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New Variants of Lepidoptericidal Toxin Genes Encoding *Bacillus thuringiensis* Vip3Aa Proteins

Diego H. Sauka Sonia E. Rodriguez Graciela B. Benintende

Insumos Bacterianos, Instituto de Microbiología y Zoología Agrícola (IMYZA), Instituto Nacional de Tecnología Agropecuaria (INTA), Castelar, Argentina

Key Words

Bacillus thuringiensis · *Vip3A* · Polymerase chain reaction-restriction fragment length polymorphism · Sequencing

Abstract

Bacillus thuringiensis is an entomopathogenic bacterium characterized by producing parasporal proteinaceous insecticidal crystal inclusions during sporulation. Many strains are capable of also expressing other insecticidal proteins called Vip during the vegetative growing phase. Particularly, Vip3A proteins have activity against certain Lepidoptera species through a unique mechanism of action which emphasized their possible use in resistance management strategies against resistant pests. The aim of the work was to develop a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method that can distinguish between *vip3A* genes from *B. thuringiensis* strains. In addition, 4 novel *vip3Aa* genes were cloned and sequenced. The method was originally based on amplification of a single PCR amplicon and the use of 2 restriction enzymes with recognition sites that facilitate simultaneous detection. Subsequently, a third restriction enzyme was used to distinguish between *vip3A* variants. Thirteen *vip3Aa* genes were identified in strains belonging to 10 different *B. thuringiensis* serovars. Three intra-subclass variants of *vip3Aa* genes could be differentiated. The presented method can serve as an invaluable tool for the investigation of known and novel *vip3A* genes in *B. thuringiensis* strains. To the best of our knowledge, this is the first report where variants of a same subclass of insecticidal genes could be distinguished following PCR-RFLP.

Introduction

The entomopathogenic bacteria *Bacillus thuringiensis* is characterized by its ability to produce parasporal crystalline inclusions during sporulation containing insecticidal proteins such as Cry and Cyt proteins [Sauka and Benintende, 2008]. In addition, many *B. thuringiensis* strains are known to produce other kinds of insecticidal proteins during the vegetative growing phase that do not form crystals which are called Vip (vegetative insecticidal proteins) [Estruch et al., 1996]. This type of proteins includes Vip1, Vip2 and Vip3. Vip1 and Vip2 are the components of binary toxins that have coleopteran specificity, whereas Vip3 proteins have activity against a wide variety of lepidopteran pests [Estruch et al., 1996; Warren, 1997]. To date, these three groups of Vip proteins can also be classified into 9 subgroups, 27 classes and 92 subclass-

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Diego H. Sauka
Insumos Bacterianos, Instituto de Microbiología y Zoología Agrícola (IMYZA)
Instituto Nacional de Tecnología Agropecuaria (INTA)
De los Reseros y Las Cabañas s/nro. C.C. 25, Castelar 1712 (Argentina)
E-Mail dsauka@cnia.inta.gov.ar

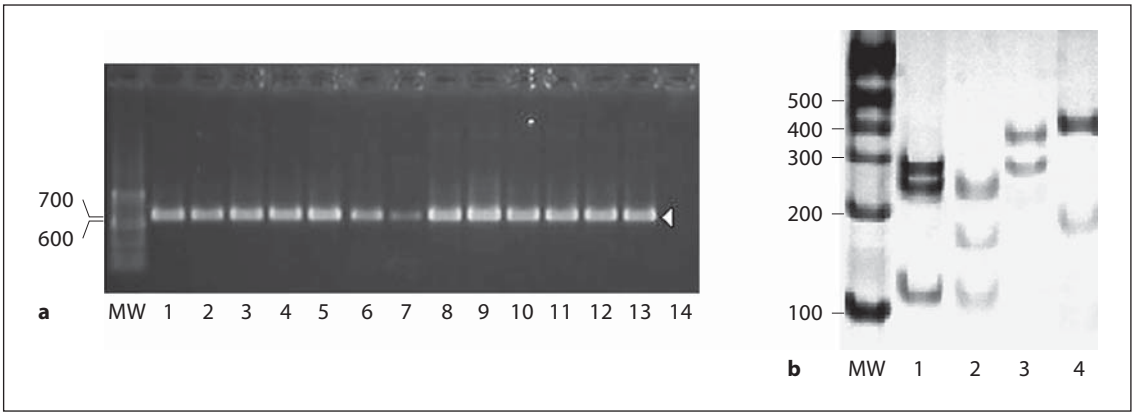

Fig. 1. a PCR amplification with oligonucleotide primers DS3AF and DS3AR of *vip3A* genes in *B. thuringiensis* strains. Lane 1: HD-133; lane 2: HD-137; lane 3: T07001; lane 4: HD-4; lane 5: HD-110; lane 6: T05001; lane 7: HD-155; lane 8: 4AT1; lane 9: HD-542; lane 10: HD-125; lane 11: 4AO1; lane 12: 4T1; lanes 13–14: HD-1 and HD-73 used as positive and negative controls, respectively. **b** Representative PCR-RFLP patterns of *vip3Aa, vip3Ab*/*vip3Aa40–41* and *vip3Aa37*-type genes digested with *Dde*I (lanes 1, 2 and 3, respectively), and *vip3Aa40–41*-type genes digested with *Fok*I (lane 4). MW = Molecular weight marker with sizes indicated on the left (bp).

es according to their amino acid sequence similarity (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/vip.html).

Among these proteins, Vip3A do not share homology with other known proteins and act against lepidopteran larvae through a unique mechanism of action [Bhalla et al., 2005; Estruch et al., 1996; Lee et al., 2003, 2006]. These proteins act by binding to specific receptors, different when compared to that of Cry, located in the midgut epithelium of susceptible insect species. Then, cation-specific pores are formed that disrupt ion flow in the midgut, causing paralysis and death [Lee et al., 2006]. All these factors emphasized the possible benefit of use of Vip3A in resistance management strategies against Cry proteins, since novel *vip3A* genes that are discovered from *B. thuringiensis* strains could be useful as biological control agent against resistant or insensitive pests [Liu et al., 2007].

Some polymerase chain reaction (PCR)-based methods have been developed to detect *vip3A* genes from *B. thuringiensis* strains [Asokan et al., 2012; Beard et al., 2008; Bhalla et al., 2005; Hernández-Rodriguez et al., 2009; Liu et al., 2007; Rice, 1999; Yu et al., 2011]. The present study reported a new PCR-restriction fragment length polymorphism (RFLP) method that can distinguish between known and novel *vip3A* genes from *B. thuringiensis* strains. Furthermore, 4 novel *vip3Aa* genes were cloned and sequenced.

Results and Discussion

In order to identify genes within the *vip3A* gene family, a new strategy based on PCR-RFLP was used. The specific primers used for PCR hybridize in highly conserved sequences flanking a highly variable region of *vip3A* genes. These produced amplification in 12 *B. thuringiensis* strains (fig. 1). *B. thuringiensis* serovar *kurstaki* strains HD-1 and HD-73 were used as positive and negative controls, respectively. Polyacrylamide gel data confirmed that 10 strains had a *vip3Aa*-type RFLP pattern, with 3 main bands of 240, 233, and 111 bp when the product was digested with *Dde*I conforming to the predicted fragments of *vip3Aa* genes. Two *vip3Ab*-type RFLP patterns were identified in *B. thuringiensis* serovar *sumiyoshiensis* 4AO1 and *japonensis* 4AT1, showing 3 main bands of 233, 159 and 111/105 bp. Moreover, a novel *vip3A*-type pattern with 2 bands of 344 and 264 bp was identified in *B. thuringiensis* serovar *entomocidus* HD-110. Representative restriction patterns of *vip3A* genes are shown in figure 1.

Afterwards, *vip3A* genes of *B. thuringiensis* serovar *kurstaki* HD-1, *entomocidus* HD-110, *sumiyoshiensis* 4AO1 and *japonensis* 4AT1 were chosen for cloning and sequencing by using specific and vector primers (table 1). To our knowledge, there are no previously published studies concerning *vip* genes with a full description of all the primers needed for sequencing. The oligonucleotide primers, SRF and SRRI, were successfully used for the amplification of the full length of *vip3A* genes. All the

Table 1. Primers for sequencing of *vip3A* genes

vip3A primers[a]	Sequence[b]	Tm, °C[c]	Position[d]	Gene/s recognized
SRF, reg 5′	ATG AAC AWG AAT AAT ACT AAA TTA ARC RCA	52.4	1–30	*vip3Aa, vip3Ab, vip3Ad, vip3Ae, vip3Af, vip3Ag, vip3Ah*
SRR I, reg 3’	TTA CTT AAT AGA GAC ATC GTA AAA ATG TAC	51.8	2341–2370	*vip3Aa*
SRR II, reg 3′	TTA CTT AAT TGA AAA ATC TCG GAA AKT TAT	51.7	2335–2364	*vip3Ab, vip3Ae, vip3Ag*
SRR III, reg 3′	TTA TTT AAT AGA AAC GTT TTC AAA TGA TAT	49.6	2338–2367	*vip3Af*
SRR IV, reg 3′	TTA TTT AAT AGA RAA ATY ATA AAA RTR TRC	47.4	2332–2361 2359–2388	*vip3Ad* *vip3Ah*
3A1F, reg 5′	ACT GAG TTA ACT GAA CTA GCG	52.0	627–648	*vip3Aa, vip3Ad, vip3Ah*
3A1R, reg 3′	GCT GAA CGC CCG AAT AAA TTA	53.4	723–741	*vip3Aa, vip3Ab, vip3Ae, vip3Af, vip3Ad, vip3Ah*
3A2F, reg 5′	GCT AAG CTA AAA C AAA ATT ATC AAG	49.3	1124–1147	*vip3Aa, vip3Ab, vip3Ae, vip3Af, vip3Ad, vip3Ah*
3A2R, reg 3′	ATT TGT TCA GAT TGA TCT GGR CA	53.6	1201–1223	*vip3Aa, vip3Ab, vip3Ae, vip3Af, vip3Ad, vip3Ah*
3A3FI, reg 5′	GGA ACT TAG AGG GAG AAA ACT TAG	52.7	1625–1648	*vip3Ab, vip3Ae*
3A3FII, reg 5′	GGT CCA TAG AAG AGG ACA ATT TAG	52.8	1625–1648	*vip3Aa, vip3Af*
3A3FIII, reg 5′	GAA ATA TAG AAA TGG ACA CCT TAG	49.1	1625–1648	*vip3Ad*
3A3FIV, reg 5′	GGA TAT TGA GGC TGA CAA CAT AG	53.1	1625–1648	*vip3Ah*
3A3RI, reg 3′	TGT GAG AAC TCA CCA TCC TTA TG	54.3	1726–1746	*vip3Ab, vip3Ad, vip3Ae*
3A3RII, reg 3′	TGT GAW AWT CCT CCG TCC TTA TG	54.3	1726–1746	*vip3Aa, vip3Af*
3A3RIII, reg 3′	TGT GAA AAT TCC CCA TCA TCT TG	53.6	1726–1746	*vip3Ah*

[a] Reg refers to the coding strand. [b] Abbreviation for wobble positions: W, A/T; R, A/G; K, G/T; Y, C/T. [c] Tm = Melting temperature of the primer. [d] The A of the first ATG is numbered 1.

Table 2. Expected restriction fragment sizes of digested *vip3A* genes

Gene	Fragment size (bp) with:		
	*Dde*I	*Fok*I	*Alu*I
vip3Aa	240, 233, 111, 24		
vip3Aa37	344, 264		
vip3Aa40–41	233, 159, 111, 105	439, 169	
vip3Ab	233, 159, 111, 105	608	
vip3Ad	264, 122, 111, 111		
vip3Ae	608		260, 198, 77, 58, 58, 15
vip3Af	264, 233, 111		190, 159, 77, 70, 58, 39, 15
vip3Ag	264, 233, 111		
vip3Ah	497, 111		

obtained sequences have in common that they were 2,370 nucleotides long, encoding 790 amino acid residues. The genes from these strains were named *vip3Aa33, vip3Aa37, vip3Aa40* and *vip3Aa41,* respectively, by the Bt Pesticidal Crystal Protein Nomenclature Committee (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).

The nucleotide sequence of the gene *vip3Aa33* from serovar *kurstaki* HD-1 strain was identical to *vip3Aa7, 9, 10, 12, 15* and *21*. A substitution at position 891 of C for G (transversion) was found to be present in the almost identical gene *vip3Aa37* from serovar *entomocidus* HD-110 strain. This point mutation constituted a silent mutation that did not result in amino acid substitutions, but generated a new *Dde*I restriction site. Consequently, this new restriction site produced a novel RFLP pattern totally different to that of the other *vip3A* genes (table 2). On the other hand, nucleotide sequences of *vip3A* genes from serovar *japonensis* 4AT1 and *sumiyoshiensis* 4AO1 strains showed to be the most different in comparison to other *vip3Aa* genes. They seem to be more similar to *vip3Ab, d, e, f, g* and *h,* and sequence data revealed that they were not *vip3Ab* genes either as suggested by *Dde*I restriction analysis. The restriction analysis for identification of *vip3A* genes was originally designed as a 2-step process. As was

Table 3. The *vip3A* gene content of exotic *B. thuringiensis* strains

B. thuringiensis strain	Source	Gene subclass	*B. thuringiensis* strain	Source	Gene subclass
aizawai HD-133	USDA	*vip3Aa*	*kenyae* HD-5	USDA	–
aizawai HD-137	USDA	*vip3Aa*	*kim* 4BP1	BGSC	–
aizawai T07001	Pasteur	*vip3Aa*	*konkukian* 4AH1	BGSC	–
alesti HD-4	USDA	*vip3Aa*	*kumamotoensis* 4W1	BGSC	–
argentinensis 4BV1	BGSC	–	*kurstaki* HD-1	USDA	*vip3Aa*
asturiensis 4BQ1	BGSC	–	*kurstaki* HD-73	USDA	–
azorensis 4CB1	BGSC	–	*kyushuensis* 4U1	BGSC	–
balearica 4BK1	BGSC	–	*leesis* 4AK1	BGSC	–
cameroun 4AF1	BGSC	–	*medellin* B-23135	USDA	–
canadensis 4H2	BGSC	–	*monterrey* 4AJ1	BGSC	–
chanpaisis 4BH1	BGSC	–	*morrisoni* T08003	Pasteur	–
colmeri 4X1	BGSC	–	*morrisoni* 4AB1	BGSC	–
dakota 4R1	BGSC	–	*morrisoni* 4K1	BGSC	–
darmstadiensis 4M1	BGSC	–	*morrisoni* 4K3	BGSC	–
darmstadiensis 4M2	BGSC	–	*muju* 4BL1	BGSC	–
entomocidus HD-110	USDA	*vip3Aa37*	*navarrensis* 4BM1	BGSC	–
entomocidus 4I1	BGSC	–	*neoleonensis* 4BE1	BGSC	–
entomocidus 4I3	BGSC	–	*nigeriensis* 4AZ1	BGSC	–
finitimus HD-3	USDA	–	*novosibirsk* 4AX1	BGSC	–
fukuokaensis 4AP1	BGSC	–	*ostriniae* 4Z1	BGSC	–
galleriae T05001	Pasteur	*vip3Aa*	*oswaldocruzi* 4AS1	BGSC	–
galleriae HD-155	USDA	*vip3Aa*	*pakistani* 4P1	BGSC	–
graciosensis 4CD1	BGSC	–	*palmanyolensis* 4BS1	BGSC	–
higo 4AU1	BGSC	–	*pingluonsis* 4BX1	BGSC	–
huazhongensis 4BD1	BGSC	–	*pirenaica* 4BU1	BGSC	–
iberica 4BW1	BGSC	–	*poloniensis* 4BR1	BGSC	–
indiana 4S2	BGSC	–	*pulsiensis* 4CC1	BGSC	–
israelensis HD-500	USDA	–	*rongseni* 4BT1	BGSC	–
israelensis HD-567	USDA	–	*roskildiensis* 4BG1	BGSC	–
israelensis HD-522	UNAM	–	*seoulensis* 4AQ1	BGSC	–
israelensis IPS-82	CINVESTAV	–	*shanongiensis* 4AN1	USDA	–
israelensis T014001	Pasteur	–	*silo* 4AG1	BGSC	–
japonensis 4AT1	BGSC	*vip3Aa40*	*sylvestriensis* 4BY1	BGSC	–
jegathesan B-23141	BGSC	–	*sotto* HD-6	USDA	–
jinghongiensis 4AR1	BGSC	–	*sooncheon* 4BB1	BGSC	–
tenebrionis DSM2803	CINVESTAV	–	*sumiyoshiensis* 4AO1	BGSC	*vip3Aa41*
thompsoni HD-542	USDA	*vip3Aa*	*vazensis* 4CE1	BGSC	–
thuringiensis HD-2	USDA	–	*wratislaviensis* 4BJ1	BGSC	–
thuringiensis T01001	Pasteur	–	*wuhanensis* 4T1	BGSC	*vip3Aa*
thuringiensis T01026	Pasteur	–	*xiaguangiensis* 4BN1	BGSC	–
tochigiensis 4Y1	BGSC	–	*yosoo* 4CA1	BGSC	–
tohukensis 4V1	BGSC	–	*yunnanensis* 4AM1	BGSC	–
tolworthi HD-125	USDA	*vip3Aa*	*zhaodongensis* 4BZ1	BGSC	–
toumanoffi 4N1	BGSC	–			

– = No positive PCR signal obtained with primers DS3AF/DS3AR.

shown, the PCR product is first digested with *Dde*I and if necessary with *Alu*I (table 2). Based on these sequences and using *in silico* restriction analysis with *Fok*I as a second step first, we could experimentally differentiate later *vip3Aa40–41* genes from *vip3Ab* genes.

The nucleotide sequence of *vip3Aa40* from serovar *japonensis* 4AT1 and *vip3Aa41* from serovar *sumiyoshiensis* 4AO1 shared the highest similarity (98 and 97%) with the *vip3Aa19* (DQ241674) gene. The deduced Vip3Aa40 protein sequence was compared with other

Table 4. The differences of amino acid residues among Vip3Aa40 and 41 compared to Vip3Aa19

Protein	Amino acid residue														
	3	9	16	244	355	396	442	470	498	543	546	547	553	571	584
Vip3Aa40	K	S	F	F	H	M	S	K	E	N	G	E	I	R	F
Vip3Aa41	M	S	L	L	H	M	S	K	E	N	G	E	I	R	S
Vip3Aa19	K	N	F	F	Y	T	F	N	G	S	E	D	K	K	F
	611	627	663	681	685	686	695	704	706	722	726	755	760	761	780
Vip3Aa40	N	E	T	V	I	R	N	G	N	N	T	I	L	L	L
Vip3Aa41	N	E	T	V	I	R	Y	G	N	N	T	I	G	G	L
Vip3Aa19	D	K	I	T	T	S	N	R	I	Y	S	M	F	E	I

Vip3 proteins, and showed the highest sequence similarity shared with Vip3Aa41 (99%). They differed at 5 amino acid residues: Met (3) for Lys, Leu (16) for Phe, Leu (244) for Phe, Ser (584) for Phe and Tyr (695) for Asn. Both sequences shared high similarity with Vip3Aa19 (97 and 96%, respectively). There were different amino acid residues between these 3 deduced proteins (table 4).

Some previous reports have shown that changes in amino acid residues in Vip3A proteins could affect their insecticidal activity [Doss et al., 2002; Estruch et al., 1996; Liu et al., 2007; Selvapandiyan et al., 2001]. Some of the differences in 19 residues between Vip3Aa19 and Vip3Aa11 were important for lack of toxicity of Vip3Aa19 against *Plutella xylostella* [Liu et al., 2007]. Moreover, differences in 2 residues between Vip3Aa9 and Vip3Aa1 proteins, and in only 1 residue between Vip3Aa9 and Vip3Aa10 proteins, showed to be involved in the ineffectiveness of Vip3Aa9 protein against *Agrotis ipsilon* [Doss et al., 2002; Estruch et al., 1996; Selvapandiyan et al., 2001]. Further studies are needed to express *vip3Aa40* and *vip3Aa41* genes, and to determine toxicity of their products against certain lepidopteran pest that have shown to be insensitive or poorly sensitive to other known Vip3A proteins.

The *vip3A* gene content of *B. thuringiensis* strains is listed in table 3. We did not find any strain that harbored *vip3Ab, vip3Ad, vip3Ae, vip3Af, vip3Ag, vip3Ah* or combinations between any one of this class of genes.

It is known that *vip3Aa* genes are widely distributed among *B. thuringiensis* [Bhalla et al., 2005; Hernández-Rodriguez et al., 2009; Yu et al., 2011]. To the best of our knowledge, just one study has reported the detection of *vip3A* genes of strains representing different serovars of standard *B. thuringiensis* [Bhalla et al., 2005]. These investigators analyzed 24 *B. thuringiensis* strains obtained from Bacillus Genetic Stock Center (USA).

The *B. thuringiensis* serovar *thompsoni* HD-542 was reported to lack a *vip* gene [Bhalla et al., 2005], but here we showed that it harbors a *vip3Aa* gene. This discrepancy could be due to the primers used in that study, which were designed based on the nucleotide sequence of *vip3Aa1* only and did not recognize the *vip3Aa* gene of this strain. In contrast, the serovar *pakistani* HD-395 (= 4P1) strain was reported to contain a *vip3A* gene [Bhalla et al., 2005], but here we could not confirm the presence of this class of genes in this strain.

We identified *vip3Aa* genes in serovar *kurstaki* HD-1, *alesti* HD-4 and *wuhanensis* HD-525 strains. In that previous work, these *B. thuringiensis* strains produced PCR products that detected a *vip3* gene [Bhalla et al., 2005].

A strain from the mosquitocidal serovar *israelensis* was reported to contain a *vip3A* gene [Bhalla et al., 2005], but here we showed the lack of this class of genes in strains belonging to this serovar and in others with recognized toxicity for mosquitoes such as *kyushuensis, medellin, morrisoni, neoleonensis* and *jegathesan* serovars.

The differences between Vip3Aa40 and 41 and the Vip3A proteins are spread throughout the sequence, but the C-terminal end of Vip3A40 and 41 is highly conserved in comparison with the other Vip3Aa proteins and has been reported to be crucial for resistance to proteases [Estruch et al., 1996]. On the other hand, the N-terminal end of Vip3A40 and 41 is highly conserved, but in comparison with other non-Vip3Aa-type proteins.

The N-terminus of the putative signal sequence of Vip3Aa40 and 41 is, with the exception of some amino acids, identical to that of Vip3A toxins except for other

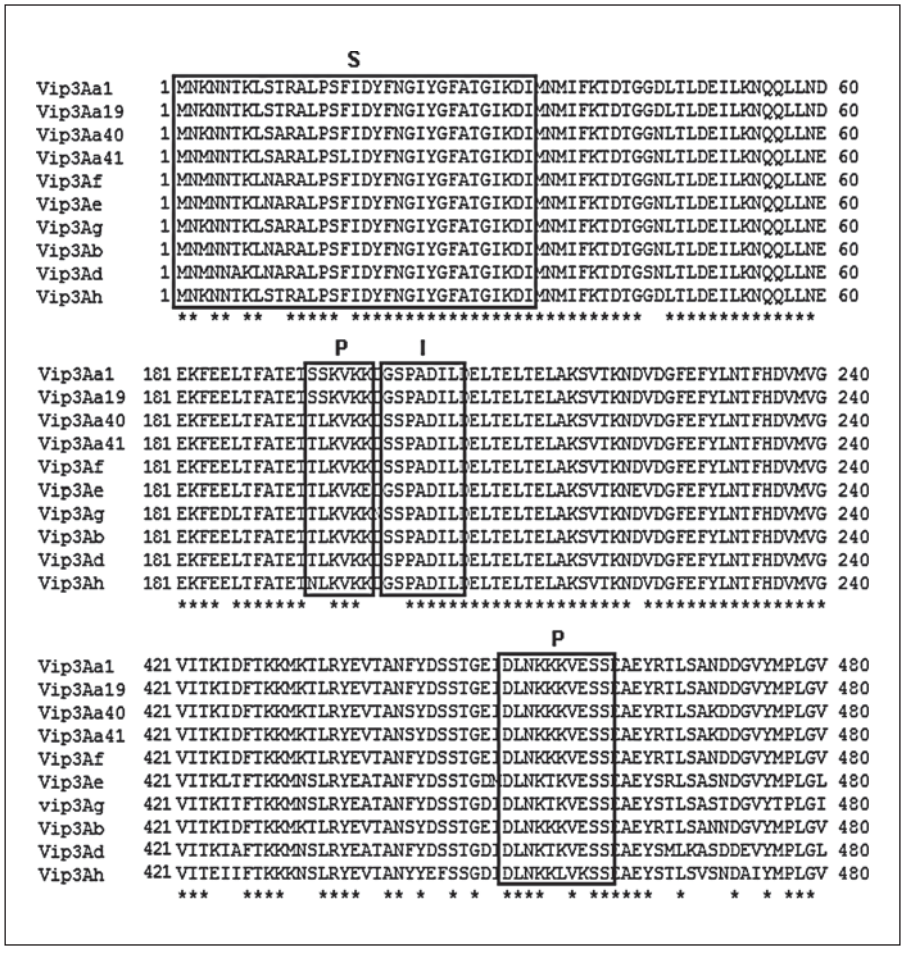

Fig. 2. Alignment of the Vip3Aa40 and 41 sequences with the sequence of a representative of each subclass of Vip3A toxins. Asterisks below the sequences indicate conserved amino acids. The black boxes indicate regions described in the text. The black box labeled S indicates the signal peptide sequence, the one labeled P indicates the proteolytic processing sites characterized in Vip3Aa and the box labeled I indicates the beginning of the 66-kDa insecticidal fragment characterized in Vip3Aa.

Vip3Aa and Vip3Ah sequences (fig. 2). Although different, these sequences have the high asparagine content that characterizes the N-terminal end of signal sequences in *Bacillus* [Pugsley, 1993; Rang et al., 2005]. The last 17 amino acids of the signal sequence are identical in all Vip3A sequences (fig. 2). The complete putative signal sequences of Vip3Aa40 and 41 are therefore MNKNNTKLSARALPSFIDYFNGIYGFATGIKDI and MNMNNTKLSARALPSLIDYFNGIYGFATGIKDI, respectively.

It is known that Vip3Aa proteins are processed at two lysine-rich sites [Estruch et al., 1996, 1998]. The first processing site is located in Vip3Aa at Lys 198 in the sequence 192-SSKVKK-199 (fig. 2). This region is significantly different in Vip3Aa40 and 41, in which it is replaced by the sequence 192-TLKVKK-199 being also highly similar to other non-Vip3Aa-type proteins (fig. 2). The 66-kDa insecticidal moiety of Vip3Aa starts at Gly 200 and contains the sequence 199-GSPADIL-207 [Estruch et al., 1998]. The corresponding region in Vip3Aa40 and 41 proteins is 199-SSPADIL-207 (fig. 2). The substitution of Ser (200) for Gly is also conserved in other non-Vip3Aa-type proteins. The second processing site in Vip3Aa is located at amino acid 455 and results in the release of a 33-kDa fragment ranging from residue 200 to residue 455 [Estruch et al., 1998]. In Vip3Aa proteins, the sequence of this second processing site is DLNKKKVESS, DLNKTKVESS or DLNKKLVKSS (fig. 2). In this case, Vip3Aa40 and 41 contains the sequence DLNKKKVESS which is present in most of the Vip3A proteins.

Vip3Aa40 and 41 contain the same 3 Cys residues in the proteins as most of the Vip3A proteins, except Vip3Aa14 (just 2) and Vip3Ad, g and h (the 3 Cys plus another at the C-terminus). It is likely that some of these cysteines are involved in disulfide bridges and may play a role in the stability of Vip3A proteins.

PCR-RFLP is important to identify the existence of known insecticidal genes and detect novel ones in *B. thuringiensis* strains. Novel insecticidal genes were dis-

covered by this method, e.g. the novel *cry1Ie1* from *B. thuringiensis* isolate Btc007 [Song et al., 2003]. Even though a PCR-RFLP method can be considered old, as shown through the course of this study, we conclude that it is still useful for the identification of novel genes. This is the first study where variants of the same subclass of insecticidal genes could be distinguished. As seen in table 2, *vip3Aa37, vip3Aa40–41* and the rest of *vip3Aa* genes could be differentiated using *Dde*I restriction analysis.

Experimental Procedures

B. thuringiensis *Strains*

Eighty-seven *B. thuringiensis* strains were kindly provided by different institutions around the world (table 3).

Detection of vip3A *Genes*

Novel primers for the specific detection of *vip3A* genes were designed following a previously described methodology [Sauka et al., 2006, 2007]. Primers used for amplification of a 608-bp DNA fragment of *vip3Aa, vip3Ab, vip3Ad, vip3Ae, vip3Af, vip1Ag* and *vip1Ah* genes were as follows: DS3AF (forward; 5′-GTG AAA ACA AGT GGC AGT G-3′) and DS3AR (reverse; 5′-TCC GCT TCA CTT GAT TCT ACT-3′).

The DNA templates for PCR were obtained as previously described [Sauka et al., 2006]. Five microliters of supernatant was used in each reaction. 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 of each deoxynucleoside triphosphate (dATP, dTTP, dGTP, and dCTP), 16 pmol of each primer, and 2.5 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 49°C for 45 s, and a chain elongation step at 72°C 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 vip3A *Genes*

For the identification of different *vip3A* genes, 10 μl of positive PCR product was digested with 2.5 U *Dde*I according to manufacturer's instructions (Promega, Madison, Wisc., USA), analyzed by 12% polyacrylamide gel electrophoresis and stained with ethidium bromide. If necessary, samples were digested with 2.5 U *Fok*I or *Alu*I. Expected restriction fragment sizes of known and novel *vip3A* genes were determined by *in silico* digestion of their available sequences on the *B. thuringiensis* toxin nomenclature website (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/) with the software 'RestrictionMapper' (http://www.restrictionmapper.org/) (table 2).

Amplification, Cloning and Nucleotide Sequencing of Full-Length vip3A *Genes*

Primers used for the amplification of the whole open reading of *vip3A* genes (table 1) were designed following a previously described methodology [Sauka et al., 2006, 2007]. The forward primer is class specific and was designed to begin at the ATG initiation codon. The reverse primers were designed from sequences at the 3′ end of all *vip3A* genes. Manual 'hot start' PCR was performed with a final volume of 50 μl containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 200 mM of each deoxynucleoside triphosphate (dATP, dTTP, dGTP, and dCTP), 1 mM of each primer and 2 mM $MgCl_2$. The PCR amplification consisted of an initial denaturation step of 2 min at 94°C, followed by 29 cycles of 5 s at 95°C, 20 s at 47°C, 4 min at 68°C and a final elongation step of 5 min at 68°C in a thermocycler (Eppendorf Mastercycler gradient). Five units of *Taq* DNA polymerase (Invitrogen) were added after the first denaturation step. Finally, the PCR product was analyzed by 1.0% agarose gel electrophoresis stained with ethidium bromide. The PCR product of 4 *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 *vip3A* gene of each clone was identified by the PCR-RFLP method described above. Three clones harboring *vip3A* genes were sequenced in both directions by primer walking using vector (SP6 and T7) and specific primers detailed in table 1 in the 'Unidad de Genómica' (INTA, Argentina).

Sequence Comparisons

These *vip3A* 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 *vip3A* genes were accessed through the *B. thuringiensis* toxin nomenclature website and used as references.

Nucleotide Sequence Accession Numbers

The DNA nucleotide sequences of *vip3A* genes from *B. thuringiensis* serovar *entomocidus* HD-110, *japonensis* 4AT1, *sumiyoshiensis* 4AO1 and *kurstaki* HD-1 have been deposited in the GenBank databases (http://www.ncbi.nlm.nih.gov) under the accession numbers HM132041, HM132042, HM132043 and GU073128, respectively.

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