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 phenotypically and genotypically 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 30°C, during 72h or until complete autolysis was observed. Spore-crystal complexes were obtained by centrifugation at 12,000 g and 4°C for 15 min, and pellets were freeze-dried. Powders of spore-crystal complexes were kept at -20°C 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 cry1I 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/scitoools/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’-CGGCCAACATTTAGTGGCRTGTCTATC-3’) and 9GN (reverse, 5’-AATTCAAGATTCTTACGTGC-3’). This PCR was carried out as described previously (Sauka et al 2006), but using 3 mM MgCl₂ 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).
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

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

Furthermore, crystalline inclusions of INTA TA24-6 were observed under phase contrast microscopy showing bipyramidal crystals typical of Lepidoptera-active \textit{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 \textit{B. thuringiensis} serovars that express Cry1 proteins such as \textit{kurstaki}, \textit{aizawai}, \textit{alesi}, \textit{thuringiensis}, \textit{kenyae} and \textit{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 \textit{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 \textit{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.
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](image-url) 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 cryIAc 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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