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Short Communication

PCR-based prediction of type I β-exotoxin production in *Bacillus thuringiensis* strains

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ABSTRACT

Some *Bacillus thuringiensis* strains secrete type I β-exotoxin, which is a non-specific insecticidal and thermostable adenine nucleoside oligosaccharide. Toxicity bioassays and HPLC are traditional methods for detecting β-exotoxin. With the aim of establish a first rapid approach for prediction of type I β-exotoxin production, two PCR-based methods were successfully evaluated in *B. thuringiensis* strains and native isolates. In order to validate a reliable technology, results obtained by this method were correlated with that obtained from *Musca domestica* bioassays.

1. Introduction

Bacillus thuringiensis Berliner is a gram-positive bacterium which synthesizes insecticidal virulence factors. The most important are the Cry proteins, which are produced during sporulation as crystal parasporal inclusions. These proteins along with the spores constitute the active ingredients of the most widely used biological pesticide to control insect pests that affect agriculture, forestry and human and animal vectors (Sauka and Benintende, 2008).

In some *B. thuringiensis* strains there are other virulence factors such as β-exotoxin, non proteinaceous and thermostable toxins that, unlike Cry toxins, are secreted during vegetative bacterial growth (De Barjac and Dedonder, 1965). Two types of β-exotoxin (type I and II) have been described by Levinson et al. (1990). The type I β-exotoxin (also called thuringiensin) has been considered for years a toxin with structural similarity to ATP, that is thought to inhibit RNA polymerase by competing with ATP for binding sites (Sebesta and Horska, 1970). However, Liu et al. (2010) proposed that it is an adenine nucleoside oligosaccharide rather than an adenine nucleotide analog, based on the predicted functions of some key enzymes. In insects, it affects molting and pupation, causing teratological effects (Burgerjon et al., 1969). It is active against dipteran, coleopteran, lepidopteran and some nematode species (Gevrey and Euzéby, 1966). Type I β-exotoxin also affects mammals and is persistent in the environment (Beebee et al., 1972; Mackedonski et al., 1972). Thus, the regulation authorities in accordance with World Health Organization recommendations banned the use of strains that produce this metabolite as the basis of bioinsecticidal formulations (World Health Organization, 1999).

Type I β-exotoxin production has often been linked to the presence of plasmids of various sizes bearing *cry* and *vip1*/*vip2* genes (Espinasse et al., 2002, 2003), but it was not till recently that genes involved in β-exotoxin synthesis were cloned and sequenced. The cluster comprised 11 ORFs that were located on a 110-kb plasmid bearing insecticidal crystal protein gene *cry1Ba* in strain CT-43 (Liu et al., 2010). One of these ORFs encodes the *thuE* protein that was proved to be responsible for the phosphorylation of a β-exotoxin precursor at the last step of their biosynthesis process (Liu et al., 2010).

There are different kind of methods to detect β-exotoxin such as High Performance Liquid Chromatography (HPLC), bioassays with *Musca domestica* Linneaus larvae and other insects (Espinasse et al., 2003; Hernández et al., 2001; Levinson et al., 1990; Mac Innes and Bouwer, 2009). All these methods have in common that they are laborious and time-consuming. In that sense, we think that a Polymerase Chain Reaction (PCR) method for rapid prediction of type I β-exotoxin production in *B. thuringiensis* sounds imperative as a first approach. The objective of this study was to establish a simple PCR method for detection of a gene (*thu*E) strongly associated to the synthesis of type I β-exotoxin. The obtained information by this method was correlated with that

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from *M. domestica* bioassays in order to validate a reliable technology.

2. Materials and methods

2.1. Bacillus thuringiensis strains

Thirty exotic *B. thuringiensis* strains were kindly provided by the Agricultural Research Culture Collection (Peoria, IL), Institut Pasteur (France), Bacillus Genetic Stock Center (Columbus, OH) and the stock collection of Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (Irapuato, Mexico). One hundred and seven native *B. thuringiensis* isolates collected from soils, dead insect larvae, leaves, spider webs and stored product dust from different ecological regions of Argentina were selected from the bacterial collection of the Instituto de Microbiología y Zoología Agrícola–Instituto Nacional de Tecnología Agropecuaria (IMYZA-INTA). Twin isolates collected from the same sample were not included in order to avoid overestimation of distribution frequencies (Sauka et al., 2011).

2.2. PCR primers

Novel specific primers for the detection of *thu*E genes were designed to anneal nucleotide sequences identified in the genome of *B. thuringiensis* serovar *chinensis* CT-43 (GeneBank locus tag CT43_P127037) using Oligoanalyzer 3.1 (available at: http://scitools.idtdna.com/scitools/Applications/OligoAnalyzer/). Primers BEF (forward; 5′-GCGGCAGCCGTTTATTCAAA-3′) and BER (reverse; 5′-CCCCTTCCCATGGAGAAACA-3′) amplify a 406-bp DNA fragment of *thu*E between nucleotides 373 and 778, whereas primers BEF1 (forward; 5′-CAGTAAAAGGCCCATTGGAA-3′) and BER1 (reverse; 5′-TTCCATGAATTTGCCGATTT-3′) amplify a 385-bp DNA fragment of the aforementioned gene between nucleotides 263 and 647 (Fig. 1). Oligonucleotides were synthesized in a DNA synthesizer as specified by the manufacturer (Alpha DNA, Montreal, Canada).

2.3. PCR-based prediction of type I β-exotoxin production

All *B. thuringiensis* strains were grown on nutrient agar plates for 16 h. A loopful of cells was transferred to 100 μl H_2O and boiled for 10 min to make DNA accessible for PCR amplification. The lysate was centrifuged briefly (5 s at 20,000*g* in a Sigma model 1–15PK centrifuge), and 5 μl supernatant was used as a DNA template in each polymerase chain reaction. This was performed with a final volume of 25 μl containing 2.5 μl 10× reaction buffer, 0.5 μl 50 mM $MgCl_2$, 0.5 μl 100 mM deoxynucleoside triphosphate mixture, 8 pmol each primer, and 1 U of Taq polymerase (Invitrogen). The PCR amplification consisted of DNA denaturation at 94 °C for 2 min followed by 25 cycles of amplification with a thermocycler (Eppendorf Mastercycler gradient). Each cycle consisted of a denaturation step at 94 °C for 1 min, an annealing step at 54 °C for 1 min, and a chain elongation step at 72 °C for 1 min. The final elongation step was extended for an additional 5 min. Subsequently, 10 μl PCR product was analyzed by 1.0% agarose gel electrophoresis.

2.4. Qualitative detection of β-exotoxin using a M. domestica bioassay

The strains were cultured for 72 h at 30 °C in 50 ml polypropylene tubes containing 10 ml of nutrient agar slants. Then, ten third-stage *M. domestica* larvae were placed in each tube and covered with a fine mesh. The *B. thuringiensis* serovar *thuringiensis* HD-2 and serovar *kurstaki* HD-1 were used as positive and negative controls respectively. These tubes were incubated in darkness at 30 °C and after 8 days the number of emerged adult flies was quantified. A strain is considered to produce β-exotoxin (positive (+) strain) when 20% or less of adults emerge (Bravo et al., 2001).

3. Results and discussion

Several methods have been described for the detection of β-exotoxin in *B. thuringiensis* (Espinasse et al., 2003; Hernández et al., 2001; Levinson et al., 1990; Mac Innes and Bouwer, 2009), of which HPLC and *M. domestica* bioassays are the most widely used. However, these methods are laborious and time-consuming. Therefore, a rapid test that can exactly detect a key gene involved in β-exotoxin production will greatly help in the potential production of this metabolite. We considered that the *thu*E gene, one of the ORFs that proved to be involved in the type I β-exotoxin biosynthesis, could be a good candidate for prediction of their production in *B. thuringiensis* by PCR.

The novel specific primers (BEF/BER) for *thu*E genes produced amplification in 13.3% (4/30) of the exotic *B. thuringiensis* strains, and in 3.7% (4/107) of our native isolates suggesting the possibility of being type I β-exotoxin-producing strains. *B. thuringiensis* serovar *kurstaki* HD-1, used as negative controls, failed to produce any amplification. Fig. 1 shows the result of electrophoresis analysis of these amplifications.

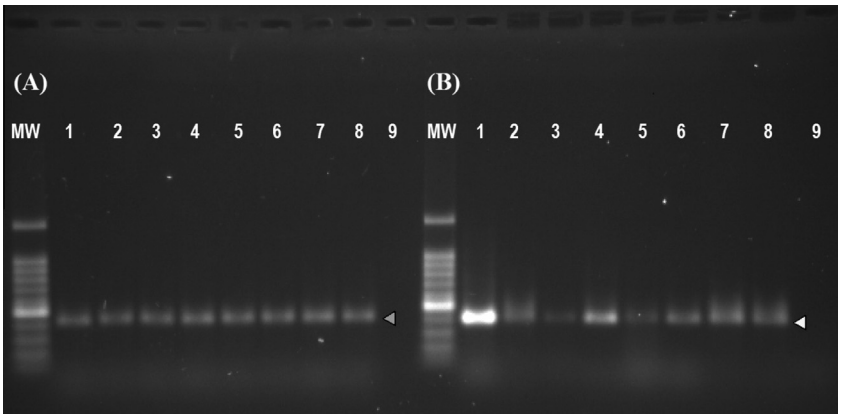

Fig. 1. Agarose gel electrophoresis analysis on 1.0% agarose gel of DNA sequences amplified by PCR assay by using primers BEF/BER (A) and BEF1/BER1 (B) targeted *thu*E genes. Lanes: 1, *B. thuringiensis* INTA H3–3; 2, INTA H4–3; 3, INTA H48–5; 4, INTA Mo14–1; 5, *B. thuringiensis* serovar *darmstadiensis* 4M1; 6, *B. thuringiensis* serovar *darmstadiensis* 4M2; 7, *B. thuringiensis* serovar *tolworthi* HD-125; 8, *B. thuringiensis* serovar *thuringiensis* HD-2; 9, *B. thuringiensis* svar *kurstaki* HD-1. MW, molecular weight marker (100 bp DNA ladder Promega).

Table 1
The *thuE* gene content of *B. thuringiensis* strains.

B. thuringiensis strain	Source[a]	*thu*E gene [b]	β-exotoxin [c]	*B. thuringiensis* strain	Source[a]	*thu*E gene [b]	β-exotoxin [c]
aizawai HD-133	A	–	–	*tolworthi* HD-125	A	+	+
aizawai HD-137	A	–	–	*tochigiensis* 4I1	B	–	–
alesti HD-4	A	–	–	*toumanoffi* 4N1	B	–	–
asturiensis 4BQ1	B	–	–	*kenyae* HD-5	A	–	–
canadensis 4H2	B	–	–	*kumamotoensis* 4W1	B	–	–
darmstadiensis 4M1	B	+	+	*kurstaki* HD-1	A	–	–
darmstadiensis 4M2	B	+	+	*kurstaki* HD-73	A	–	–
entomocidus HD-110	A	–	–	*kyushuensis* 4U1	B	–	–
entomocidus 4I1	B	–	–	*morrisoni* 4AB1	B	–	–
galleriae T05001	C	–	–	*morrisoni* 4K1	B	–	+
israelensis IPS-82	A	–	–	*morrisoni* 4K3	B	–	–
japonensis 4AT1	B	–	–	*pakistani* 4P1	B	–	–
tenebrionis DSM2803	D	–	–	*roskildiensis* 4BG1	B	–	–
thompsoni HD-542	A	–	+	*sotto* HD-6	A	–	–
thuringiensis HD-2	A	+	+	*wuhanensis* 4T1	B	–	–

[a] A, USDA; B, BGSC; C, Institut Pasteur; D, CINVESTAV.
[b] No positive PCR signal obtained with primers BEF/BER and BEF1/BER1.
[c] Presence (+) or absence (–) of β-exotoxin determined using bioassays with *Musca domestica*.

In order to rule out possible variation in *thu*E genes that could have yielded a false negative using BEF/BER pair, a second pair of primers were tested. Once again, PCRs with the BEF1/BER1 pair revealed identical results as obtained previously with the BEF/BER pair (Fig. 1). The use of this second primer pair would minimize the possibility of not detecting *thu*E gene variants that may not be amplified using a single pair of primers.

To confirm the specificity of the amplification, the positive PCR products were purified, sequenced and analyzed using BLAST (http://blast.ncbi.nlm.nih.gov). As expected, BLAST hits confirmed the specifity of the amplification. This analysis revealed that the sequences are 100% identical to the *thu*E gene from strain CT-43. Partial DNA sequences of the *thuE* gene from these strains obtained with primers BEF/BER and BEF1/BER1 have been deposited in the GenBank databases under accession Nos. KF934417 to KF934424 and KJ933694 to KJ933701 respectively.

Since the *thu*E gene has been associated with type I β-exotoxin biosynthesis, we expected that these strains would be also toxin producers. That is, the four exotic strains (Table 1) and the four native isolates (Fig. 1) that harbor the *thuE* gene also showed to produce β-exotoxin according to the results obtained in bioassays with *M. domestica*. In addition, two exotic strains (*B. thuringiensis* serovar *morrisoni* 4K1 and *thompsoni* HD-542) that were negative in PCR were positive by *M. domestica* bioassays (Table 1). That toxicity in 4K1 (HD-12) strain is known to be due to type II β-exotoxin (Levinson et al., 1990). We suggest that toxicity in HD-542 may be also due to this type of β-exotoxin. This toxin production is not being predicted by our PCR method since type II β-exotoxin producer strains do not harbor a *thu*E gene. Type II β-exotoxin would be encoded by other genes different from that requires for type I β-exotoxin biosynthesis.

Although type I β-exotoxin production is an intrinsic property of any *B. thuringiensis* strain (Ohba et al., 1981), a strong correlation between its production or lack of production exists with the type of serovar too (Hernández et al., 2003). The detection of potential production of β-exotoxin by PCR in exotic *B. thuringiensis* strains belonging to serovar *tolworthi*, *thuringiensis* and *darmstadiensis* is in agreement with previous studies that showed that these serovars are common producers (Ohba et al., 1981; Hernández et al., 2001, 2003; Levinson et al., 1990). Moreover, as mention before, the *thu*E gene was detected and β-exotoxin production confirmed in four native isolates. These isolates have showed a Rep-PCR pattern identical to the anti-lepidopteran strain *kurstaki* HD-1, indicating that may belong to the serovar *kurstaki* according to the conclusions drawn during the development of this technique (Sauka et al., 2011). This is also in agreement with a previous study that classified strains belonging to serovar *kurstaki* as seldom β-exotoxin producers (Hernández et al., 2003).

In summary, the current study shows that PCR amplification with primers BEF/BER and BEF1/BER1 is a useful assay for the prediction of type I β-exotoxin production in *B. thuringiensis*. This methodology may be used as a first rapid approach at large screenings of type I β-exotoxin producer strains in *B. thuringiensis* collections. However, since our PCR procedure detect the presence of one of the genes required for type I β-exotoxin biosynthesis, the mutations in the *thu*E gene or other genes involved in the biosynthesis may prevent the production of this metabolite.

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