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Identification of toxin genes encoding Cyt proteins from standard and Argentine strains of *Bacillus thuringiensis*

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ABSTRACT

Polymerase chain reaction-restriction fragment length polymorphism methods for identification of *cyt* subclasses from *Bacillus thuringiensis* were established. Eight of 68 standard and ten of 107 Argentine *B. thuringiensis* strains harbor at least one *cyt* gene. The combination of *cyt1Aa*/*cyt2Ba* genes was identified in four standard and in ten native strains, whereas the *cyt1Ba*, *cyt2Aa* and *cyt2Bb* genes alone and the *cyt1Ab*/*cyt2Bc* genes together were found in four different standard strains respectively. The *cyt2Ba* genes from three *B. thuringiensis* svar *israelensis* strains and two Argentine *B. thuringiensis* strains were cloned, sequenced and designated as *cyt2Ba10* to *cyt2Ba14* respectively. These results suggest that these methods are relevant tools for identification of toxin genes encoding Cyt proteins in *B. thuringiensis*.

Key words: *Bacillus thuringiensis*, *cyt1*, *cyt2*, PCR-RFLP

Introduction

Bacillus thuringiensis is a Gram-positive bacterium characterized by its ability to produce proteinaceous crystalline inclusions during sporulation. According to their amino acid similarity, these crystal proteins are classified in two major families (Cry and Cyt) (Crickmore et al. 1998). These proteins may own a specific toxic activity against insect larvae that affect agriculture and forestry, and/or that transmit animal and human pathogens (Sauka and Benintende 2008). In particular, antidipteran *B. thuringiensis* strains commonly produce Cyt proteins which also harbor cytolytic and hemolytic activities against a broad range of cells in vitro (Thomas and Ellar 1983). To date, a variety of different Cyt proteins that range from 25 to 29 kDa have been found, and genes encoding some of these toxins have been identified and sequenced (Koni and Ellar 1983, Guerchicoff et al. 1997). These genes are *cyt1Aa1*, *2* and *5*, *cyt2Ba1*, *2* and *9*, and *cyt1Ca1* from *B. thuringiensis* svar *israelensis*, *cyt1Aa3* and *4*, *cyt2Ba4* and *5* from *B. thuringiensis* svar *morrisoni*, *cyt1Aa6*, *cyt2Aa3*, *cyt2Ba7* and *8* from different *B. thuringiensis* strains from China, *cyt1Ab1* and *cyt2Bc1* from *B. thuringiensis* svar *medellin*, *cyt1Ba1* from *B. thuringiensis* svar *neoleonensis*, *cyt2Aa1* from *B. thuringiensis* svar *kyushuensis*, *cyt2Aa2* from *B. thuringiensis* svar *darmstadiensis*, *cyt2Ba3* from *B. thuringiensis* svar *fukuokaensis*, *cyt2Ba6* from *B. thuringiensis* svar *tenebrionis*, *cyt2Bb1* from *B. thuringiensis* svar *jegathesan*, *cyt2Ca1* from a *B. thuringiensis* strain from Monsanto Company, and *cyt3Aa* from *B. thuringiensis* TD516 (Waalwijk et al. 1985, Koni and Ellar 1993, Cheong and Gill 1997, Guerchicoff et al. 1997, Thiery et al. 1997, Crickmore et al. 1998, Juárez-Pérez et al. 2002, Yu et al. 2002)(See *B.t.* toxin nomenclature) http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/).

Cry and Cyt proteins have different modes of action. Whereas Cry toxins bind to specific receptors in the microvillus of midgut epithelial cells, the Cyt toxins do not bind to receptors and they directly interact with membrane lipids inserting into the membrane and forming pores or destroying the membrane by a detergent like interaction (Soberon et al. 2007). In addition, mosquitocidal Cry and Cyt proteins show a high synergistic effect in combination by functioning as a Cry membrane-bound receptor (Soberon et al. 2007). Therefore, Cyt proteins may be useful for managing insecticide resistance and for increasing the toxic activity of microbial insecticides. In this study, it was of interest to detect *cyt* genes from standard and Argentine *B. thuringiensis* strains. First of all, it is imperative to have a dependable method for detection and identification of *cyt* genes. Polymerase chain reaction (PCR)-based methods have been developed to detect *cyt* genes (Guerchicoff et al 1997, Ibarra et al. 2003, Promdonkoy et al. 2003, Salehi Jouzani et al. 2008, Vidal-Quist et al. 2009). However, to our knowledge, just one work has described the distribution of different *cyt* genes subclasses profiles in *B. thuringiensis* (Wu et al. 2008).

In the present study, we developed strategies for the detection and identification of the mosquitocidal toxin genes encoding Cyt proteins from *Bacillus thuringiensis* based on PCR-restriction fragment length polymorphism (RFLP) methods. We also cloned and sequenced the *cyt2Ba* genes from three *B. thuringiensis* svar *israelensis* strains and two Argentine *B. thuringiensis* strains.

Materials and methods

***Bacillus thuringiensis* strains:** Sixty eight standard *B. thuringiensis* strains were provided by the United States Department of Agriculture, Agricultural Research Service (Peoria, IL), Bacillus Genetic Stock Center (Columbus, OH), Institut Pasteur (France), Instituto de Biotecnología-Universidad Nacional Autónoma de Mexico (Cuernavaca, Mexico) and Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional's stock collection (Irapuato, Mexico). One hundred and seven native *B. thuringiensis* strains collected from soils, stored product dust and leaves from different regions from Argentina were obtained from the Instituto de Microbiología y Zoología Agrícola–Instituto Nacional de Tecnología Agropecuaria (IMYZA-INTA) bacterial collection.

PCR primers: Specific primers for the detection of *cyt1* and *cyt2* genes were designed based on the analysis of conserved regions by multiple alignments of DNA sequences in the *B. thuringiensis* toxin nomenclature website using ClustalW (http://www.ebi.ac.uk/clustalw/) and Oligoanalyzer 3.0 (http://scitools.idtdna.com /scitools/Applications/OligoAnalyzer/) (Table 1). The *cyt3* gene was not included in this study because their nucleotide sequences are not freely available yet.

DNA preparation and detection of *cyt* genes. The DNA templates for PCR were obtained as previously described (Sauka et al. 2006). Five µl of supernatant was used in each reaction. These were performed in the same way for the detection of *cyt1* and *cyt2* genes. All the reactions were performed with a final volume of 25 µl containing final concentrations of 50 mM KCl, 2.0 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 µM each deoxynucleoside triphosphate (dATP, dTTP, dGTP, and dCTP), 16 pmol each primer, and 2.5 U of Taq polymerase (Invitrogen). The PCR amplification consisted of DNA denaturation at 94ºC for 2 min followed by 25 cycles of amplification with a thermocycler (Eppendorf Mastercycler gradient). Each cycle consisted of a denaturation step at 94ºC for 1 min, an annealing step at 46ºC for 45 s, and a chain elongation step at 72ºC for 45 s. The final elongation step was extended for an additional 5 min. Finally, 10 µl PCR product was analyzed by 1.5% agarose gel electrophoresis.

Identification of *cyt* genes. For the identification of different *cyt1*and *cyt2* genes, 10 µl of positive PCR product was digested with *Rsa*I and a mixture of *Alu*I + *Rsa*I (Promega) respectively according to manufacturer's instructions, analyzed by 10% polyacrylamide gel electrophoresis and stained with ethidium bromide. The expected restriction fragment sizes of the known *cyt* genes were determined by *in silico* digestion of their available sequences in the *B. thuringiensis* toxin nomenclature website with the software 'RestrictionMapper' (Table 2).

Amplification, cloning and nucleotide sequencing of *cyt2Ba* genes. Manual ''hot start'' PCR was performed with a final volume of 50 µl containing the same final concentrations described for the reactions used in the detection of *cyt* genes. Ten µl of supernatant was used as the DNA template in the reactions. Five U of Taq DNA polymerase (Invitrogen) were added after the first denaturation step. Finally, the PCR product was analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide.

The PCR product of three exotic and two native *B. thuringiensis* strains were purified from the agarose gel matrix using Wizard SV Gel and PCR Clean-Up System (Promega), cloned in pGEM-T Easy vector (Promega) and then transformed into competent *Escherichia coli* JM109 strain following the manufacturer protocols. Fifteen white colonies were selected on X-gal IPTG containing selective LB agar plates. Verifying whether the clones contained inserts was accomplished by PCR of recombinant plasmid DNA using vector primer SP6 and T7. Afterwards, the *cyt2Ba* gene of each clone was identified by the PCR-RFLP method described above.

Three clones harboring *cyt2Ba* genes from each *B. thuringiensis* were sequenced in both directions using vector primers (SP6 and T7) in the 'Unidad de Genómica' (Instituto de Biotecnología, INTA). These *cyt2Ba* nucleotide sequences and their translations into amino acid sequences (with the ExPASy translate tool; http://www.expasy.org/tools/dna.html) were aligned separately with ClustalW (Thompson et al. 1994). The deposited sequences in GenBank of all *cyt2Ba* genes were accessed through the *B. thuringiensis* toxin nomenclature website (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/) and used as references.

Table 1: Characteristics of primers used in this study

Primer pair	Sequence	Tm (°C)	Gene	Position	Size (bp)	Accession no.
cyt1F	5'CAATCAACAGCAAGRGTT ATT	50.0	*cyt1Aa*	71-549	478	X03182
			cyt1Ab	71-542	471	X98793
cyt1R	5' GRA TTG CAA ACA GGA CAT TRT A	51.3	*cyt1Ba*	100-577	477	U37196
			cyt1Ca	71-524	453	AL731825
cyt2F	5'CAAATTGCAAATGGTMTT CC	50.3	*cyt2Aa*	244-592	348	Z14147
			cyt2Ba	246-594	348	U52043
cyt2R	5'AACATCYACAGTAATYTC AAATGC	49.9	*cyt2Bb*	237-585	348	U82519
			cyt2Bc	244-592	348	CAC80987
			cyt2Ca	171-519	348	AAK50455
Scyt2BaF	5'ATGCACCTTAATAATTTG AATAATTT	48.5	*cyt2Ba*	1-792	792	U52043, AF020789,
Scyt2BaF	5'TTACGATTTTATTGGATTA ACATTC	48.1				AF022884, AF022885, AF022886, AF034926, AF215645, AF215646, AL731825

Table 2. Expected restriction fragment sizes of digested *cyt* genes

Genes	Fragment size (bp) with *Rsa*I	Genes	Fragment size (bp) with *Alu*I + *Rsa*I
cyt1Aa	478	*cyt2Aa*	25, 141, 182
cyt1Ab	226, 245	*cyt2Ba*	107, 241
cyt1Ba	140, 337	*cyt2Bb*	25, 75, 107, 141
cyt1Ca	26, 55, 112, 120, 140	*cyt2Bc*	348
		cyt2Ca	66, 100, 182

Results and Discussion

The specific oligonucleotide primers for *cyt1* (cyt1F/cyt1R) produced amplification in five standard (Fig. 1) and ten Argentine *B. thuringiensis* strains, and for *cyt2* (cyt2F/cyt2R) in six (Fig. 1) and 10 respectively. *B. thuringiensis* svar *kurstaki* HD-1 and HD-73, used as negative controls, failed to produce any amplification, as expected. Those native isolates, that were been collected from the same sample, were analyzed by sodium dodecyl sulphate–polyacrylamide gels and PCR to discard twin strains and did not overestimate distribution frequencies (data not shown) (Sauka et al. 2005, 2006). Identification of *cyt* genes was determined in these bacteria according to restriction analysis of PCR products digested as previously mentioned. Just three classes of *cyt1*and four of *cyt2* genes were successfully identified during this study. Overall, the combination of *cyt1Aa*/*cyt2Ba* genes was the most frequent identified in four standard and in ten native *B. thuringiensis* strains, whereas the *cyt1Ba*, *cyt2Aa* and *cyt2Bb* genes alone and the *cyt1Ab*/*cyt2Bc* genes together were found in four different standard strains respectively.

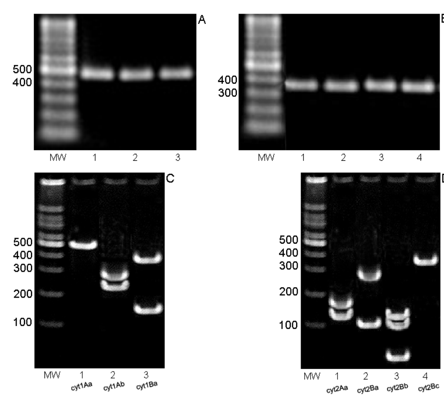

Fig 1. PCR amplification with oligonucleotide primers cyt1F/cyt1R (A) and cyt2F/cyt2R (B), and representative PCR-RFLP patterns of *cyt1*(C) and *cyt2* (D) genes of *B. thuringiensis* strains. (A and C) Lanes: 1, svar *israelensis* HD-567; 2, svar *medellin* B-23135; 3, svar *neoleonensis* 4BE1. (B and D) Lanes: 1, svar *kyushuensis* 4U1; 2, svar *israelensis* HD-567; 3, svar *jegathesan* B-23141; 4, svar *medellin* B-23135; MW, molecular weight marker with sizes indicated on left (bp).

Table 3. The *cyt* gene content of exotic *B. thuringiensis* strains

B. thuringiensis strain	Source	*cyt* gene profile	*B.thuringiensis* strain	Source	*cyt* gene profile
aizawai HD-133	USDA	-	*kenyae* HD-5	USDA	-
aizawai HD-137	USDA	-	*kim* 4BP1	BGSC	-
aizawai T07001	Pasteur	-	*konkukian* 4AH1	BGSC	-
alesti HD-4	USDA	-	*kumamotoensis*	BGSC	-
argentinensis	BGSC	-	4W1	USDA	-
4BV1	BGSC	-	*kurstaki* HD-1	USDA	-
asturiensis 4BQ1	BGSC	-	*kurstaki* HD-73	BGSC	*cyt2Aa*
azorensis 4CB1	BGSC	-		BGSC	-
balearica 4BK1	BGSC	-	*kyushuensis* 4U1	USDA	*cyt1Ab/*
cameroun 4AF1	BGSC	-	*leesis* 4AK1		*cyt2Bc*
canadensis 4H2	BGSC	-		BGSC	-
chanpaisis 4BH1	BGSC	-	*medellin* B-23135	Pasteur	-
colmeri 4X1	BGSC	-		BGSC	-
dakota 4R1	BGSC	-	*monterrey* 4AJ1	BGSC	-
darmstadiensis	USDA	-	*morrisoni*	BGSC	-
4M2	USDA	-	T08003	BGSC	*cyt1Ba*
entomocidus HD-	Pasteur	-	*morrisoni* 4AB1	BGSC	-
110	BGSC	-	*muju* 4BL1	BGSC	-
finitimus HD-3	BGSC	-	*navarrensis*	BGSC	-
galleriae T05001	BGSC	-	4BM1	BGSC	-
graciosensis 4CD1	BGSC	-	*neoleonensis*	BGSC	-
higo 4AU1	BGSC	-	4BE1	BGSC	-
huazhongensis	USDA	*cyt1A*	*nigeriensis* 4AZ1	BGSC	-
4BD1		*a/*	*novosibirsk*	BGSC	-
iberica 4BW1	USDA		4AX1	BGSC	-
indiana 4S2		*cyt2B*	*ostriniae* 4Z1	BGSC	-
israelensis HD-500	UNAM	*a*	*oswaldocruzi*	BGSC	-
		cyt1A	4AS1	BGSC	-
israelensis HD-567	CINVESTAV	*a/*	*pakistani* 4P1	BGSC	-
		cyt2B	*palmanyolensis*	USDA	-
israelensis HD-522	BGSC	*a*	4BS1	BGSC	-
	USDA	*cyt1A*	*pingluonsis* 4BX1	BGSC	-
israelensis IPS-82	BGSC	*a/*	*pirenaica* 4BU1	BGSC	-
	BGSC	*cyt2B*	*poloniensis* 4BR1	BGSC	-
japonensis 4AT1	USDA	*a*	*pulsiensis* 4CC1	BGSC	-
jegathesan B-	USDA	*cyt1A*	*rongseni* 4BT1	BGSC	-
23141	USDA	*a/*	*roskildiensis*		
jinghongiensis		*cyt2B*	4BG1		
4AR1		*a*	*seoulensis* 4AQ1		
shanongiensis		-	*sotto* HD-6		
4AN1		*cyt2B*	*sotto* 4E2		
thompsoni HD-542		*b*	*sumiyoshiensis*		
thuringiensis HD-2		-	4AO1		
tolworthi HD-125		-	*wratislaviensis*		
		-	4BJ1		
		-	*wuhanensis* 4T1		
		-	*xiaguangiensis*		
			4BN1		
			yunnanensis		
			4AM1		

- no positive PCR signal obtained with primers cyt1F/cyt1and cyt2F/cyt2R.

The *cyt* genes content of standard *B. thuringiensis* strains is listed in Table 2. We did not find any bacteria harboring *cyt1Ca* or *cyt2Ca* genes. Representative profiles of *cyt1Aa*, *cyt1Ab*, *cyt1Ba*, *cyt2Aa*, *cyt2Ba*, *cyt2Bb* and *cyt2Bc* genes are shown in Fig. 1. We just detected *cyt* genes in the mosquitocidal *B. thuringiensis* strains belonging to svar *israelensis*, *jegathesan*, *medellin*, *neoleonensis* and *kyushuensis*. These findings are in agreement with the knowledge that Cyt proteins are part of ovoid crystal inclusions typical of strains that belong to these serovars (Waalwijk et al. 1985, Koni & Ellar 1993, Cheong & Gill 1997, Guerchicoff et al. 1997, Thiery et al. 1997, Juarez-Perez et al. 2002), and strongly suggest that the genes that encode these kind of proteins are restricted to a few *B. thuringiensis* serovars and that are not very common in *B. thuringiensis*. It is also known, as it has been previously shown by other investigators, that *cyt* genes are distributed among some *B. thuringiensis* strains from other serovars like *canadensis*, *colmeri*, *darmstadiensis*, *fukuokaensis*, *kumamotoensis*, *morrisoni*, *thompsoni*, *ostriniae* and *tenebrionis* (Guerchicoff et al. 1997, 2001, Promdonkoy et al. 2003, Wu et al. 2008). However, some discrepancies exist with these studies. *B. thuringiensis* svar *kumamotoensis* and *colmeri* were reported to harbor *cyt1* and *cyt2* genes (Wu et al. 2008); however, in agreement with previous observations (Guerchicoff et al. 2001), we could not detect any class of *cyt* genes.

In contrast, *B. thuringiensis* svar *ostriniae* and *canadensis* were also reported to contain a *cyt2* gene (Guerchicoff et al. 2001), but here we showed the lack of this class of gene in strains 4Z1 and 4H2 as has already been reported by Wu et al. (2008). Strains from svar *morrisoni* seem to represent a special case, since some of them were reported to contain *cry2Ba* genes (e.g., HD-12 and HD-518); others are known to not harbor any (Guerchicoff et al. 2001). Strains T08003 and 4AB1 from this serovar seem to fall into this last group.

Many studies have reported PCR-based methods to detect *cyt1* and *cyt2* genes (Guerchicoff et al. 1997, Ibarra et al. 2003, Promdonkoy et al. 2003, Salehi Jouzani et al. 2008, Vidal-Quist et al. 2009), but, to the best of our knowledge, just one study has reported a detailed identification of *cyt* subclasses of native *B. thuringiensis* strains (Wu et al. 2008). The investigators analyzed 143 *B. thuringiensis* isolates from soil samples of China by PCR amplification using two pairs of primers previously described by Ibarra et al. (2003), which showed a great diversity compared with our identified *cyt* genes profiles. However, the investigators failed to inform if twin strains were discarded in order to avoid overestimating the genetic diversity of the sampled areas and to get a closer estimate of *cyt* genes diversity (Sauka et al. 2005, 2006).

The DNA nucleotide sequences of *cyt2Ba* genes and their deduced amino acid sequences from *B. thuringiensis* svar *israelensis* HD-567, HD-522 and IPS82, and *B. thuringiensis* INTA H41-1 and 160-2 have been deposited in the GenBank databases (http://www.ncbi.nlm.nih.gov) under the accession numbers GQ919039, GQ919040, FJ205866, GQ919041 and FJ205865 respectively. These genes were named by the Bt Pesticidal Crystal Protein Nomenclature Committee as *cyt2Ba10* to *cyt2Ba14* (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).

The search for sequence similarity with the previously known Cyt sequences using ClustalW revealed that different *B. thuringiensis* strains may contain very similar to identical Cyt toxins. The nucleotidic sequences of these five new *cyt2Ba* genes were identical between them and to the *cyt2Ba1*, *cyt2Ba4*, *cyt2Ba5*, *cyt2Ba6* and *cyt2Ba9* genes. There is only a three-bp difference with *cyt2Ba7* and *cyt2Ba8*, one with *cyt2Ba2* and *cyt2Ba3*. However, the amino acid sequences are also identical to Cyt2Ba7; they differ by a single amino acid from Cyt2Ba2 and Cyt2Ba3, and by two from Cyt2Ba8.

We presented the establishment of a novel PCR-RFLP method, initially developed using *in silico* design of PCR primers and predictions of restriction fragment sizes, which could detect and identify existing *cyt* genes. The expected PCR product size and restriction fragment patterns of *cyt1Aa, cyt1Ab*, *cyt1Ba*, *cyt2Aa*, *cyt2Ba*, *cyt2Bb* and *cyt2Bc* genes were confirmed experimentally in standard and Argentine *B. thuringiensis* strains. Since the methodology was also developed to detect and identify *cyt1Ca* and *cyt2Ca* genes, an experimental future confirmation would be required. However, there should be understood that getting all the strains containing such genes sometimes is very difficult. Besides increasing our general understanding of their distribution, these results suggest that this method is a relevant tool for identification of toxin genes encoding Cyt proteins from *B. thuringiensis*.

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