bacillus thuringiensis* · Fingerprinting · Intra-serovar diversity · Rep-PCR

Abstract

Bacillus thuringiensis is classified into serovars on the basis of H-flagellar antigens. Several alternative typing methods have been described. Among them, a *B. cereus* group-specific repetitive extragenic palindromic (Rep)-PCR fingerprinting technique was shown to be discriminative and able to identify *B. thuringiensis* serovars. The aim of this study was to investigate the genomic diversity and relationship among *B. thuringiensis* strains collected from different Argentinean ecosystems. Thirty-seven *B. thuringiensis* reference strains and 131 Argentinean isolates were analyzed using a *B. cereus* group-specific Rep-PCR. Fourteen different patterns were identified among the Argentinean isolates. Eight could not be associated to any pattern obtained from a reference strain. The pattern identical to the serovar *kurstaki* HD-1 strain was the most frequently identified in 68 native isolates. The profiles allowed tracing a single dendrogram with two groups and eight main lineages. Some strains showed distinctive patterns despite belonging to the same serovar. An intraspecific diversity resulted from this analysis that was highlighted by this technique since strains from a given serovar showed distinct profiles. This study may help to establish a system of *B. thuringiensis* classification with a higher discrimination level than established by the H antigen serotyping.

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Introduction

Bacillus thuringiensis is a rod-shaped, Gram-positive and spore-forming bacterium. It is a member of the so-called *B. cereus* group together with *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis* and the recently reported *Bacillus gaemokensis* [Jung et al., 2010]. However, *B. thuringiensis* is mainly characterized by the production of parasporal crystals composed of insecticidal proteins (Cry proteins) during sporulation [Sauka et al., 2008]. Different groups of these proteins possess highly specific insecticidal activity against lepidopteran, coleopteran, dipteran, hemipteran and hymenopteran larvae [Sauka et al., 2008]. The use of *B. thuringiensis* is considered worldwide a viable alternative for the insect pest control in agriculture and public health, and com-

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patible with sustainable and environmentally friendly practices.

Since *B. thuringiensis* was described as a new species in 1915, some methods to classify *B. thuringiensis* into subspecies have been put forward [de Barjac and Bonnefoi, 1962, 1973; Sauka et al., 2008]. A useful classification of *B. thuringiensis* was developed on the basis of H-flagellar antigens by de Barjac and Bonnefoi [1962] and has been used ever since [Lecadet et al., 1999]. *B. thuringiensis* has been classified into 84 serovars [Lecadet et al., 1999], including the latest described serovar *jordanica* (H serotype 71, H71) [Khyami-Horani et al., 2003]. However, serotyping is useless for non-motile and/or autoagglutinated strains, besides the agglutination found in some *B. cereus strains* with specific *B. thuringiensis* H antigens [Lecadet et al., 1999; Ohba and Aizawa, 1986].

Several alternative typing methods for *B. thuringiensis* strains, mostly based on molecular techniques, have been recently described [Cherif et al., 2007; Gaviria Rivera and Priest, 2003a, b; Joung and Côte, 2001; La Duc et al., 2004; Lima et al., 2002; Manzano et al., 2009; Peruca et al., 2008; Reyes-Ramirez and Ibarra, 2005, 2008; Xu and Côte, 2006; Yu et al., 2002]. Among them, a *B. cereus* group-specific repetitive extragenic palindromic (Rep) sequence-based PCR fingerprinting technique was shown to be highly discriminative, fast, easy, and able to identify *B. thuringiensis* serotypes, including non-flagellar and self-agglutinated strains [Reyes-Ramirez and Ibarra, 2005].

The aim of this study was to characterize *B. thuringiensis* strains collected from different Argentinean ecosystems through a *B. cereus* group-specific Rep-PCR. The results are analyzed to aid discussions concerning the classification and ecology of this micro-organism.

Results and Discussion

After Rep-PCR conditions were established and tested, 37 *B. thuringiensis*, one *B. megaterium*, one *B. subtilis* and one *B. sphaericus* reference strains were analyzed in order to obtain representative electrophoretic patterns. In agreement with the results of Reyes-Ramirez and Ibarra [2005], most of the strains (95%) showed different Rep-PCR patterns. Also, T08 003 and HD-12 strains, despite belonging to the same serovar *(morrisoni)*, showed distinctive Rep-PCR patterns (fig. 1). In contrast to previous observations, serovars *aizawai* (H7) and *galleriae* (H5a, 5b) showed the same pattern (fig. 1). On the other hand, slight differences between electrophoretic patterns of serovars *sotto* (H4a, 4b) and *dakota* (H15) were observed (fig. 1). It is interesting to point out that serovar *kurstaki* HD-1 and HD-73 strains, despite belonging to the same serovar, showed similar but distinctive Rep-PCR patterns (fig. 1). Genomic heterogeneity may occur in this group, similar to that observed with the serovar *israelensis* group. Although IPS82 and HD-522 strains showed the same pattern, it was different from that of HD-567 strain (fig. 1). The differences found between Rep-PCR patterns of strains belonging to serovars *kurstaki*, *israelensis* and *morrisoni* are not detected by using serotyping and confirm the suggestions by Reyes-Ramirez and Ibarra [2005] about the ability of this *B. cereus* group-specific Rep-PCR technique to discriminate between strains from the same *B. thuringiensis* serovar.

A total of 131 selected native isolates from the IMYZA-INTA collection were also analyzed by Rep-PCR. They include 73 selected native isolates with lepidopteran, 18 with dipteran and 40 with neither lepidopteran nor dipteran activity. Isolates collected from the same sample were analyzed by SDS-PAGE and PCR (data not shown) in order to identify and discard twin isolates (isolates with identical insecticidal crystal protein profiles and *cry* gene profiles). Exclusion of twin isolates is important in order to get a real estimate of the genetic diversity of the analyzed areas [Sauka et al., 2005, 2006]. Thus, 14 different Rep-PCR patterns were successfully identified during this part of the study. Eight of them could not be associated to any pattern obtained from a *B. thuringiensis* reference strain and were classified into A to H Rep-PCR patterns groups. These patterns obtained from native isolates could be associated to a novel serovar, or simply to one not analyzed during this study.

The pattern identical to the anti-lepidopteran strain *kurstaki* HD-1 was the most frequently identified in 68 native *B. thuringiensis* isolates with lepidopteran/dipteran activity (64.8%). In addition, the Rep-PCR pattern identical to other lepidopteran-toxic strains as serovar *kenyae* and serovar *aizawai/galleriae* were found in four (3.8%) and one (1.0%) isolates with lepidopteran activity, respectively. On the other hand, seven (6.6%) and two (1.9%) mosquitocidal native isolates showed a Rep-PCR pattern identical to that of the mosquitocidal serovar *israelensis* IPS82 and HD-567 strains, respectively. Also, nine (8.5%) of the neither lepidopteran nor dipteran activity isolates showed a Rep-PCR pattern identical to that of the anti-coleopteran serovar *kumamotoensis*, while one (1.0%), two (1.9%), three (2.8%), two (1.9%), three (2.8%), one (1.0%), one (1.0%) and one (1.0%) showed one pattern classified into groups A to H, respectively (fig. 2).

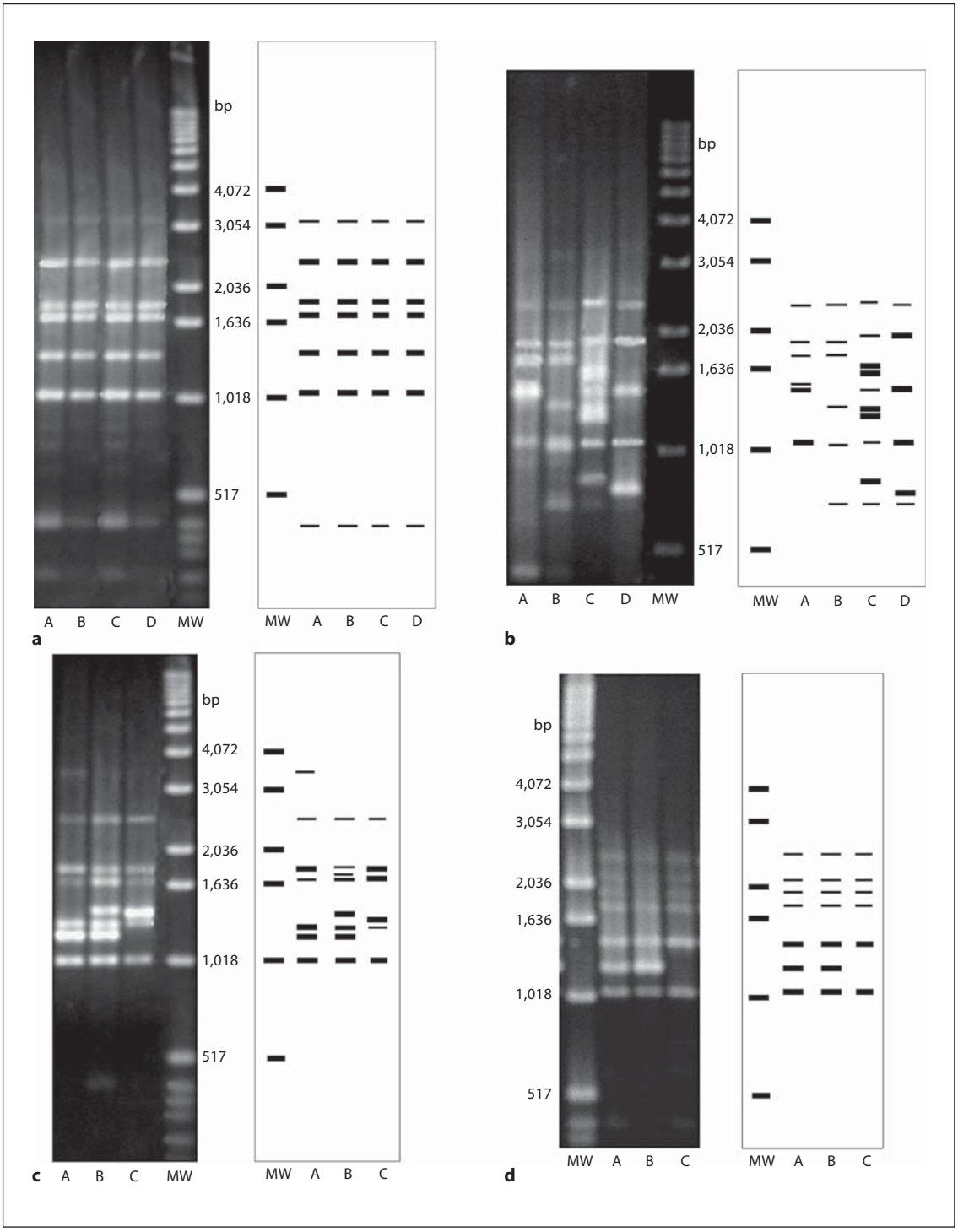

Fig. 1. Rep-PCR fingerprinting of some *B. thuringiensis* reference strains. **a** A: serovar *galleriae* HD-8; B: serovar *galleriae* T05 001; C: serovar *aizawai* HD-133; D: serovar *aizawai* T07 001. **b** A: serovar *dakota* HD-511; B: serovar *sotto* HD-6; C: serovar *morrisoni* HD-12; D: serovar *morrisoni* T08 003. **c** A: serovar *kurstaki* HD-1; B: serovar *kurstaki* HD-73; C: serovar *kenyae* HD-5. **d** A: serovar *israelensis* IPS82; B: serovar *israelensis* HD-522; C: serovar *israelensis* HD-567. MW = Molecular weight marker.

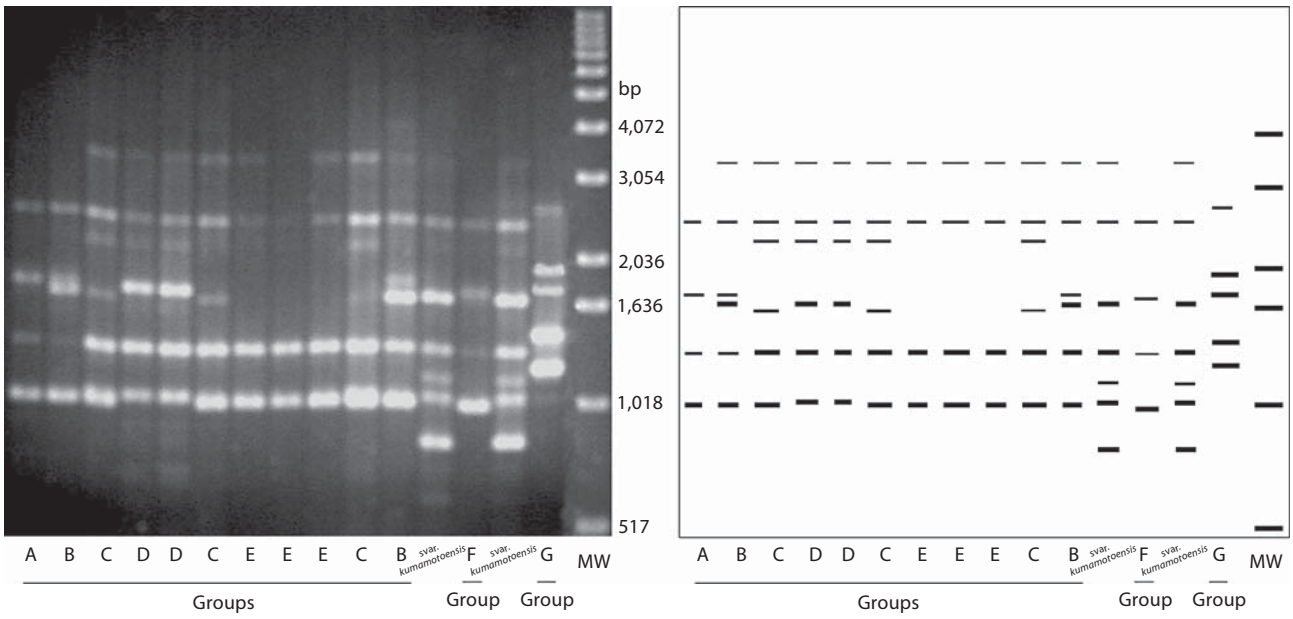

Fig. 2. Representative Rep-PCR patterns of *B. thuringiensis* native isolates that could not be associated with that belonging to one serovar of *B. thuringiensis*. MW = Molecular weight marker.

Table 1. Distribution of Rep-PCR patterns of native to Argentina *B. thuringiensis* isolates according to the sample source

Rep-PCR pattern identical to:	Sample source					
	soils	stored product dust	dead insect larvae	leaves	spider webs	total
Serovar *kurstaki* HD-1	9	20	7	25	7	68
Serovar *kenyae*	0	1	3	0	0	4
Serovar *aizawai/galleriae*	0	1	0	0	0	1
Serovar *israelensis* IPS82	5	0	0	2	0	7
Serovar *israelensis* HD-567	0	0	0	2	0	2
Serovar *kumamotoensis*	1	1	0	7	0	9
Group A	0	0	0	1	0	1
Group B	2	0	0	0	0	2
Group C	1	0	0	1	1	3
Group D	1	0	0	1	0	2
Group E	0	1	0	2	0	3
Group F	0	0	0	1	0	1
Group G	0	1	0	0	0	1
Group H	1	0	0	0	0	1
Total	20	25	10	42	8	105

When we analyzed the distribution of Rep-PCR patterns of *B. thuringiensis* isolates according to the sample source, we found that isolates with a Rep-PCR pattern identical to that of the anti-lepidopteran serovar *kurstaki* was the most frequently identified irrespective of the kind of sample (table 1). *B. thuringiensis* isolates collected from phylloplane samples showed the most diverse Rep-PCR patterns (9 of 14). It is worth pointing out the highest frequencies of detection of Rep-PCR patterns identical to that of the mosquitocidal serovar *israelensis* IPS82 (25.0%) and serovar *kumamotoensis* (16.6%) strains in isolates collected from soils and phylloplane, respectively. These kinds of ecological niches might be those preferred for these bacteria, but more studies are needed before affirming this possibility.

In order to analyze the genetic relationship between *B. thuringiensis* strains and isolates with atypical Rep-PCR patterns that could not be associated with that belonging to one *B. thuringiensis* serovar, a dendogram was generated by UPGMA (fig. 3). Shown are two groups (BT1 and BT2) where eight main lineages, each sharing more than 40% similarity, could be defined. BT1 group encompassed: lineage 1 including *B. thuringiensis* serovars *kenyae*, *aizawai*, *galleriae*, *sooncheon*, *indiana*, *tol-*

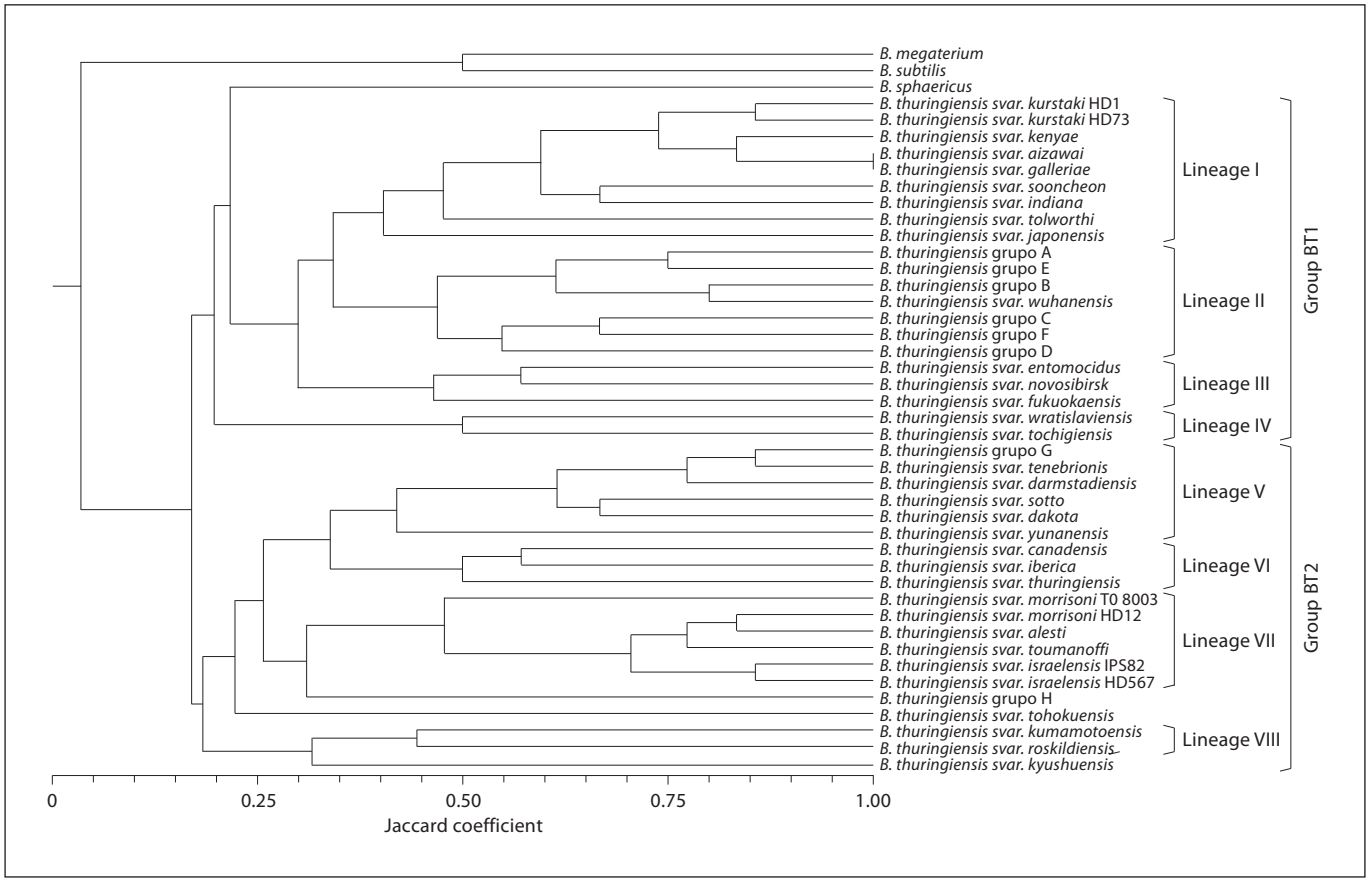

Fig. 3. Dendogram estimated from the Rep-PCR patterns obtained from *B. subtilis*, *B. megaterium*, *B. sphaericus*, *B. thuringiensis* strains and atypical isolates, using the Jaccard coefficient and UPGMA.

worthi, *japonensis*, *kurstaki* HD-1 and HD-73; lineage 2 represented by native isolates belonging to groups A, B, C, D, E, F and serovar *wuhanensis*; lineage 3 containing serovars *entomocidus*, *novosibirsk* and *fukuokaensis*; lineage 4 included serovars *wratislaviensis* and *tochigiensis*. BT2 group encompassed: lineage 5 containing *B. thuringiensis* serovars *tenebrionis*, *darmstadiensis*, *sotto*, *dakota*, *yunanensis* and a native isolate grouped as G; lineage 6 represented by serovars *canadensis*, *iberica* and *thuringiensis*; lineage 7 included serovars *alesti*, *toumanoffi*, *morrisoni* T0 8003 and HD-12, and *israelensis* IPS82 and HD567; lineage 8 represented by serovars *kumamotoensis* and *roskildiensis*. Other *B. thuringiensis* strains and isolates showed less than 40% similarity and were not included in any lineage. *B. megaterium*, *B. subtilis* and *B. sphaericus* strains were used to establish the outgroup.

The intraspecific diversity is an interesting issue resulting from the Rep-PCR analysis and is highlighted by this fingerprinting technique since strains from a given serovar showed distinct Rep-PCR profiles. Lineage 1 encompassed serovar *kurstaki* HD-1 and HD-73 strains, which despite belonging to the same serovar, showed distinctive Rep-PCR patterns. Similar results were obtained by other authors based on different methodologies [Cherif et al., 2007; Lima et al., 2002]. *B. thuringiensis* serovars *kenyae*, *aizawai*, *galleriae*, *sooncheon*, *indiana*, *tolworthi* and *japonensis* were also included in the same lineage. In disagreement with previous results [Cherif et al., 2007; Joung and Côte, 2001; Lima et al., 2002; Reyes-Ramirez and Ibarra, 2005], strains that belong to serovars *galleriae* and *aizawai* clustered together. Lineage 7 encompassed strains, which despite belonging to the same serovar, showed similar but not identical Rep-PCR patterns. That is the case of strains IPS82 and HD-567 from serovar *is-*

Table 2. Reference bacterial strains used in this study

Micro-organism	Source	Micro-organism	Source	Micro-organism	Source
B. thuringiensis serovars		*entomocidus* HD-110	B	*aizawai* TO7 001	A
thuringiensis HD-2	B	*tochigiensis* HD-868	C	*darmstadiensis* HD-146	C
alesti HD-4	B	*yunnanensis* T020 001	C	*dakota* HD-511	C
kurstaki HD-1	B	*roskildiensis* T045 001	C	*indiana* HD-521	C
kurstaki HD-73	D	*kumamotoensis* HD-867	C	*wratislaviensis* PO12	C
kenyae HD-5	B	*toumanoffi* HD-201	C	*tohokuensis* 78-FS-29-17	C
sotto HD-6	B	*tolworthi* HD-125	B	*fukuokaensis* T03C 001	C
galleriae HD-8	B	*wuhanensis* HD-525	B	*novosibirsk* T24A 001	C
galleriae T05 001	A	*israelensis* IPS82	D	*sooncheon* T41 001	C
canadensis HD-224	C	*israelensis* HD-522	E	*iberica* L60	C
morrisoni HD-12	C	*israelensis* HD-567	B	*B. megaterium* B-14308	B
morrisoni TO8 003	A	*japonensis* T023 001	C	*B. sphaericus* SO9002	A
morrisoni DSM2803	D	*entomocidus* HD-10	C	*B. subtilis* NRS-1315	B
kyushuensis HD-541	C	*aizawai* HD-133	B		

A: Institut Pasteur; B: United States Department of Agriculture; C: Bacillus Genetic Stock Center; D: Centro de Investigación y Estudios Avanzados; E: Instituto de Biotecnología, Universidad Nacional Autónoma de México.

raelensis and T0 8003 and HD-12 from serovar *morrisoni*. In addition, taking in account that strain *tenebrionis* from serovar *morrisoni* is included into another lineage (lineage 5), a highly genomic heterogeneity may occur in this serovar. Similar results were obtained by Reyes-Ramirez and Ibarra [2005]. These kinds of observations may be indicating the limits of the classification based on flagellar antigen of *B. thuringiensis*.

Lineage 2 included most of the native *B. thuringiensis* isolates that showed a Rep-PCR pattern that could not be associated with that belonging to one *B. thuringiensis* strain. The highest similitude were detected between *B. thuringiensis* isolates belonging to groups A and E, and between those belonging to groups C and F; isolates belonging to group B cluster together to serovar *wuhanensis* HD-525 strain, while isolates belonging to group D together to that of groups C and F. A tight phylogenetic relationship exists between a native isolate from group G and the coleopteran-active strain *tenebrionis* that belong to serovar *morrisoni* was observed in lineage 5. This same lineage included serovars *sotto* and *dakota* that were shown to be closely related, too. This observation is in agreement with previous studies [Cherif et al., 2007; Joung and Côte, 2001; Lima et al., 2002], but different from that obtained by Reyes-Ramirez and Ibarra [2005] who found identical Rep-PCR patterns for serovars *sotto* and *dakota*. It is interesting to point out that despite using the same methodology developed by Reyes-Ramirez and Ibarra [2005] we obtained different results, indicating lack of reproducibility between laboratories. This kind of reproducibility problem seems to be a recurring issue that, despite recent efforts at standardizing procedures, has yet to be overcome [Deplano et al., 2000]. Lack of reproducibility is a surprising and disappointing conclusion for Rep-PCR, which historically has been touted as a method that is far superior to RAPD or other similar PCR genotyping approaches [Manzano et al., 2009].

In summary, this study may help in the establishment of a new system of *B. thuringiensis* classification with a higher discrimination level than established by the H antigen serotyping. The results also confirm that this *B. cereus* group-specific Rep-PCR is a rapid and reliable way of identifying *B. thuringiensis* serovars and of providing information on phylogenetic relationships.

Experimental Procedures

Bacterial Isolates and Strains

The 37 *B. thuringiensis*, one *B. megaterium*, one *B. subtilis* and one *B. sphaericus* strains used in this study were kindly provided by different stock collections around the world (table 2). Another 131 native *B. thuringiensis* isolates used in this study, collected from stored product dust, leaves, soils, spider webs and dead insect larvae from different ecological regions of Argentina, were obtained from the IMYZA-INTA Bacterial Collection.

Rep-PCR Amplification Conditions and Electrophoretic Analysis

Rep-PCR amplification conditions and electrophoretic analysis were carried out following the previously described methodology [Reyes-Ramirez and Ibarra, 2005].

Phylogenetic Analysis

Polymorphic bands range between 0.5 and 3.6 kb from all the Rep-PCR profiles were individually identified by their specific migration rates in the electrophoretic analyses. Once bands were properly and distinctively identified, binary (0/1) matrices were constructed to compare the profiles. Jaccard's similarity coefficients were generated by the SIMQUAL subroutine from the NTSYS-pc 2.02g package (Applied Biostatistics, Inc.). Finally, cluster analyses along with their corresponding dendrograms were generated by the unweighted-pair group method using average linkages (UPGMA), with the SAHN and TREE subroutines from the NTSYS-pc package.

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