BIOLOGICAL CONTROL

Characterization of an Argentine Isolate of *Bacillus thuringiensis* Similar to the HD-1 Strain

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ABSTRACT - We report the characterization of an Argentine isolate of *Bacillus thuringiensis* (INTA TA24-6) similar to the HD-1 strain, which harbors a cryptic *cry2Ab* gene that apparently is transcribed but not translated into a protein. INTA TA24-6 showed a Rep-PCR pattern identical to the HD-1 strain, a plasmid pattern that resembled that of this strain and *cry1* and *cry2* genes as HD-1. Screening of *cry1* and *cry2* genes showed that INTA TA24-6 harbors only *cry1Ac* and *cry2Ab* genes. Furthermore, crystalline inclusions of INTA TA24-6 exhibit a bipyramidal shape, typical of Lepidoptera-active *B. thuringiensis* strains, containing a major protein of *ca*. 130 kDa toxic to *Epinotia aporema* Wals. (Lepidoptera: Tortricidae) larvae. Neither the flat-square to cuboidal crystal nor a *ca*. 65 kDa protein typical of strains expressing Cry2 proteins were detected in INTA TA24-6. In agreement with this information, parasporal crystals of INTA TA24-6 did not show toxicity to *Aedes aegypti* L. (Diptera: Culicidae) larvae. Gene transcription analyses suggested that the *cry2A* gene might be cryptic in INTA TA24-6 despite its transcription at different sporulation stages.

KEY WORDS: Cryptic gene, cry1Ac, cry2Ab, *Epinotia aporema*

The main feature of the Gram-positive spore-forming bacterium *Bacillus thuringiensis* is the production of proteinaceous crystalline inclusions (crystals) during sporulation, which are responsible for its toxicity towards a variety of invertebrates, especially insects (Sauka & Benintende 2008). These proteins (Cry proteins) are classified according to their amino acid similarity in 59 major groups divided into different classes and subclasses (*B. thuringiensis* toxin nomenclature website at http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/) (Crickmore *et al* 1998). Genes coding for the Cry proteins (*cry* genes) follow the protein classification.

Genes within the *cry1*, *cry2* and *cry9* groups encode δ-endotoxins toxic to lepidopteran larvae (Bravo 1997). Generally, *B. thuringiensis* strains toxic to lepidopteran larvae produce 130-140 kDa proteins contained in bipyramidal crystals and also synthesize 65 kDa proteins contained in smaller cuboidal crystals which have a somewhat extended toxicity spectrum, as some are also mildly toxic to mosquito larvae (Sauka & Benintende 2008). Still, some Lepidoptera-active *B. thuringiensis* strains can produce 130 kDa proteins that occur as spherical inclusions (Wasano & Ohba 1998). Most *B. thuringiensis* strains harbor complex *cry* gene combinations (Juarez-Perez *et al* 1997, Bravo *et al* 1998, Uribe *et al* 2003), such as the well-known HD-1 strain (Höfte & Whiteley 1989), whereas some others can bear a single *cry* gene, such as the strain HD-73, with only a *cry1Ac* gene (Gonzalez *et al* 1981). *Bacillus thuringiensis* svar *kurstaki* HD-1 strain is the most useful strain as an insecticide, because it exhibits powerful toxicity to various lepidopteran larvae (Li *et al* 2002). Formulations of HD-1 strain typically contain Cry1Aa, Cry1Ab, Cry1Ac, and Cry2A proteins, all of which have relatively high levels of toxicity to over 100 species of Lepidoptera (Glare & O'Callaghan 2002).

A screening program of *B. thuringiensis* isolates native to Argentina has led to the finding of some atypical isolates (Benintende *et al* 1999, 2000, Franco-Rivera *et al* 2005). Many of these isolates, all collected from areas where no commercial *B. thuringiensis*- based products had been used before, showed to be phenotipically and genotipically highly similar to the *B. thuringiensis* HD-1 strain (Franco-Rivera *et al* 2004, Sauka *et al* 2007). In this study, we report the characterization of a new Argentine isolate of *B. thuringiensis* similar to the HD-1 strain, which harbors a cryptic *cry2Ab* that apparently is transcribed, but not translated into a protein.

Material and Methods

Bacterial strains, culture conditions. *Bacillus thuringiensis* INTA TA24-6 was isolated from a spider web collected

in Paraná, province of Entre Ríos, Argentina, in PEMBA medium (Holbrook & Anderson 1980). This isolate was primarily identified by the presence of parasporal inclusions observed under phase-contrast microscopy. Two *B. thuringiensis* reference strains, *B. thuringiensis* svar *kurstaki* HD-1 and HD-73, were kindly provided by the United States Department of Agriculture (USDA), Agricultural Research Service (Peoria, USA). INTA TA24-6 and reference strains were grown in 100 ml of BM medium (Benintende & Cozzi 1996), at 340 rpm and 30ºC, during 72h or until complete autolysis was observed. Spore-crystal complexes were obtained by centrifugation at 12,000 *g* and 4ºC for 15 min, and pellets were freeze-dried. Powders of spore-crystal complexes were kept at -20ºC until further use.

Fingerprinting by Rep-PCR analysis. Fingerprinting specific for strains within the *Bacillus cereus* group using Rep-PCR analysis was carried out on INTA TA24-6 and reference strains, following previously described methods (Reyes-Ramirez & Ibarra 2005). Rep-PCR patterns obtained by electrophoresis in 1.2% agarose gels were compared.

Plasmid patterns. Plasmid extracts were obtained by following a technique described previously (Ibarra *et al* 2003). Plasmid patterns were obtained on 0.6% agarose gel electrophoresis carried out for 12 h at 30 V.

Detection and identification of *cry* genes. INTA TA24-6 was grown on nutrient agar plates for 16h. A loopful of vegetative cells was transferred to 100 µl of water and boiled for 10 min to make bacterial DNA accessible for PCR amplification. The lysate was briefly centrifuged (5 s at 15,700 *g*; Eppendorf model 5415R centrifuge), and 5 µl of supernatant was used as a DNA template for PCR. Detection of *cry1* genes was carried out following conditions essentially as described by Juarez-Perez *et al* (1997), using the I(+) and I(-) group primers. Further identification of *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ad*, *cry1B*, *cry1C*, *cry1D*, *cry1F* and *cry1G* was conducted as previously described (Ceron *et al* 1994, 1995, Juarez-Perez *et al* 1997). PCR–restriction fragment length polymorphism (RFLP) methods previously described (Sauka et al 2005, 2006) were used to detect and identify *cry2* and *cry11* genes. For the detection of *cry9* genes, novel specific primers were designed based on the analysis of conserved regions by multiple alignments of DNA sequences in the "Bt toxin nomenclature website" using ClustalW (http://www.ebi.ac.uk/clustalw/) and Oligoanalyzer 3.0 (http://scitools.idtdna.com/scitools/Applications/OligoAnalyzer/). Primers used for the amplification of a DNA fragment of 643 bp in size of *cry9Aa*, *cry9Ba*, *cry9Da* and *cry9Eb*, and of 640 bp in size of *cry9Bb*, *cry9Ca*, *cry9Ea* and *cry9Ec* were as follow: 9GP (forward; 5'-CGGCAAATTTAGTGTCRTGCTTATC-3') and 9GN (reverse; 5'-AATTCAAGATTTCCTARCGTCGC-3'). This PCR was carried out as described previously (Sauka *et al* 2006), but using 3 mM $MgCl_2$ per reaction and each cycle consisting of an annealing step at 50°C for 1 min.

Electron microscopy. Scanning and transmission electron microscopy images of INTA TA24-6 were obtained by following the procedure described by Benintende *et al* (2000).

Crystal purification. Crystals of INTA TA24-6, HD-1 and HD-73 strains were purified by continuous NaBr gradient differential centrifugation (Ibarra & Federici 1986). Pure, freeze-dried crystals were kept at -20ºC until further use.

Crystal protein composition. The protein composition of pure crystals of INTA TA24-6 was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) conducted as described by Laemmli & Favre (1973) with a 3% stacking gel and 10% resolving gel in a Bio-Rad Mini Protean 3 Cell system. Electrophoresis was carried out at 50 V for 15 min and 100 V for 2 h. Gels were stained with Coomassie Brilliant Blue. A high molecular weight standard mixture (Sigma SDS-6H) was used to estimate molecular masses of crystal proteins.

Solubilization and trypsinization of crystal proteins. Pure crystals of *B. thuringiensis* of INTA TA24-6, HD-1 and HD-73 strains were solubilized and digested by trypsin according to Lopez-Meza & Ibarra (1996).

Insect bioassays. Toxicity of INTA TA24-6 spore-crystal suspensions was qualitatively and quantitatively analyzed by bioassays against neonate larvae of *Epinotia aporema* Wals. (Lepidoptera: Tortricidae) and qualitatively against fourth-instars of *Aedes aegypti* L. (Diptera: Culicidae). Highly concentrated spore-crystal suspensions (final concentration of 100 µg/ml and 1 mg/ml, respectively) were incorporated into polypropylene conical tubes containing a thermostatized (40°C) artificial diet for *E. aporema* and poured into each well of a 24-well plates (Nunc 143982) (Greene *et al* 1976), or into plastic cups containing dechlorinated water for *A. aegypti* (Ibarra *et al* 2003). Distilled water was added to the controls. Twenty four *E. aporema* larvae and 20 *A. aegypti* larvae were used per assay (three replicates). Mortality was registered after five days at 29ºC in *E. aporema* and one day at 29ºC in *A. aegypti* bioassays. Quantitative bioassays were conducted in the same way, except that a series of six concentrations (concentration range: 0.22-3.75 µg/ml) was prepared in order to establish the concentration–response relationship by probit analysis. Twenty four larvae were tested for each concentration. Statistical restrictions were followed as mentioned earlier (Ibarra & Federici 1987).

RNA purification and Reverse Transcriptase-PCR (RT-PCR) analysis. Total RNA from INTA TA24-6 and HD-1 strain were extracted at the T2 and T5 stages using SV Total RNA Isolation System (Promega). Ten µl of total RNA was used as a template in RT-PCRs using Access RT-PCR System (Promega). Primers described previously were used for the amplification of the transcribed *cry* genes (Ceron *et al* 1994, Masson *et al* 1998). Control tubes without reverse-transcriptase were also included.

Results and Discussion

As a part of a nationwide program focused to the finding of *B. thuringiensis* isolates native to Argentina with increased toxic activity against some critical lepidopteran pests, crystal

morphology and the *cry1* and *cry2* gene content of several isolates were investigated. *Bacillus thuringiensis* INTA TA24-6, isolated from a spider web, was one of those isolates, which was further characterized by Rep-PCR typing, plasmid pattern and *cry* gene content, followed by composition of their parasporal inclusions, toxicological properties and gene transcription analysis.

Bacillus thuringiensis INTA TA24-6 showed a Rep-PCR pattern identical to the reference strain HD-1 and similar to HD-73 (Fig 1), indicating that this isolate may belong to the serovar *kurstaki*, according to the conclusions drawn during the development of this technique (Reyes-Ramirez & Ibarra 2005). Furthermore, plasmid profiling of INTA TA24-6 in agarose gels revealed a very similar pattern, although not identical to that of the HD-1 reference strain (Fig 1). Plasmid patterns have been widely used in discriminating isolates, even within the same serovar (Gonzalez *et al* 1981, Benintende *et al* 1999, 2000). Evidence supporting this assertion was the clear difference shown by the plasmid pattern of the reference strain HD-73, which also belongs to the same serovar as the HD-1 strain (*kurstaki*) (Fig 1).

On the other hand, the *cry* gene content of *B. thuringiensis* strains is known to be related to their toxicity and the detection of *cry* genes by PCR analysis has been exploited to predict their insecticidal activity (Juarez-Perez *et al* 1997, Bravo *et al* 1998, Uribe *et al* 2003). To some extent, *cry* gene content is useful to the characterization of an isolate too (Höfte & Whiteley 1989). After the PCR analysis was carried out using the general primers for the identification of *cry1*, *cry2* and *cry9* genes, positive results were obtained for the first two primers pairs, but not for the third one. That is, INTA TA24-6 contains *cry1* and *cry2* genes, but not *cry9* genes.

All the information accumulated so far about INTA TA24-6 indicated similarity with the HD-1 strain; however, differences started to appear when specific *cry* genes were detected. Screening of *cry1* genes using the specific primers described (Ceron *et al* 1994, 1995, Juarez-Perez *et al* 1997, Sauka *et al* 2006) showed that INTA TA24-6 harbors only a *cry1Ac* gene. No amplification was detected for *cry1Aa*, *cry1Ab*, *cry1Ad*, *cry1B*, *cry1C*, *cry1D*, *cry1F*, *cry1G*, and *cry1I* genes. Also, the specific primers for *cry2* genes amplified a *ca*. 1.5 kb amplicon which was subjected to

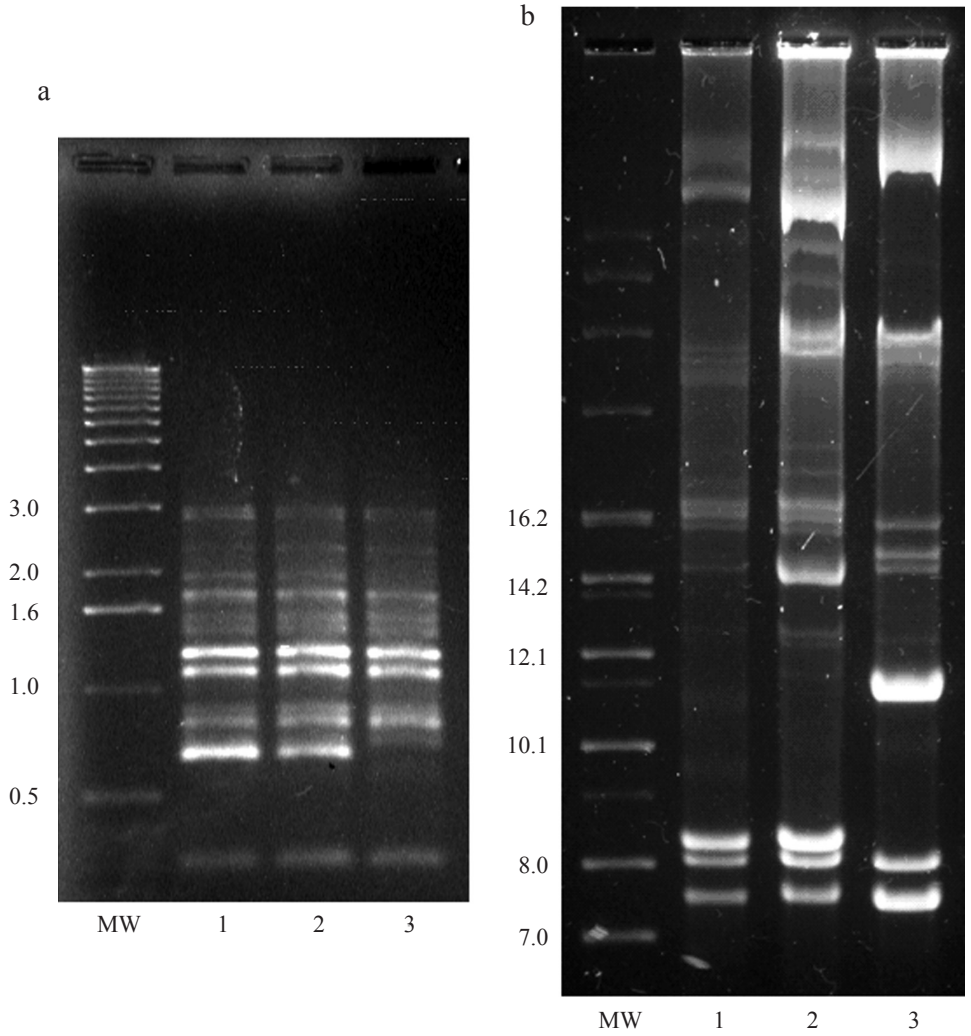

Fig 1 a) Fingerprinting using Rep-PCR; b) plasmid patterns. Lanes: 1, *Bacillus thuringiensis* svar *kurstaki* HD-1; 2, *B. thuringiensis* INTA TA24-6; 3, *B. thuringiensis* svar *kurstaki* HD-73. MW with sizes indicated on left (kb).

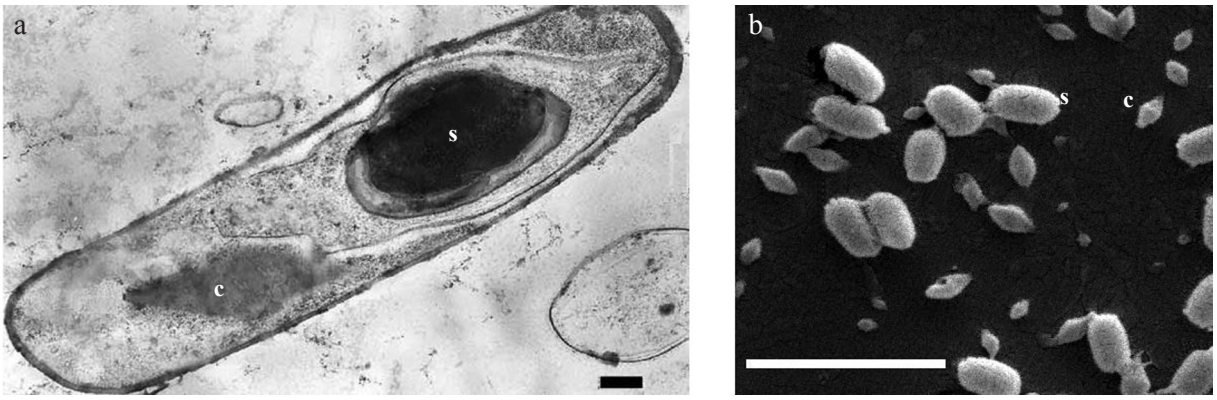

Fig 2 Electron micrographs of *Bacillus thuringiensis* INTA TA24-6. a) Transmission electron micrograph of a sporulating cell, showing a spore (S) and a bipyramidal crystalline inclusion (C). Bar: 0.2 μm; b) Scanning electron micrograph of spores (S) and crystals (C). Bar: 5 μm.

restriction analysis. This indicated the presence of a *cry2Ab* gene in this isolate (Sauka *et al* 2005). It is well known that *B. thuringiensis* svar. *kurstaki* HD-1 contains at least six *cry* genes, including *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ia*, *cry2Aa* and *cry2Ab* (Höfte & Whiteley 1989), whereas the strain HD-73 present only a *cry1Ac* gene (Gonzalez *et al* 1981). The INTA TA24-6 *cry* gene profile, with *cry1Ac* and *cry2Ab* genes, is not frequently detected among *B. thuringiensis* and differs from both reference strains (Ben-Dov *et al* 1997).

Furthermore, crystalline inclusions of INTA TA24-6 were observed under phase contrast microscopy showing bipyramidal crystals typical of Lepidoptera-active *B. thuringiensis* strains (Sauka & Benintende 2008). When ultra-thin sections of sporulating cells were analyzed under transmission electron microscopy, obvious uniform bipyramidal crystals appeared, completely separated from the spores, and ranging in size from 1.2 μm to 1.5 μm long by 0.4 μm to 0.7 μm wide (Fig 2). These dimensions were verified under scanning electron microscopy (Fig 2) where, again, only bipyramidal crystals were observed. This morphology resembled to the parasporal crystals of *B. thuringiensis* serovars that express Cry1 proteins such as *kurstaki*, *aizawai*, *alesti*, *thuringiensis*, *kenyae* and *entomocidus*, among others (Höfte & Whiteley 1989). However, the flat-square to cuboidal crystal typical of strains expressing Cry2 proteins (Höfte & Whiteley 1989) was not detected in INTA TA24-6. This is one other difference between this isolate and the HD-1 strain, as this reference strain is known to contain both bipyramidal and cuboidal crystals (Höfte & Whiteley 1989).

When continuous NaBr gradients were used to purify INTA TA24-6, HD-1 and HD-73 crystals, separation of bipyramidal crystals from the cuboidal inclusions was achieved to some extent in the HD-1 strain, as indicated by the formation of two bands, and corroborated by phase-contrast microscopy and SDS-PAGE analysis (data not shown). However, gradients with INTA TA24-6 and HD-73 samples showed the formation of a single band only, corresponding to bipyramidal crystals, as observed under phase-contrast microscopy. Furthermore, crystals from the INTA TA24-6 band were subjected to SDS-PAGE analysis showing a unique band of *ca*. 130 kDa (Fig 3), which co-migrated with the Cry1A proteins from HD-1 and HD-73 reference strains. HD-1 crystals also showed the expected *ca*. 65 kDa band corresponding to the Cry2 proteins from the cuboidal inclusions. As expected from the previous results on the electron microscopy and NaBr gradients of INTA TA24-6, no *ca*. 65 kDa band was observed in this strain.

Additionally, because insecticidal activity of *B. thuringiensis* crystals is based on their solubility under alkaline conditions, followed by proteolytic activation by gut proteases, parasporal crystal solubility and proteolysis were tested *in vitro*. INTA TA24-6, HD-1 and HD-73 crystals successfully dissolved and were digested with trypsin under conditions early described. SDS-PAGE resolved co-migrating 65 kDa protease-resistant peptides, as expected

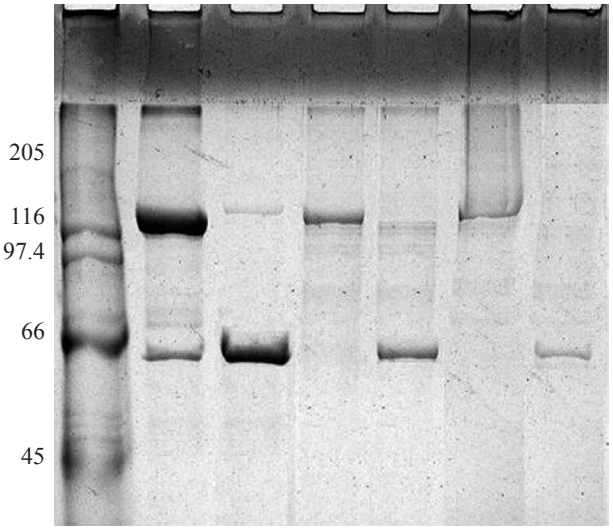

Fig 3 Electrophoretic analysis of crystal proteins (lanes 1, 3 and 5) and trypsin digests of solubilized proteins (lanes 2, 4 and 6) of *Bacillus thuringiensis* svar *kurstaki* HD-1 (lanes 1 and 2), *B. thuringiensis* svar *kurstaki* HD-73 (lanes 3 and 4), and *B. thuringiensis* INTA TA24-6 (lanes 5 and 6); MW with sizes indicated on left (kDa).

Table 1 Dose/response insecticidal activity of *Bacillus thuringiensis* strains to neonate larvae of *Epinotia aporema*.

Strain	50% lethal concentration (µg/ml) (95% confidence interval)	Coefficient of variation (%)	Slope
HD-1	0.81 (0.60-0.99)	9.23	2.14
INTA TA24-6	1.34 (1.03-1.93)	9.91	1.78
HD-73	3.29 (2.57-4.21)	15.46	1.89

from the partial digestion and activation of the Cry1 protoxins of all three strains (Fig 3). *In vivo* activity of INTA TA24-6 was tested by qualitative bioassays carried out using neonate larvae of *E. aporema*, which showed 100% mortality after five days of assay. As expected, no activity was detected when INTA TA24-6 was tested against 4th instars of the yellow fever mosquito *A. aegypti*, as the toxins encoded by the two *cry* genes detected in this native isolate (*cry1Ac* and *cry2Ab*) are known for their toxic activity only against lepidopteran larvae (Höfte & Whiteley 1989). Insecticidal activities of *B. thuringiensis* INTA TA24-6, serovar *kurstaki* HD-1 and HD-73 are shown in Table 1. Mean lethal concentrations were obtained by quantitative bioassays using neonate larvae of *E. aporema*. HD-1 strain showed the highest activity. INTA TA24-6 insecticidal activity was almost three fold-higher when compared with HD-73 strain. This may be due to an undetected factor or protein, or some differences in the expressed Cry proteins.

However, the *cry2Ab* gene identified in INTA TA24-6 may not contribute to its toxicity due to a lack of expression. This assertion is based on the lack of cuboidal inclusions, the lack of cuboidal inclusions-enriched band during crystal purification, and the lack of a *ca*. 65 kDa band at the SDS-PAGE gel. Therefore, transcription of the *cry2Ab* gene was tested by RT-PCR analysis at stages T2 and T5, which indicated that this gene is transcribed into an mRNA at both stages (Fig 4). Additionally, the *cry1Ac* gene showed positive transcription as well (Fig 4). Interestingly, these results suggest that the *cry2A* gene might be cryptic in INTA TA24-6 despite its transcription. It is noteworthy that other *B. thuringiensis* strains that harbor a *cry2Ab* gene contain little or no Cry2Ab protein in their crystalline inclusions (Dankocsik *et al* 1990). Lack of expression of *cry2* genes has been related to mutations that lead to a lost in the coding frame (Crickmore & Ellar 1992) and to the lack of a functional promoter (Jain *et al* 2006), leaving this as the first report of a lack of expression due to a post-transcriptional factor. Further studies are necessary to validate our observations. However, expression of *cry2Ab* at high levels has been achieved when a strong promoter leads its transcription, indicating that low levels of expression are probably the result of a weak promoter (Dankocsik *et al* 1990).

In summary, this report presents the characterization of an Argentine *B. thuringiensis* isolate that is similar to the HD-1 strain, and harbors an uncommon *cry* gene profile and a cryptic *cry2Ab* gene. Despite of its genetic background, INTA TA24-6 seems to be, at least in part, also similar to the

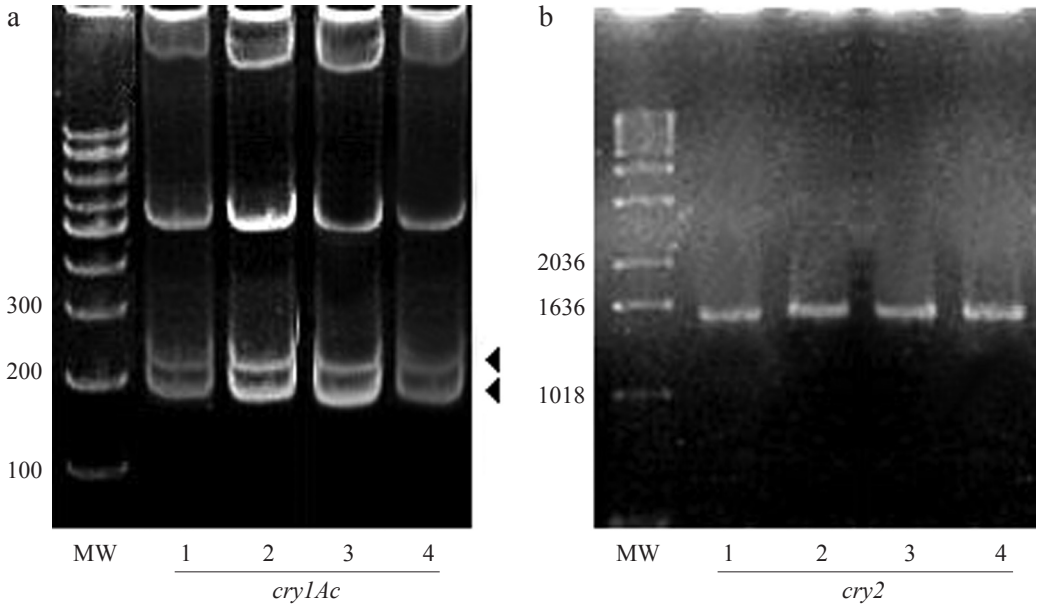

Fig 4 The *cry* genes transcription analysis of *Bacillus thuringiensis* svar *kurstaki* HD-1 and INTA TA24-6 by RT-PCR. The *cry*-type genes analyzed each time are shown at the bottom of black numbers. a and b: Lanes: 1 and 3, HD-1 and INTA TA24-6 at T2 stage, respectively; 2 and 4, HD-1 and INTA TA24-6 at T5 stage, respectively. Black arrows indicate the bands that correspond to the *cry1Ac* gene. MW with sizes indicated on left (bp).

HD-73 strain, as this native strain only expresses the *cry1Ac* gene. The presence of untranslated *cry* genes may hamper the PCR ability as a predictive tool for insecticide activity in *B. thuringiensis* strains.

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