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ORIGINAL ARTICLE

Screening and identification of *vip* genes in *Bacillus thuringiensis* strains

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Abstract

Aims: To identify known *vip* genes and to detect potentially novel *vip* genes in a collection of 507 strains of *Bacillus thuringiensis*.
Methods and Results: Following a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) strategy, four restriction patterns were found within the *vip1* family: *vip1Aa1*, *vip1Ba1/vip1Ba2* and *vip1Ca*. In the screening of *vip2* genes, patterns similar to those of *vip2Aa1*, *vip2Ba1/vip2Ba2* and *vip2Ac1* genes were observed. Patterns for *vip3Aa1*, *vip3Ae2* and *vip3Af1* were found among *vip3* genes. Two new patterns revealed novel *vip1* and *vip3A* genes. The observed frequency of genes belonging to *vip1* and *vip2* families was around 10%, whereas 48·9% of the strains showed amplification of *vip3* genes. A tendency of *vip* and *cry* genes to occur together has been observed in this collection of *B. thuringiensis* strains.
Conclusions: Ten different patterns of *vip* genes belonging to the three *vip* families and two novel *vip* genes have been identified in this study.
Significance and Impact of the Study: This is the first time that *vip1* and *vip2* genes have been identified by PCR-RFLP. Furthermore, the results show that the strategy used in this study can lead to the classification of known *vip* genes as well as the identification of novel *vip* genes.

Introduction

Under adverse conditions, the gram-positive entomopathogenic bacterium *Bacillus thuringiensis* forms endospores and, simultaneously, parasporal crystalline inclusions containing one or more insecticidal proteins such as Cry and Cyt proteins. These proteins are primarily toxic to a wide range of insect orders, but also to nematodes, mites and protozoa (Feitelson *et al.* 1992; Schnepf *et al.* 1998). Cry proteins are toxic by ingestion and their specificity is determined by their binding to receptors in the midgut epithelium cells of insects. Commercial biopesticides based on *B. thuringiensis* have been used for more than 30 years and transgenic plants expressing Cry proteins have been used since 1996 for insect pest management (Schnepf *et al.* 1998; Van Rie 2000). Some strains of *B. thuringiensis* also synthesize other insecticidal molecules that are secreted. Several types of secreted toxins with very different structure and mode of action have been described. For example, Cry1I is a lepidopteran-specific proteinaceous toxin secreted during early stationary phase (Kostichka *et al.* 1996; Tounsi and Jaoua 2002; Song *et al.* 2003). β-exotoxin is another extracellular insecticidal toxin expressed during vegetative growth (de Barjac and Dedonder 1965). This toxin is an adenine nucleotide analogue and it likely acts as an inhibitor of DNA-dependent RNA polymerases (Sebesta and Horská 1970). Because of toxicity to vertebrates, its public use is banned in many countries (WHO 1999). Several strains of both *B. thuringiensis* and *Bacillus cereus* secrete some toxic proteins during vegetative growth that do not form crystals and that do not have homology with Cry proteins. These vegetative insecticidal proteins, called Vip proteins, are the most recently discovered insecticidal proteins from *B. thuringiensis* and a system of nomenclature has been proposed

(http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/vip.html). Vip1 and Vip2 are proteins of 100 and 52 kDa, respectively, which form a binary toxin with activity against the larvae of some coleopteran species (Warren 1997). Based on their homology with other binary toxins, it has been proposed that Vip1 proteins would bind to specific receptors in the midgut cells and, as a multimer, would form a channel. Vip2 would pass through this pore and, based on its homology to ADP-ribosyltransferases, would modify monomeric actin and inhibit its polymerization (Carlier 1990; Warren *et al.* 1996). Vip3 does not share homology with any other known protein and has been shown to have a broad insecticidal spectrum (Estruch *et al.* 1996; Selvapandiyan *et al.* 2001; Doss *et al.* 2002; Bhalla *et al.* 2005; Mesrati *et al.* 2005). This toxin of 88·5 kDa is processed in the insect midgut to an active form that is toxic to several lepidopteran species after binding to specific receptors in the midgut cells (Yu *et al.* 1997; Lee *et al.* 2003, 2006).

Discovery programmes carried out in several laboratories are looking for novel insecticidal proteins from *B. thuringiensis* with toxic activity against insect pests that are not susceptible to known toxins. Furthermore, as resistance to some *B. thuringiensis* toxins has been observed in several insect species, the identification of novel toxins could be useful for resistance management programmes. Some insect species exposed to a certain toxin have developed cross-resistance to other related toxins. Therefore, novel toxins with different modes of action could be used in *B. thuringiensis* formulations and in transgenic plants to avoid the phenomenon of cross-resistance. Vip proteins seem to bind to different target sites than Cry proteins in midgut epithelium cells of the species tested (Lee *et al.* 2003, 2006); so these proteins are suitable candidates to be used together with Cry proteins in insect pest management. The use of this combination of toxins could be applied to widen the spectrum of toxicity and to minimize the risk of cross-resistance.

Detection of *vip2* and *vip3* genes in strains of *B. thuringiensis* has been performed by using polymerase chain reaction (PCR)-based methods (Selvapandiyan *et al.* 2001; Espinasse *et al.* 2002, 2003; Loguercio *et al.* 2002; Arrieta *et al.* 2004; Franco-Rivera *et al.* 2004; Bhalla *et al.* 2005; Liu *et al.* 2007). Amplification products of PCR reactions confirm the presence of *vip* genes, but this approach only allows the assignment of the gene into a *vip* family (*vip1*, *vip2* or *vip3*) and does not allow the identification of specific genes within a family. In the present study, a screening of *vip1*, *vip2* and *vip3* genes has been first performed by PCR in 507 strains of *B. thuringiensis*. Restriction fragment length polymorphism (RFLP) was then used to identify the different types of *vip* genes within a same family. This strategy has led to the discovery of two novel *vip* genes.

Materials and Methods

Bacillus thuringiensis strains

Strains used in this study belong to a collection of 507 isolates established from four collections of strains isolated in several habitats, mainly from Spain and Bolivia (Hernández-Rodríguez and Ferré 2009).

Screening of *vip1*, *vip2*, and *vip3* genes by PCR

Total DNA was extracted following the method described by Ferrandis *et al.* (1999). Screening of *vip* genes was performed with primers designed from conserved regions within the *vip* gene families. In a final volume of 25 μl, PCR mixtures included 1 μl of the DNA template, 1 U of Taq polymerase, 2·5 μl of 10× reaction buffer, 250 mmol l^{-1} (each) of deoxynucleoside triphosphate and primers for *vip* gene screening (50 pmol l^{-1} for *vip1*-forward, *vip1*-reverse, *vip2*-forward, *vip3*-forward and *vip3*-reverse primers; and 100 pmol l^{-1} for *vip2*-reverse primer; Table 1). Amplifications were carried out in an Eppendorf Mastercycler thermal cycler. Screening of *vip* genes was performed as follows: 5 min denaturation at 95°C followed by 35 cycles of amplification with 1 min denaturation at 95°C, 1 min of annealing at 45–47°C, and 1·5 min of extension at 72°C. An extra extension step of 10 min at 72°C was added after completion of 35 cycles.

Identification of *vip* gene-types by PCR-RFLP

For *vip1* and *vip2* family genes, a second PCR with 'typing' primers for the further identification of *vip* genes was performed (Table 1). PCR reactions were performed as described before using 50 pmol l^{-1} of *vip1*- and *vip2*-typing primers. PCR cycling for these reactions was as follows: 5 min denaturation at 95°C followed by 35 cycles of amplification with 1 min denaturation at 95°C, 1 min of annealing at 45–48°C and 1·5 min of extension at 72°C. An extra extension step of 10 min at 72°C was added after completion of the 35 cycles. The PCR fragment from *vip1* and *vip2* 'typing' amplification, and from *vip3* 'screening' amplification, was digested with *Alu*I and *Sau3A*I separately. The digest mixture included 5 μl of PCR product, 5 U of the enzyme and 2 μl of 10× appropriate reaction buffer in a total volume of 20 μl. The digest mixture was incubated at 37°C for 4 h and the digestion products were analysed by agarose gel electrophoresis.

Results

Detection of *vip1*, *vip2* and *vip3* genes by PCR

DNA templates from 507 *B. thuringiensis* strains were used for PCR amplification with 'screening' primers designed from conserved regions within *vip* gene families. The expected sizes for PCR products were around 585 bp for *vip1*, 845 bp for *vip2* and 1621 bp for *vip3* genes (Table 1). From the 507 strains tested, 54 strains (10·7%) gave an amplification product for *vip1* genes, 46 strains (9·1%) for *vip2* genes and 248 strains (48·9%) for *vip3* genes (Table 2). Pair-wise combinations of genes from different *vip* families was less frequent than the simultaneous occurrence of genes from the three families.

Identification of *vip* genes by PCR-RFLP

To identify the specific genes within a *vip* gene family, a strategy based on PCR-RFLP was used. A second PCR amplification with 'typing' primers for *vip1* and *vip2* genes was performed with strains that showed a PCR product of expected size with the 'screening' primers. Primers used in this round of PCR hybridize within the 5′-region of the *vip* genes, which corresponds to the most variable region among the *vip* genes. Expected sizes for typing PCR products were, approximately, 1044 bp for *vip1*, and 1107 bp for *vip2* (Table 1). The resulting amplicons from the typing PCR reaction for *vip1* and *vip2* genes, and from the screening PCR reaction for *vip3* genes, were then digested with *Alu*I and *Sau*3AI separately. The expected lengths of the PCR products and that of the RFLP digest patterns for the known *vip1*, *vip2* and *vip3* genes is shown in Table 3. Amplicons with a relative size difference of more than 10%, as compared with the expected size, were considered nonspecific amplicons and were not studied further.

Regarding the RFLP patterns of *vip1* genes, there were five strains in our collection that showed the *vip1Aa1* pattern, 18 strains showed the *vip1Ba1/vip1Ba2* pattern and 16 strains exhibited the *vip1Ca1* pattern (Fig. 1a). There were 11 strains that did not render any amplification product after typing PCR for *vip1* genes, although they had shown a positive amplification in the screening PCR. Therefore, the *vip1* gene in these strains could not be identified further. A novel restriction pattern for *vip1* genes was found in four strains from this collection. The restriction fragments obtained with *Alu*I in these strains were: 56, 84, 132, 196 and 579 bp; while *Sau*3AI did not cut in the amplified products. Partial sequencing of the *vip1* gene in these strains showed that the new pattern corresponds to a novel *vip1* gene. Alignment of a sequence of 1357 bp from this gene with known *vip1* genes gave an identity of 84% and 82% with the two more related *vip1* genes in the databases (EF442245 and AJ872073, respectively). When the comparison was made

Table 1 Primers used in this study for screening and typing *vip* genes

Primer	Sequence*	Position† in the reference gene (accession number)	Product size‡ (bp)
Screening			
vip1-sc.fw	5′ TTATTAGATAAACAACAACAAG AATATCAATCTATTMGNTGGATHGG 3′	281-865 (AJ872073)	585
vip1-sc.rev	5′ GATCTATATCTCTAGCTGCTTTTT CATAATCTSARTANGGRTC 3′		
vip2-sc.fw	5′ GATAAAGAAAAAGCAAAAG AATGGGRNAARRA 3′	205-1049 (AJ872074)	845
vip2-sc.rev	5′ CCACACCATCTATATACAGT AATATTTTCTGGDATNGG 3′		
vip3-sc.fw	5′ TGCCACTGGTATCAARGA 3′	78-1698 (AY743436)	1621
vip3-sc.rev	5′ TCCTCCTGTATGATCTACAT ATGCATTYTTRTTRTT 3′		
Typing			
vip1-tp.fw	5′ AAACGGGTGATTTYACNTT 3′	326-1369 (AJ872073)	1044
vip1-tp.rev	5′ GGGAATTAAAATCATCCAT 3′		
vip2-tp.fw	5′ AGAATGGGGGAAAGARAA 3′	222-1329 (AJ872074)	1107
vip2-tp.rev	5′ ACCTCTGTTACTTTATCDAT 3′		

*Universal code for degenerate bases: R=A, G; Y=C, T; M=A, C; S=G, C; H=A, T, C; D=G, A, T; N=A, C, G, T.

†Position at 5′ end of the forward and reverse primers for each polymerase chain reaction primer pair.

‡Using the reference gene as the template.

Table 2 *vip* and *cry* gene content in isolates of *Bacillus thuringiensis*

	cry gene content				
vip gene content	*cry1A* and *cry2*	Only *cry1*	Only *cry2*	Without *cry1A* or *cry2*	Total
Without *vip*	35	38	11	157	241
Only *vip1*	3	–	–	6	9
Only *vip2*	–	–	–	1	1
Only *vip3*	193	1	11	3	208
vip1+vip2	7	1	–	–	8
vip1+vip3	2	–	1	–	3
vip2+vip3	1	–	2	–	3
vip1+vip2+vip3	31	–	3	–	34
Total	272	40	28	167	507

with the partial translated sequence of the novel *vip1* gene, the highest identities were found with the proteins ABR68093 (84%) and CAI43278 (79%), which corresponded to the two *vip1* genes referred to before.

In the identification of *vip2* genes, the *vip2Aa1* pattern was found in 5 strains, the *vip2Ac1* pattern in 16 strains and the *vip2Ba1/vip2Ba2* pattern in 18 strains (Fig. 1b). There were seven strains that did not amplify in the typing PCR reaction in spite of their positive amplification in the screening PCR for *vip2* genes.

For *vip3* gene identification, the products from the screening PCR were directly digested with the restriction enzymes. The pattern corresponding to the *vip3Aa1* gene was found in 171 strains, 57 strains showed the *vip3Ae1* pattern and 19 exhibited strains the *vip3Af1* pattern (Fig. 1c). One strain showed a novel pattern with different *Alu*I and *Sau*3AI restriction fragments as compared with those of the known *vip3* genes. The generated fragments with *Alu*I were: 9, 93, 108, 135, 154, 440 and 682 bp; whereas with *Sau*3AI, the restriction fragments were: 15, 55, 87, 106, 129, 426 and 803 bp. The amplicon of 1645 bp from the screening PCR of this strain, slightly larger than the expected size, was cloned and sequenced. The nucleotide sequence confirmed the presence of a novel *vip3* gene in this strain. The sequenced region of this gene showed an identity of around 80% with known *vip3A* genes, and the translated sequence an identity of 77% with proteins belonging to the Vip3Aa family.

Correlation between *cry* and *vip* gene occurrence

To analyse the possible correlation between the presence of *cry* and *vip* genes, data from a previous study of *cry* gene distribution in this collection of 507 *B. thuringiensis* strains were used (Hernández-Rodríguez and Ferré 2009). The *cry* content in these strains has been related to the occurrence of *vip* genes from this study. In general, strains containing *vip* genes also carried both *cry1A* and *cry2* genes (Table 2). In this way, 79·6% of the strains with *vip1* genes, 84·8% of the strains with *vip2* genes and 91·5% of the strains with *vip3* genes, also contained both *cry1A* and *cry2* genes. Likewise, 65·1% of the strains that did not contain any *vip* gene also lacked *cry1A* and *cry2* genes.

Table 3 Restriction fragment length polymorphism (RFLP) patterns from polymerase chain reaction (PCR) fragments of *vip* genes

vip gene	Patent or accession number	Size of PCR fragment (bp)	Size of RFLP fragment (bp)	
			*Alu*I	*Sau*3AI
vip1Aa1	US 5770696	1037	459-578	33-499-505
vip1Ab1	US 5770696	1037	12-84-120-252-569	123-367-547
vip1Ba1	US 6605701	1043	12-84-114-120-138-159-416	148-348-547
vip1Ba2	AJ872073	1043	12-84-114-120-138-159-416	148-348-547
vip1Bb1	US 6656908	1052	12-84-114-120-138-159-425	31-117-357-547
vip1Ca1	AY245547	1044	27-27-38-42-91-120-212-486	60-123-377-483
vip1Da1	AJ871923	1046	6-25-59-187-331-438	499-547
vip2Aa1	US 5770696	1113	23-59-148-271-612	51-91-109-862
vip2Aa2	US 6656908	1113	23-59-80-147-191-619	50-91-109-171-698
vip2Ab1	US 6066783	1119	23-59-80-147-191-619	50-91-109-171-698
vip2Ac1	AY245547	1113	23-59-148-271-612	109-142-171-691
vip2Ad1	AJ871924	1113	442-671	207-236-251-419
vip2Ba1	US 6605701	1107	85-86-130-154-286-366	452-655
vip2Ba2	AJ872074	1107	85-86-130-154-286-366	452-655
vip3Aa1	L48811	1621	15-77-96-135-198-212-358-530	15-55-87-103-374-987
vip3Aa2	L48812	1621	15-44-77-91-96-198-212-358-530	15-55-87-103-374-987
vip3Ab1	US 6603063	1621	15-77-96-135-198-212-358-530	15-55-87-477-987
vip3Ad2	AJ872071	1621	5-15-39-44-49-72-96-135-151-159-277-286-293	142-144-172-176-987
vip3Ae1	AJ872072	1621	15-39-77-96-135-159-190-380-530	15-142-144-333-379-608
vip3Af1	AJ872070	1621	15-77-96-135-143-198-212-358-387	15-103-142-374-987

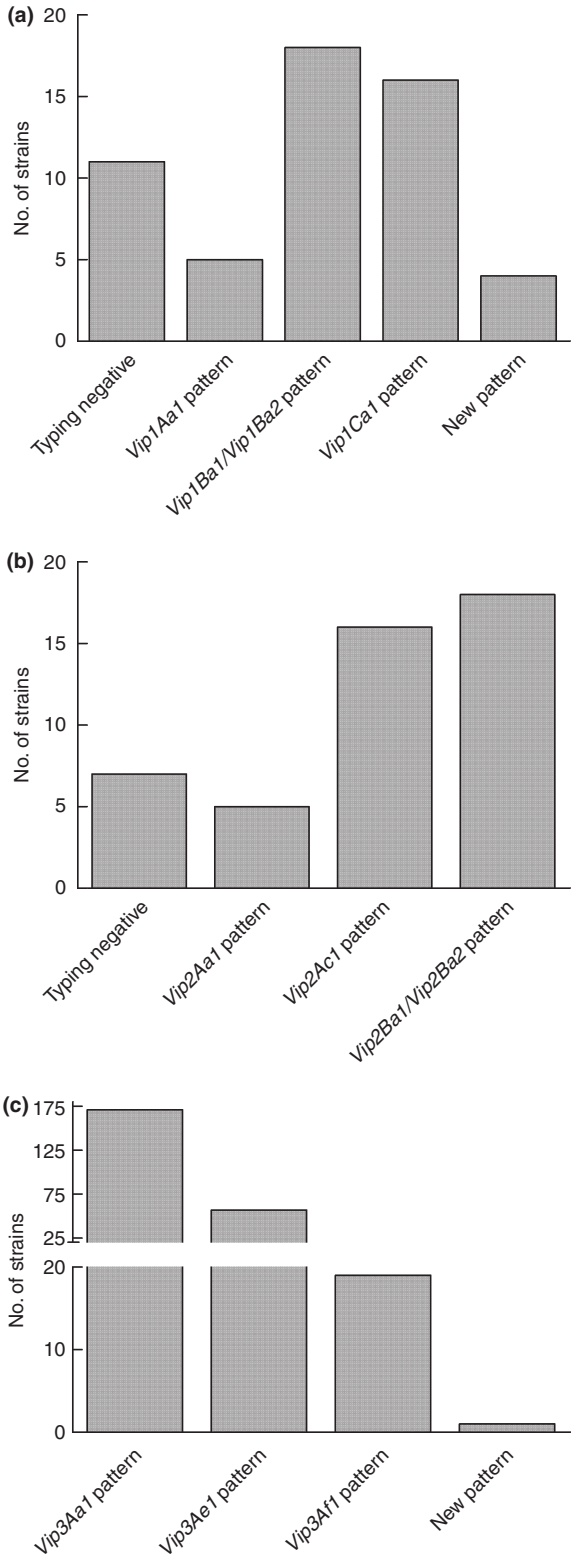

Figure 1 Distribution of *vip* genes in isolates of *Bacillus thuringiensis*: (a) *vip1*, (b) *vip2* and (c) *vip3* genes.

Discussion

Usually, the occurrence of *vip* genes in isolates of *B. thuringiensis* has been determined by PCR alone (Selvapandiyan *et al.* 2001; Espinasse *et al.* 2002, 2003; Loguercio *et al.* 2002; Arrieta *et al.* 2004; Franco-Rivera *et al.* 2004; Bhalla *et al.* 2005). This approach only allows the assignment of the amplified gene into a *vip* gene family, but it does not allow identification beyond this level. In this study, a PCR-RFLP technique has been used to detect *vip* genes and classify them within their types. A similar strategy, in which an amplified DNA region was digested with restriction enzymes, has been already used to distinguish the *cry* genes (Kuo and Chak 1996; Wang *et al.* 2003; Hernández *et al.* 2005; Beard *et al.* 2008b), and more recently *vip3A*-type genes (Liu *et al.* 2007; Beard *et al.* 2008a). In the PCR-RFLP approach used in the present study, the first step consisted in the detection of those strains that contained at least one *vip* gene belonging to the *vip1*, *vip2* and/or *vip3* families. For this purpose, specific primers in the conserved regions for each *vip* family were designed. After this initial screening (screening PCR), strains that showed positive amplification for *vip1* and *vip2* genes were subjected to a second PCR, performed with another pair of primers (typing PCR), which amplified a larger region of the gene. The amplicons resulting from the second amplification of *vip1* and *vip2* genes, and from the single amplification of *vip3* genes, were digested separately with the restriction enzymes, *Alu*I and *Sau*3AI. The observed patterns of restriction fragments were then compared with the expected patterns for the described *vip* genes. The restriction patterns that differed from the expected patterns may very likely indicate novel *vip* genes. On the other hand, identical patterns may correspond to identical, known genes, as well as to (slightly) different *vip* genes that are not distinguishable by this approach.

Strains that showed a positive amplification in the first screening PCR, but did not amplify in the second PCR with typing primers, could also harbour novel *vip* genes with variability at least in the region where typing primers hybridize. In this study, there were 11 strains that did not show amplification in the *vip1* typing PCR, and 7 strains without amplification in the *vip2* typing PCR.

Upon digesting the amplicons, four different patterns were found in the *vip1* and *vip3* gene families, and three patterns in the *vip2* family. The most frequent patterns were those similar to the *vip1Ba1/vip1Ba2* pattern, the *vip2Ba1/vip2Ba2* pattern and the *vip3Aa1* pattern. The *vip1Ba2* and *vip2Ba2* genes have been recently included in databases and *vip* nomenclature, and they are highly similar to *vip1Ba1* and *vip2Ba1* genes, respectively. The strategy

used in this study cannot differentiate between very closely related genes as cited before. In fact, the same pattern is expected for closely related genes, as occurs with most of the *vip3Aa* genes, for example. In this way, an identical pattern to *vip3Aa1* is expected for *vip3Aa7*, *vip3Aa9*, *vip3Aa12* and *vip3Aa15* (accession nos AY0044227, Y17158, AF500478 and AY295778, respectively), among others. There were two patterns that did not match the expected pattern for any known *vip* genes. Partial sequencing of these two genes successfully identified two novel genes belonging to the *vip1* and *vip3* families.

In our screening, the occurrence of *vip3* genes was more common than the occurrence of *vip1* and *vip2* genes. Whereas only around 10% of the strains showed *vip1* or *vip2* gene amplification, almost half of the strains contained genes belonging to the *vip3* family. This frequency of *vip3* genes was similar to that observed by Espinasse *et al.* (2003).

The tendency of *cry1* and *cry2* genes to occur together has been previously reported (Ben Dov *et al.* 1997; Hernández-Rodríguez and Ferré 2009). The analysis of the presence of *vip* and *cry* genes in the *B. thuringiensis* strains from this study showed that there was a tendency of *vip* genes to be present together with both *cry1A* and *cry2* genes. Correlation between the occurrence of *cry1I* and *vip3* genes has also been observed (Espinasse *et al.* 2003). It has long been known that *cry* genes are encoded by large plasmids (González *et al.* 1981), as recently reported for *vip3A*-like genes (Wu *et al.* 2004; Mesrati *et al.* 2005). In this study, the propensity of *vip* genes to occur together with *cry* genes supports the hypothesis that the *vip1, vip2* and *vip3* genes are all encoded by the plasmids that harbour *cry* genes.

The screening of *vip* genes by means of PCR-RFLP resulted in the identification and classification of *vip* genes within the *B. thuringiensis* strain collection of this study. Ten different patterns have been found among the three families of *vip* genes. There were 18 strains that did not amplify with typing primers suggesting that these could contain novel *vip* genes. Furthermore, two new patterns that corresponded to two novel *vip* genes have been found. This is the first time that *vip1* and *vip2* genes are classified within a *vip* family by PCR-RFLP, as up to now sequencing was required for their identification. These results show that the strategy carried out in this study can lead to the classification of known *vip* genes as well as the identification of novel *vip* genes.

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