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Characterization of a novel Cry9Bb δ-endotoxin from *Bacillus thuringiensis*

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ABSTRACT

The Brazilian *Bacillus thuringiensis* serovar *japonensis* strain S725 was selected for its toxicity to the velvetbean caterpillar, *Anticarsia gemmatalis*. This strain produces spherical crystals harbouring a major protein of about 130 kDa which yields fragments of between 50 and 70 kDa upon trypsin activation. The protein showed a high level of identity and immunoafinity to the Cry9 class of δ-endotoxins. The cloned *cry9*-like gene sequence contains a 3492 bp ORF, which encodes a polypeptide of 1163 amino acids, with a predicted molecular mass of 131.4 kDa. The deduced amino acid sequence is unique and shows 73% identity to Cry9Ba, 64% identity to Cry9Ea, 63% identity to Cry9Da, and 59% identity to Cry9Ca proteins. The novel δ-endotoxin was assigned to a new subclass, Cry9Bb, by the Bt Toxin Nomenclature Committee. The Cry9Bb protein was expressed in an acrystalliferous Bt strain, and exhibited activity against the tobacco hornworm, *Manduca sexta*, and the velvetbean caterpillar, *A. gemmatalis*. The biological effect of an amino acid residue change, A84P, was investigated. The LC_{50} for the Cry9Bb crystals against *M. sexta* neonate larvae was 6.84 μg/cm^2, while the LC_{50} for the mutant's Cry9Bb crystals was 0.78 μg/cm^2. PCR screening revealed that in addition to *cry9Bb*, Bt strain S725 also contains *cry1I* and *vip3* genes. Transcription analysis, using RT-PCR, showed that the *cry1I* gene was transcribed at T_2 and T_5 stages of sporulation.

1. Introduction

Bacillus thuringiensis (Bt) is an aerobic, Gram-positive bacterium that synthesizes parasporal inclusions or crystals during sporulation, which are composed of one or more Cry and/or Cyt toxins (δ-endotoxins) (Höfte and Whiteley, 1989; Schnepf et al., 1998). These proteins are toxic to a large variety of crop and forestry insect pests and disease vectors (Schnepf et al., 1998; de Maagd et al., 2001; de Maagd et al., 2003). Strains with toxic activity against other invertebrates like nematodes (Edwards et al., 1990; Feitelson et al., 1992; Wei et al., 2003; Kotze et al., 2005) and mites (Payne et al., 1994) have been identified. Cry toxin binding to insect midgut epithelial receptors is an important determinant of specificity (Pigott and Ellar, 2007).

Because of the high specificity and environmental safety of Cry proteins (Krieg and Langenbruch, 1981; Höfte and Whiteley, 1989; Schnepf et al., 1998), Bt spore-crystal mixtures have been successfully used as bioinsecticides for more than 40 years against Lepidoptera, Diptera and Coleoptera insects (Feitelson et al., 1992; Schnepf et al., 1998). The δ-endotoxins are a valuable alternative to synthetic chemical pesticides in agriculture, forest management, and mosquito control (Schnepf et al., 1998).

To date, many δ-endotoxin genes have been cloned and sequenced and the proteins are classified into 55 Cry and 2 Cyt classes, according to their amino acid sequence identity (http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/). Several *cry* genes have been introduced into plants, conferring insect resistance (Schnepf et al., 1998; Romeis et al., 2006).

In addition to the production of δ-endotoxins, many Bt strains also produce other virulence factors throughout their growth cycle that contribute to their insecticidal activity (de Maagd et al., 2001). The Vip toxins (Vegetative insecticidal proteins), for instance, are produced during the vegetative stage of growth, and are secreted into the culture supernatant by many strains of Bt (Estruch et al., 1996, 1997). These proteins are highly toxic to certain coleopteran species (Vip1 and Vip2) or to a range of lepidopteran insects (Vip3) (Espinasse et al., 2003).

Intensive screening programs are leading to a broader activity spectrum of toxins as the result of isolation and characterization of new strains with different combinations of crystal proteins, as well as the discovery of new toxins.

One Brazilian Bt strain, named S725, belonging to the Collection of entomopathogenic *Bacillus* of Embrapa Genetic Resources and Biotechnology, in Brazil, was previously selected in screening tests for its toxicity to the velvetbean caterpillar, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). This strain was isolated from a Brazilian soil sample and was serotyped as Bt subsp. *japonensis*. It was found to contain a novel Cry toxin that might have entomopathogenic

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properties. The aim of the present work was to investigate the biochemical characteristics and activity of the novel protein, and the *cry* genes content from the Bt strain S725.

2. Material and methods

2.1. Bacterial strains, culture conditions and plasmids

The *B. thuringiensis* strain S725 was isolated from a soil sample collected at "Águas Emendadas" Ecological Reserve, in the Federal District of Brazil, and was stored in the Collection of entomopathogenic *Bacillus* of Embrapa Genetic Resources and Biotechnology. The standard *B. thuringiensis* subsp. *kurstaki* (Btk) HD-1, Btk HD-73, Bt subsp. *tenebrionis* (Btt), Bt subsp *israelensis* (Bti) IPS78, Bti IPS78/11 (a plasmid-cured mutant of Bti lacking δ-endotoxin genes) (Ward and Ellar, 1983), Bt JC292, Bt 4412, and other Bt strains used as controls for PCR, in particular the HD series, were obtained from Prof. Ellar's laboratory collection and are referred to in the text. Bt strains were grown at 30 °C, in LB (Luria–Bertani) broth (Sambrook and Russell, 2001), CCY—Bt sporulation media (Stewart et al., 1981) or on LB-agar, NYSM-agar (Yousten, 1984) or CCY-agar. Antibiotics were added to their working concentrations where required. *Escherichia coli* strain XL10-Gold (Stratagene) was used as general cloning host. Strain XL1-Blue (Stratagene) was used as cloning host for mutant DNA. *E. coli* strains were grown at 37 °C in LB broth or on LB-agar supplemented with the appropriate antibiotic. Plasmid pGEM-3Zf(+) (Promega) was used for cloning and sequencing, and the Bt-*E. coli* shuttle vector pSVP27A (Crickmore and Ellar, 1992) was used for cloning and expression in Bt. Derivatives from pGEM-3Zf(+) and pSVP27A were constructed, and are detailed in the Cloning and sequencing section below.

2.2. Purification of crystals

Bt crystals were purified on discontinuous sucrose gradients according to Thomas and Ellar (1983). Protein concentration was determined by the Lowry assay (Lowry et al., 1951) with bovine serum albumin (BSA) as a standard.

2.3. Solubilization and activation of δ-endotoxin inclusions

Crystals of Cry or Cyt toxins were pelleted at 14,000*g* (Anachem Hyspin 16 K centrifuge) for 15 min at 4 °C. The pellet was resuspended in solubilization buffer [50 mM Na_2CO3, 10 mM DTT (dithiothreitol), pH range 8–11] at 1 to 5 mg/ml, and incubated at 37 °C for 1 h. The pH of the solubilization buffer and the toxin concentration depended on the toxin and the assay. Insoluble material was removed by centrifugation at 14,000*g* for 10–15 min, at RT (room temperature). Solubilized protein concentration was determined by the Bio-Rad protein assay (Bio-Rad) or the DC protein assay (Bio-Rad) with BSA as a standard. Solubilized toxins were activated by TPCK treated trypsin (Sigma) at different trypsin: toxin ratios, ranging from 1:1 to 1:100 (w/w), depending on the toxin and the experimental procedure. The sample was incubated at 37 °C for 1 h. Cyt2A toxin was activated with 0.1% (w/w) proteinase K (Sigma) at 37 °C for 30 min. The solubilized toxin from Bt strain S725 was also activated with 2.5% (v/v) *Anthonomus grandis* or *Pieris brassicae* gut extract at 37 °C for 1 h. Following incubation, insoluble material was removed and the protein concentration was determined by the Bio-Rad protein assay.

2.4. Immunoblotting analysis

Proteins separated by electrophoresis on SDS–polyacrylamide gels (SDS–12% PAGE), as described by Laemmli and Favre (1973), were electrophoretically transferred to nitrocellulose membranes (Pall corporation) by the method of Towbin et al. (1979). Cry toxins were detected with polyclonal anti-Cry1Ac, Cry1B, Cry2A and Cry9C antibodies, from laboratory stocks, as primary antibodies, followed by goat anti-rabbit IgG peroxidase-conjugated (Sigma) as the secondary antibody. Primary and secondary antibodies were diluted 1:1000 in 3% (w/v) skimmed dry milk in TTS [20 mM Tris–HCl pH 7.5, 500 mM NaCl, 0.05% (v/v) Tween 20]. Membranes were incubated in blocking buffer [3% (w/v) skimmed dry milk in TBS buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl)], and then in the antibody dilution, both for 1 h at RT. Detection was performed as described by Vilchez et al. (2004), or using DAB (Sigma Fast 3,3 Diaminobenzidine) according to the manufacturer's instructions.

2.5. N-terminal protein sequencing

Activated protein preparations were resolved by SDS-7.5% or SDS-10% PAGE. Protein fragments were then transferred to a PVDF membrane (Applied Biosystems). After washings, the blotted membrane was stained with 0.1% (w/v) Coomassie blue R-250 in 50% (v/v) methanol, 1% (v/v) acetic acid. It was destained in 50% methanol and then washed twice in dH_2O. The membrane was air dried, the selected stained protein bands were excised, and the N-terminal sequence determined at the Protein and Nucleic Acid Chemistry Facility (Department of Biochemistry, University of Cambridge).

2.6. Microscopic observations

Spore-crystal suspensions and purified crystals of the Bt strains were observed under a Nikon Optiphot phase-contrast microscope, using the 100× objective. Crystal suspensions were air-dried on aluminum stubs. Samples were sputter-coated with 10 nm Au/Pd using a Polaron E5100 Sputter Coater, then examined and photographed using a Philips XL30 FEG scanning electron microscope at 5 kV beam current.

2.7. Gut extract preparation

Gut extract was prepared from 5th instar *A. grandis* and *P. brassicae* larvae as described by Vilchez et al. (2004).

2.8. BBMV preparation

Brush border membrane vesicles (BBMV) were prepared from whole 4^{th}-5^{th} instar larvae of *A. grandis* by the Mg^{2+} precipitation method (Wolfersberger et al., 1987). BBMV from *P. brassicae* were kindly donated by Johanna Rees (Department of Biochemistry, University of Cambridge). BBMV protein concentration was measured by the BCA assay (Pierce) using BSA as a standard.

2.9. Toxin biotinylation and binding assays

Trypsin activated Cry9Bb toxin was biotinylated using the EZ-Link™ Sulfo-NHS-Biotinylation kit (Pierce), according to the manufacturer's instructions. For ligand blot assays, 17 μg of *A. grandis* BBMV and 19 μg of *P. brassicae* BBMV were separated in SDS–12% PAGE, and the proteins transferred to a nitrocellulose membrane. The membrane was blocked in 3% BSA in TBST (TBS plus 0.1% (v/v) Tween 20) for 30 min at RT. It was then incubated in biotinylated toxin at 4.3 μg/ml in blocking buffer. After two washes in TBST, the membrane was incubated in 2 μg/ml Streptavidin–HRP, 0.1% BSA in TBST. The washing step was repeated, and followed by detection of BBMV-bound toxin using DAB (Sigma).

2.10. Bioassays

2.10.1. Manduca sexta bioassays

A volume of 500 μl of artificial diet (Bell and Joachim, 1976) was dispensed in each well of a 48-well microtiter plate (Costar) and allowed to solidify. Crystal suspensions were serially diluted in dH_2O and 20 μl of each concentration were applied on the diet surface in each of 24 wells. Crystal suspensions of strains S725 and SVBt725 were assayed from 20 $\mu g/cm^2$ to 32 ng/cm^2. As positive controls, Cry1Ac crystal suspensions were used at concentrations from 100 to 0.16 ng/cm^2. For the negative control, sterile dH_2O replaced the toxin suspension. After drying, one *M. sexta* neonate larva was placed on each well. The plates were wrapped in cling film, and incubated at 26 °C, with a photoperiod of 16: 8 (Light:Dark) h, for 5 days. Mortality was assessed and the LC_{50} calculated by Probit analysis (Finney, 1971).

2.10.2. Mamestra brassicae and P. brassicae bioassays

Bioassays with *M. brassicae* and *P. brassicae* were performed using their artificial diets (Lightwood, 1999) and the procedure described for *M. sexta*, except that for *M. brassicae*, Cry1Ca crystal suspension was used as positive control.

2.10.3. Musca domestica bioassays

Bioassays with adult *M. domestica* were performed as previously described (Ruiu et al., 2006), with three replications, and the crystal suspension of strain SVBt725 at 50 μg/ml final concentration. Mortality was assessed daily for 5 days.

2.10.4. Anticarsia gemmatalis and Spodoptera frugiperda bioassays

Bioassays with *A. gemmatalis* and *S. frugiperda* larvae were performed as described by Monnerat et al. (2007), using crystal suspension of strains S725 and SVBt725 at final concentrations of 21.4 and 2.14 $\mu g/cm^2$ for *A. gemmatalis* and 17.5 and 1.75 $\mu g/cm^2$ for *S. frugiperda*.

2.10.5. Other bioassays

Bioassays with the Lepidoptera *Helicoverpa zea*, *Ostrinia nubilalis*, *Plutella xylostella* and *Agrotis ipsilon*, and the Coleoptera *Diabrotica virgifera*, *D. undecimpunctata* and *Leptinotarsa decemlineata* were kindly performed by staff at Dr A. Abad's laboratory, at Pioneer Hi-Bred, Johnston, Iowa, USA. Bioassays with the Coleoptera *A. grandis* were kindly performed by staff at Dr V. Sanchis' laboratory, at the Institute Pasteur, Paris, France. Bioassays with the Lepidoptera *Trichoplusia ni* and the Diptera *Aedes aegypti* were kindly performed by staff at Dr J. Ibarra's laboratory, at CINVESTAV-IPN, Irapuato, Mexico.

2.11. DNA preparation

Bt genomic DNA was isolated following the protocol of Pospiesch and Neumann (1995) except that the strain was grown in LB broth. Bt plasmid DNA was extracted by the alkaline lysis method (Birnboin and Doly, 1979), except that 4 mg/ml lysozyme was used to lyse the cells for 45 min, and DNA was purified on a CsCl gradient, as described (Sambrook and Russell, 2001). Plasmid DNA was extracted from *E. coli* using the QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions. DNA from Bt strains used as templates in PCR for genes screening was generally extracted by a quick extraction method described by Bravo et al. (1998). DNA from *E. coli* used as template in colony PCR for clones screening was extracted by a quick extraction method. In brief, *E. coli* clones were grown overnight at 37 °C on LB-agar containing the selective antibiotic. A colony was transferred with a tooth pick to 10 μl of dH_2O in a 500 μl PCR tube (Starlab), and boiled at 95 °C for 10 min.

2.12. PCR (Polymerase chain reaction)

Bt S725 plasmid DNA was amplified by PCR for DNA sequencing or cloning in a reaction mixture containing 1× *Taq* or *Pfu* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 μM of each primer, 20–100 ng template DNA, 1.5–2.5 U *Taq* DNA Polymerase (Invitrogen) or *Pfu* DNA Polymerase (Promega) in a final volume of 50 μl in a 500 μl PCR tube (Starlab). In PCR to detect *cry* genes, 15 μl of lysate supernatant was used as DNA template in a final volume of 50 μl. For *E. coli* colony PCR, 1 μl of the lysate was added to 25 μl of PCR reaction. In general, the PCR conditions were: initial denaturation at 94 or 95 °C for 2 min; 25 or 30 cycles at 94 or 95 °C for 1 min; annealing temperature (Table 1) for 30 s–1 min; 72 °C for 1 min (for PCR products bigger than 3 kb: 1 min per kb of expected product for *Pfu*, or 30 s per kb for *Taq*); and a final extension at 72 °C for 5 min. PCR for site-directed mutagenesis was performed as recommended by the Quikchange Site-directed Mutagenesis kit (Stratagene). PCR reactions were carried out in a Techne Progene thermal cycler. Primer sequences, genes identified, products sizes and annealing temperatures are given in Table 1.

2.13. Cloning and sequencing

A PCR amplified DNA fragment containing a *cry9*-like gene from Bt S725 was cloned into the BamHI–SphI sites of pGEM-3Zf(+) vector (Promega) to generate the construct pGEM725. Plasmid DNA from pGEM725 was subcloned into the BamHI–SphI sites of the Bt-*E. coli* shuttle vector pSVP27A (Crickmore et al., 1990; Crickmore and Ellar, 1992), containing a fragment from the upstream region of the *cyt1Aa* gene including the *cyt1Aa* promoter, to form the construct pSVP725. The *cry9*-like gene in pGEM725 was mutated (Ala84 to Pro84) and the mutant DNA was subcloned into BamHI–SphI sites of the shuttle vector pSVP27A generating pSVP725-A84P. Transformation of *E. coli* strains XL10-Gold (Stratagene) and XL1-Blue (Stratagene) with DNA containing the wild-type and the mutant gene, respectively, was performed using standard techniques (Sambrook and Russell, 2001). Transformants of *E. coli* XL10-Gold were selected on LB plates containing 250 μg/ml carbenicillin plus X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) for cloning in pGEM-3Zf(+), or 250 μg/ml carbenicillin for cloning in pSVP27A. Transformants of XL1-Blue were selected on 50 μg/ml carbenicillin. Plasmid DNA of selected clones was analysed by digestion with restriction enzymes. DNA sequencing was carried out on a model 3700 Automated DNA Analyser (Applied Biosystems, Inc.), at the DNA Sequencing Facility (Department of Biochemistry, University of Cambridge). The sequences were analysed with the GCG package (Accelrys), BLAST (Altschul et al., 1997) and Clustal W (Thompson et al., 1994).

2.14. Electrotransformation of Bt

Transformation of Bti IPS78/11 (Ward and Ellar, 1983) cells was carried out by electroporation as described by Bone and Ellar (1989), using a gene pulser (Bio-Rad). The transformants were selected onto LB-agar containing 6 μg/ml chloramphenicol, at 30 °C.

2.15. Site-directed mutagenesis

Point mutation in the DNA of the *cry9Bb* gene (CCG instead of GCG) to replace Ala84 by Pro84 in the protein sequence was carried out according to the Quikchange Site-directed Mutagenesis protocol (Stratagene). The primers used in the PCR were 7A84P:

Table 1
Characteristics of general and specific primers used to screen for *cry* and *vip3* genes in the DNA of *B. thuringiensis* strain S725

Primer pair	Gene identified	Product size (bp)	Ta* (°C)	Sequence (5′ → 3′)	Original primer name and reference
cry1(d)	*cry1*	543	52.0	CTGGATTTACAGGTGGGGATAT	gral-cry1(d)/(r)
cry1(r)		594		TGAGTCGCTTCGCATATTTGAT	Bravo et al. (1998)
CJ1	*cry1Aa*	246	47.0	TTATACTTGGTTCAGGCCC	CJ1/CJ2
CJ2	*cry1Ad*			TTGGAGCTCTCAAGGTGTAA	Cerón et al. (1994)
CJ3	*cry1Ad*	171	47.0	CAGCCGATTTACCTTCTA	CJ3/CJ2
CJ2				TTGGAGCTCTCAAGGTGTAA	Cerón et al. (1994)
CJ4	*cry1Ab*	216	47.0	AACAACTATCTGTTCTTGAC	CJ4/CJ5
CJ5	*cry1Ac*			CTCTTATTATACTTACACTAC	Cerón et al. (1994)
CJ6	*cry1Ac*	180	47.0	GTTAGATTAAATAGTAGTGG	CJ6/CJ7
CJ7				TGTAGCTGGTACTGTATTG	Cerón et al. (1994)
1BCJ8	*cry1B*	367	47.0	CTTCATCACGATGGAGTAA	CJ8/CJ9
1BCJ9				CATAATTTGGTCGTTCTGTT	Cerón et al. (1994)
CJ10	*cry1C*	130	47.0	AAAGATCTGGAACACCTTT	CJ10/CJ11
CJ11				CAAACTCTAAATCCTTTCAC	Cerón et al. (1994)
CJ12	*cry1D*	290	47.0	CTGCAGCAAGCTATCCAA	CJ12/CJ13
CJ13				ATTTGAATTGTCAAGGCCTG	Cerón et al. (1994)
CJ14	*cry1E*	147	52.0	GGAACCAAGACGAACTATTGC	CJ14/CJ15
CJ15				GGTTGAATGAACCCTACTCCC	Cerón et al. (1995)
CJ16	*cry1F*	177	52.0	TGAGGATTCTCCAGTTTCTGC	CJ16/CJ17
CJ17				CGGTTACCAGCCGTATTTCG	Cerón et al. (1995)
CJ18	*cry1G*	235	52.0	ATATGGAGTGAATAGGGCG	CJ18/CJ19
CJ19				TGAACGGCGATTACATGC	Cerón et al. (1995)
1I(d)	*cry1I*	1137	47.0	ATGAAACTAAAGAATCCAGA	V (+)/V(−)
1I(r)				AGGATCCTTGTGTTGAGATA	Masson et al. (1998)
cry2A(d)	*cry2A*	308	45.0	CCTCCCTCCTTTATCAAC	Cry2A/Cry2B
cry2A(r)				GCACAGATACCAAATAGGC	De-Souza et al. (1999)
cry3(d)	*cry3*	652	48.0	TTAACCGTTTTCGCAGAGA	CJIII20/CJIII21
cry3(r)		733		TCCGCACTTCTATGTGTCCAAG	Cerón et al. (1995)
cry7(d)	*cry7Aa*	535	48.0	CAATCCCAGTGTTTACTTGGAC	CJIIIcte22/ CJIIIC25
cry7Aa				CCTATTCTTTCATTTTGACC	Cerón et al. (1995)
cry7(d)	*cry7Ab*	211	48.0	CAATCCCAGTGTTTACTTGGAC	CJIIIcte22/ CJIIICg26
cry7Ab				AGTGGAGAGTTTACGGTAGCC	Cerón et al. (1995)
cry8(d)	*cry8*	373	49.0	ATGAGTCCAAATAATCTAAATG	gral-cry8(d)/ (r)
cry8(r)		376		TTTGATTAATGAGTTCTTCCACTCG	Bravo et al. (1998)
cry9gen (d)	*cry9*	504	51.0	CACCATCATAAAGTCCATCTTGTG	cry9gen(d)/(r)
cry9gen (r)				GACAAGATTTTGAGCGTCCATAAT	This work
spe-cry9A (d)	*cry9A*	571	50.0	GTTGATACCCGAGGCACA	spe-cry9A(d)/spe-cry9(r)
spe-cry9(r)				CCGCTTCCAATAACATCTTTT	Bravo et al. (1998)
spe-cry9B (d)	*cry9B*	402	50.0	TCATTGGTATAAGAGTTGGTGATAGAC	spe-cry9B(d)/spe-cry9(r)
spe-cry9(r)				CCGCTTCCAATAACATCTTTT	Bravo et al. (1998)
spe-cry9C (d)	*cry9C*	306	50.0	CTGGTCCGTTCAATCC	spe-cry9C(d)/spe-cry9(r)
spe-cry9(r)				CCGCTTCCAATAACATCTTTT	Bravo et al. (1998)
cry9E(d)	*cry9Ea*	452	51.0	ACAGCTCCAACAACTAATAGC	cry9E(d)/(r)
cry9E(r)				CTATCCGCAGTAATTGTGTTC	This work
cry9Hinks (d)	*cry9Hinks*	351	51.0	TGGTGGATAAGTTGGTACG	cry9Hinks(d)/(r)
cry9Hinks (r)				CCTCTCACCTGAGCTTACA	This work
vip3-F	*vip3*	678	53.0	ACATCCTCCCTACACTTTCTAATAC	vip3-fw/vip3-rev
vip3-R				TCTTCTATGGACCCGTTCTCTAC	Espinasse et al. (2003)

* T_a = annealing temperature.

5′ GGTACTTTAGGTGGACCGGTTGGTGGC 3′ (forward) and 7A84P(r): 5′ GCCACCAACCGGTCCACCTAAAGTACC 3′ (reverse). The cycling conditions were initial denaturation at 95 °C for 1 min, 25 cycles at 95 °C for 30 s, 55 °C for 1 min, 68 °C for 9 min, and a final extension at 68 °C for 5 min. Plasmid DNA of the selected clone was purified and then sequenced using the T7 universal primer to confirm that the mutation was in place.

2.16. Bacterial RNA isolation

Total RNA of Bt strains was isolated using the hot acid phenol method as follows. A 50 ml sample of the culture in LB was collected at the mid exponential phase, T_2 and T_5 stages of sporulation (determined from their growth curves), and added to an equal volume of hot acid phenol: chloroform (5:1) pH 4.5 (Ambion), in a water bath at 95–100 °C. Tubes were mixed intermittently for 10 min, then cooled on ice for 10 min. After centrifugation at 8000*g* (Beckman Coulter centrifuge, JA 14 rotor) for 20 min, at 4 °C, the aqueous layer was extracted with 1 volume of phenol: chloroform: isoamyl alcohol (25:24:1) pH 6.6 (Ambion). The centrifugation step was repeated. The aqueous layer was transferred to a fresh tube and precipitated by 1/10 volume of 3 M sodium acetate, pH 5.5, and two volumes of 100% ethanol. After overnight incubation at −20 °C, the samples were centrifuged at 9000*g* (Beckman Coulter centrifuge, JA 14 rotor) for 30 min, at 4 °C. The pellet was washed in 70% ethanol in DEPC (Diethyl pyrocarbonate)-treated dH_2O, air dried and dissolved in DEPC-treated dH_2O. RNA samples were then treated with RQ1-RNase free Dnase (Promega), and purified using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions.

2.17. Reverse transcriptase-PCR (RT-PCR) for cry1I

For *cry1I* cDNA synthesis, 1 μg of total RNA was mixed with 10 μM of primer 1I(r) (specific for *cry1I*) in DEPC-treated dH_2O, denatured at 70 °C for 10 min and rapidly cooled on ice. Then, M-MLV-RT buffer, 10 mM dNTPs, M-MLV-RT RNase H minus (Promega) and DEPC-treated dH_2O were added, and the reaction incu-

bated at 42 °C for 1 h. For the PCR, 2 μl of cDNA was used as template, with the primers 1I(d)/1I(r) (Table 1), in a 25 μl reaction.

3. Results

3.1. Bt strain S725 crystal protein analysis

The sucrose gradient purified crystals of the *B. thuringiensis* subsp. *japonensis* strain S725 were observed under phase-contrast light microscopy at 1000× magnification and looked spherical. Scanning electron microscopy (SEM) of the crystals at 10,000× magnification confirmed that the strain S725 produced only spherical crystals (not shown). The crystals were analysed by SDS–12% PAGE and showed a major protein band around 130 kDa, which was solubilized in 50 mM Na_2CO3, 10 mM DTT, at pH 8.5 or pH 9.0 (Fig. 1). Activation by trypsin at 1:1, 1:2 or 1:10 (w/w) trypsin: toxin ratio, following solubilization, produced a range of fragments between 50 and 70 kDa, as analysed by SDS–PAGE (Fig. 1).

3.2. Immunodetection of Cry toxins in Bt strain S725

Firstly, immunodetection was performed to detect Cry1Ac, Cry1B and Cry2A δ-endotoxins in the crystal preparations of Bt strain S725. Crystals of the reference strains Btk HD-1, which produces Cry1Aa, Cry1Ab, Cry1Ac and Cry2A proteins, Btk HD-73, which produces only Cry1Ac, and Bt subsp. *thuringiensis* 4412, which produces Cry1B, were solubilized at pH 10.5 and activated at 1:1 (w/w) trypsin: toxin ratio and used as controls. Crystals of the strain S725 were solubilized at pH 9 and activated at 1:1 (w/w) trypsin: toxin ratio. Proteins were separated by SDS-12% PAGE and immunoblotted. No reaction was observed for S725 with any of these antibodies (data not shown), indicating that the strain does not produce these δ-endotoxins. For detection of Cry9, crystals, solubilized (pH 9.5) and trypsin activated toxins from the Bt strain JC292, which produces Cry9 and Cry2 proteins (Hinks, 1998), were used as positive controls. Strong positive reactions were observed for JC292 and the strain S725 (data not shown).

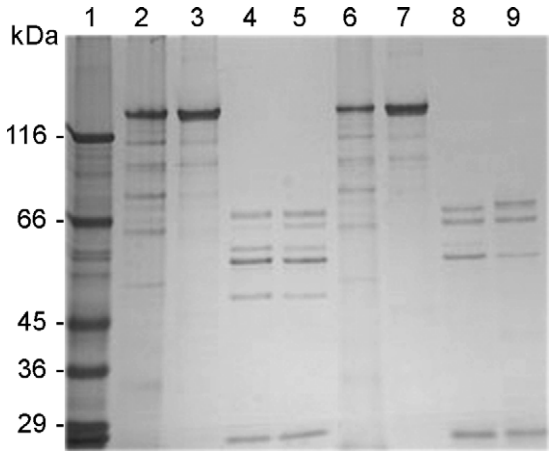

Fig. 1. SDS–PAGE profile of products of solubilization and activation of crystals from *B. thuringiensis* strain S725. Crystals were solubilized at pH 9 (lanes 3–5) or pH 8.5 (lanes 7–9), and activated at 1:1 (lanes 4 and 8) or 1:2 (lanes 5 and 9) (w/w) trypsin: toxin ratio. A total of 7.5 μg of purified crystals or solubilized toxin, or 3.5 μg of activated toxin were loaded per well, resolved in SDS–12% PAGE and coomassie blue stained. Lane 1: molecular mass markers (kDa); lane 2: S725 purified crystals; lane 3: S725 solubilized toxin; lane 4: S725 activated toxin; lane 5: S725 activated toxin; lane 6: S725 purified crystals; lane 7: S725 solubilized toxin; lane 8: S725 activated toxin; lane 9: S725 activated toxin.

3.3. N-terminal sequencing of Cry proteins in the strain S725

Solubilized and trypsin activated proteins of S725 were separated by SDS–10% PAGE and transferred to a PVDF membrane. A 50 kDa and a 56 kDa activated fragments and the two solubilized fragments around 130 kDa were N-terminal sequenced and the sequences analysed by BLAST search. High levels of homology with Cry9Ca, Cry9Da, Cry9Ea and Cry9Eb were found, ranging from 70% to 100% identity.

3.4. Detection of the cry9 gene in Bt strain S725

Multiplex PCR to detect the *cry9*-like gene in the DNA of the strain S725 was performed using general primers for *cry9* genes [cry9-gen(d)/(r)] and specific primers for *cry9Ea* [cry9E(d)/(r)] and *cry9-Hinks* [cry9Hinks(d)/(r)], designed using Vector NTI. These primers sequences and features are described in Table 1. The expected product for *cry9Ea* was obtained for the strain S725. PCR with the set of primers spe-cry9A, spe-cry9B and spe-cry9C, specific for *cry9A*, *cry9B* and *cry9C*, respectively (Bravo et al., 1998) was also performed, but no product was amplified. When the general primers cry9-gen(d)/(r) were used in a single primer pair reaction, the expected product was also amplified for S725 (not shown).

3.5. Detection of other cry and vip3 genes in Bt strain S725

In addition to *cry9*, genomic DNA of the strain S725 was screened by PCR for the genes *cry1, cry1Aa, cry1Ab, cry1Ac, cry1Ad, cry1B, cry1C, cry1D, cry1E, cry1F, cry1G, cry1I, cry2A, cry3, cry7Aa, cry7Ab, cry8* and *vip3* using the primers described in Table 1. Standard Bt strains were used as positive and negative controls. Amplification products were obtained with the general primers for *cry1 genes*, and primers for *cry1I* and *vip3* genes (not shown).

3.6. Cloning and sequencing of the cry9-like gene

The full-length of the *cry9-like* gene of S725 was amplified by PCR with primers End9 (d) (CACAGGAGGATCCTAAGATTG) and End9 (r) (GAATTCTTTCTTTATAAGG), designed based on the *cry9Ea* sequence and positioned −123 bp upstream and +3656 bp downstream of the start codon, respectively. The PCR product was purified and then sequenced by primer walking. A 3791 bp sequence containing the *cry9-725* gene was obtained and analysed. A 3492 bp ORF was identified and a restriction map obtained. In order to clone the *cry9-725* gene, the primers Up725 (5′ ATGGATCCAAGAGTGTGTCAGG 3′) and Sph725 (5′ CCCCCGCATGCTCCTTCTACC 3′) were designed to introduce a BamHI site 77 bp upstream of the start codon and a SphI site 98 bp downstream of the stop codon, respectively. PCR with these primers was performed using CsCl gradient purified plasmid DNA from the strain S725 and *Pfu* DNA polymerase (Promega). The 3678 bp PCR product was purified, double digested with BamHI and SphI and cloned into pGEM-3Zf(+), generating the plasmid pGEM725. The *cry9-725* gene was then subcloned into the shuttle vector pSVP27A, creating the plasmid pSVP725, and sequenced. The 3951 bp sequence contained a 3492 bp ORF, which encodes a polypeptide of 1163 amino acids, with a predicted molecular mass of 131.4 kDa. Nucleotide sequence was deposited into GenBank with the accession number AY758316 and was classified as *cry9Bb* by the Bt Toxin Nomenclature Committee (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt).

3.7. Expression of the cry9-725 gene

The acrystalliferous Bti IPS78/11 transformed with the plasmid pSVP725 expressed the *cry9Bb* gene. Crystals of the selected re-

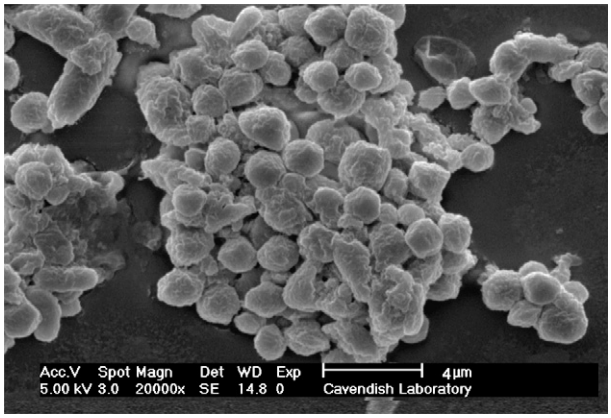

Fig. 2. Scanning electron micrograph of crystals from *B. thuringiensis* recombinant strain SVBt725.

combinant Bt clone SVBt725 were purified by discontinuous sucrose gradient. The strain produced spherical crystals, similar to the ones produced by strain S725, as viewed by phase-contrast microscopy and by SEM (Fig. 2).

3.8. Protein sequence analyses

BLASTx homology search of the deduced Cry9 protein sequence showed that the highest identities in the databases were: 73% identity with CryX (Shevelev et al., 1993), currently classified as Cry9Ba, 64% identity with Cry9Ea (Midoh and Oyama, 1998), 63% identity with Cry9Da (Asano, 1996), and 59% identity with Cry9Ca (Lambert et al., 1996). The protein sequence and its putative structural domains sequences were aligned with Cry9 holotype proteins by Clustal W method. The five conserved blocks of sequences identified by Höfte and Whiteley in several Cry toxins (Höfte and Whiteley, 1989) were localised (data not shown). Analysis of sequence similarity for each of the three structural domains showed that the Cry9-like protein from S725 (Cry9-725) is most closely related to Cry9Ba in domains I and II (72.4% and 61% similarity respectively), while closer to Cry9Da in domain III (47.3%). A phylogenetic tree was obtained through the Mega 2.1 program, using the minimum evolution method (Fig. 3). It can be observed that the Cry9-725 is more closely related to the Cry9Ba protein.

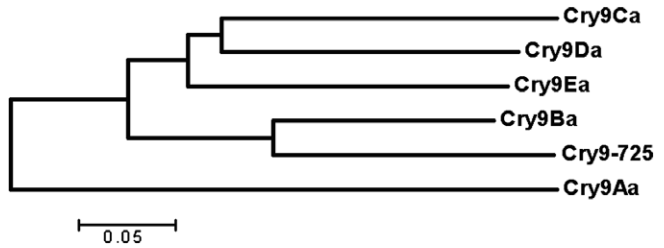

Fig. 3. Cry9 proteins phylogenetic tree. Phylogenetic distances performed by Mega 2.1 program, using the minimum evolution method. The Cry9-like protein from *B. thuringiensis* strain S725 is represented as Cry9-725.

3.9. Characterization of the novel Cry9 protein

3.9.1. Solubilization and activation of the novel Cry9 protein

SDS-PAGE analysis of the inclusions of the recombinant SVBt725 showed that the expressed Cry9Bb has molecular mass about 130 kDa (Fig. 4). Solubilization of crystals in 50 mM Na_2CO_3, 10 mM DTT, at varying pH values (pH 8.0; 8.5; 9.0; 9.5; 10; 10.5 and 11), resulted in a main band around 130-kDa at all pH range, although it was stronger between pHs 8.0 and 9.0, and at pH 11. Fig. 4 (lane 4) shows the solubilized toxin at pH 8.5. Activation of the toxin was performed either by trypsin at different trypsin: toxin ratios [1:1, 1:2, 1:5, 1:10, 1:20, 1:30, 1:40 and 1:50 (w/w)] or by insect gut extract [2.5% (v/v) *A. grandis* or *P. brassicae* gut extract]. Activation of the Cry9Bb toxin with trypsin produced a profile of two main fragments around 69 and 58 kDa that varied slightly with the trypsin: toxin ratio. Fig. 4 (lane 7) shows the protein profile after activation with trypsin at 1:10 (w/w) trypsin: toxin. Activation with *A. grandis* gut extract produced a single protein band around 70 kDa (Fig. 4, lane 5), while activation with *P. brassicae* gut extract produced a fragment around 66 kDa (not shown).

3.9.2. Immunoblotting for Cry9

Immunodetection of Cry9 in crystals, solubilized-, trypsin activated- and *A. grandis* gut extract activated-toxin from the recombinant strain SVBt725 was performed as described previously. Crystals of the strain S725 were used as positive control. Strong positive reactions were observed for crystals of both strains and for the soluble SVBt725 toxin (Fig. 4). Only a band below 25 kDa (not visible on the coomassie stained gel), was detected among the products of the trypsin-activated protein (Fig. 4).

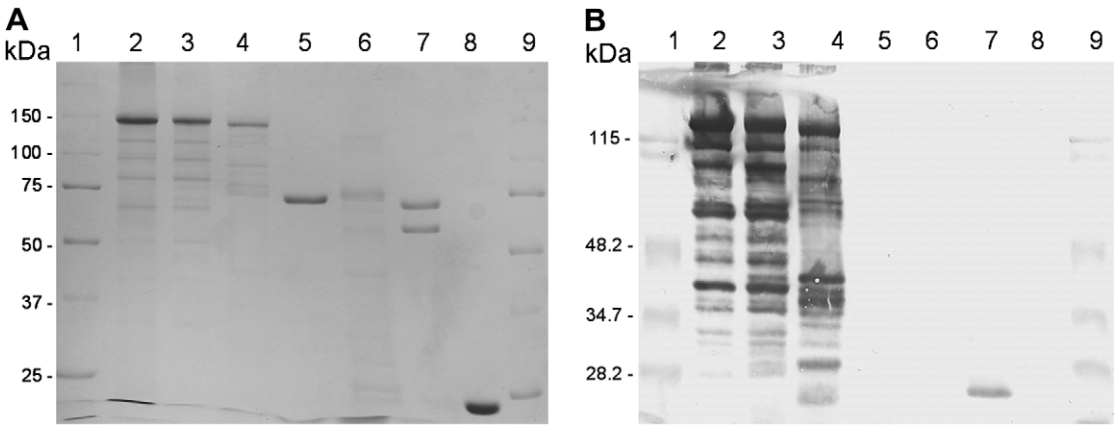

Fig. 4. Immunoblotting of *B. thuringiensis* proteins from strains S725 and SVBt725 for Cry9 δ-endotoxin. Crystals from SVBt725 were solubilized at pH 8.5 and the protein activated with *A. grandis* gut extract or trypsin. (A) The protein bands were resolved in SDS–12% PAGE and stained with coomassie blue. Ten micrograms of purified crystals or 8 μg of solubilized or activated toxin were loaded per well. Lanes 1 and 9: molecular mass markers (kDa); lane 2: S725 crystals; lane 3: SVBt725 crystals; lane 4: SVBt725 solubilized toxin; lane 5: SVBt725 toxin activated by 2.5% (v/v) *A. grandis* gut extract; lane 6: *A. grandis* gut extract proteins; lane 7: SVBt725 toxin activated with trypsin at 1:10 (w/w) enzyme: toxin; lane 8: TPCK treated trypsin (8 μg). (B) Blot of a gel identical to A incubated with polyclonal antibody to Cry9 protein. Legend as in (A).

3.9.3. Bioassays

The protein in the crystals from Bt strain S725 and the recombinant strain SVBt725 (Cry9Bb protein) was active against the lepidopterans *M. sexta* (>70% mortality), *A. gemmatalis* (90–100% mortality) and *S. frugiperda* (<30% mortality) at the highest doses tested. The protein showed no toxicity to the lepidopterans *M. brassicae*, *P. brassicae*, *H. zea*, *O. nubilalis*, *P. xylostella*, *A. ipsilon* and *T. ni*, the coleopterans *A. grandis*, *D. virgifera*, *D. undecimpunctata* and *L. decemlineata*, and the dipterans *M. domestica* and *A. aegypti*. Table 2 shows the average LC_{50} values, calculated from six bioassays for purified Cry9Bb and from three bioassays for Cry1Ac crystals from the strain Btk HD-73 against neonate larvae of *M. sexta*. The Cry9Bb toxin was approximately 1700 times less toxic to *M. sexta* than Cry1Ac.

3.10. RT-PCR for cry1I-type genes analysis

As a *cry1I* gene was detected in the DNA from the strain S725, its transcription was analysed by RT-PCR. It was performed with total RNA from S725 and the control Bt subsp. *aizawai* strain HD-133 (Masson et al., 1998) extracted at three different growth stages. Transcription of the *cry1I* gene was observed at the T_2 and T_5 stages of sporulation (2 and 5 h after the beginning of sporulation, respectively) for S725 and HD-133 (data not shown).

3.11. Mutant analysis

Using a predicted structural alignment of Cry proteins, Pro84 was observed to be a conserved residue in all Cry9 toxins, but Cry9Bb, as well as Cry1, Cry3, Cry7 and Cry8. It was seen to have been substituted by Ala in domain I of Cry9Bb in the region between helix α1 and helix α2A. A point mutation in the DNA of the *cry9Bb* gene was then performed to replace Ala84 by Pro84 in the Cry9Bb protoxin, in order to test the effect on biological activity. The mutated gene in the construct pSVP725-A84P was expressed in Bti IPS78/11. The selected Bt clone Bt7ASV produced spherical crystals, similarly to the parental strain SVBt725. The crystals were solubilized in solubilization buffer at three different pHs (8.5, 9.0 and 9.5) and the protein activated with trypsin at 1:100 (w/w) trypsin: toxin ratio, in parallel with Cry9Bb. Solubilization of the mutant Cry9BbA84P protein yielded a main band around 130 kDa, identical to Cry9Bb. Activation resulted in two main fragments around 69 and 58 kDa for both proteins (data not shown). The concentrations obtained for solubilized and activated proteins at the different pHs suggested that the mutant toxin was slightly more soluble than the parental under the conditions used (data not shown). Bioassays of Cry9BbA84P crystals against *M. sexta* neonate larvae were performed. The average LC_{50} of three distinct bioassays for the mutant toxin and of six bioassays for Cry9Bb are shown on Table 2. The LC_{50} of crystals of the mutant toxin for *M. sexta* was 8.77-fold smaller than the LC_{50} for Cry9Bb. Thus, the toxin with the mutation $A_{84}P$ is almost 9 times more potent than the wild-type toxin Cry9Bb against *M. sexta* neonate larvae.

4. Discussion

Lepidoptera-specific Japanese strains producing spherical crystals composed of protein bands around 130–140 kDa have been reported (Wasano et al., 1998). These strains belong to multiple H serovars, including the type strain of Bt *japonensis* (strain 84-F-31-31), and are comprised of Cry9D or a novel Cry9-like protein (Wasano and Ohba, 1998; Wasano et al., 2001). Later on, the latter protein, from a Bt serovar *galleriae* strain, was characterised and classified as Cry9Ec1 (Wasano et al., 2005). Spherical crystals are also produced by the Coleoptera (Scarabaeidae)-specific strain Buibui of the serovar *japonensis*, which are composed of Cry8Ca proteins (Wasano et al., 1998; Wasano et al., 2000). In this work, we have shown that the Bt subsp. *japonensis* strain S725 produced spherical crystals which showed a main protein band around 130 kDa. The immunodetection analysis showed that the strain S725 produced a Cry9-like protein and did not produce Cry1Ac, Cry2A or Cry1B toxins.

The crystals of the strain S725 were solubilized and activated at different conditions. Trypsin activation of the protein, for one hour, yielded several fragments between 50 and 70 kDa. Wasano and collaborators (Wasano et al., 1998) also found a similar proteolysis profile for the parasporal inclusion proteins of the lepidopteran-active type strain of Bt serovar *japonensis*.

BLAST searches of N-terminal sequences of solubilized and trypsinized fragments of the crystal protein from the strain S725 showed high levels of homology with Cry9Ca, Cry9Da, Cry9Ea and Cry9Eb δ-endotoxins. The Cry9 class of δ-endotoxins comprises the subclasses Cry9A to Cry9E (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt), mostly Lepidoptera active. As an exception into this class, Asano (1996) and Iizuka et al. (1996) reported the cloning of a δ-endotoxin gene, now designated *cry9Da*, from the strain N141 of *B. thuringiensis* serovar *japonensis*, coding for a Coleoptera (scarabaeid) toxic protein.

The PCR method has been widely used for the detection and identification of novel and known *cry* genes in *B. thuringiensis* strains (Cerón et al., 1995; Bravo et al., 1998; Ibarra et al., 2003). The genomic DNA of the strain S725 was screened for genes that code for lepidopteran and coleopteran-active toxins and showed the presence of *cry9*-like, *cry1I* and *vip3* genes. Some Cry1I proteins have been reported to have dual activity against Lepidoptera and Coleoptera species (Tailor et al., 1992; Ruiz de Escudero et al., 2006). However, most of the Cry1I proteins have shown activity only against lepidopteran insects (Gleave et al., 1993; Shin et al., 1995; Kostichka et al., 1996; Choi et al., 2000; Song et al., 2003). The occurrence of *cry1I* genes among Bt strains, as deduced from PCR-based studies, is very high, with frequencies of around 50% or more, and they frequently occur when other *cry1*-type genes are present (Martínez and Caballero, 2002; Porcar and Juárez-Pérez, 2003). On the other hand, the frequency of *cry9* genes found in Bt collections has been low. Wang and collaborators found the lowest frequency for *cry9* containing isolates (15.5%) in a Chinese collection (Wang et al., 2003). Bravo and co-workers found *cry9* genes in only 2.6% of a Mexican Bt collection (Bravo et al., 1998). Ben-Dov and co-workers found that 10.2% Bt isolates from a collection from Israel, Kazakhstan, and Uzbekistan contained a *cry9* gene. All of the *cry9* containing isolates found by Ben-Dov *et al.* also harboured *cry1* and *cry2* genes (Ben-Dov et al., 1999). Interestingly, the strain S725 contains a combination of *cry1I* and *cry9* genes. Investigation data have shown that while the *cry1* and *cry2* genes have a high tendency to occur together, there is no such association between *cry9* genes and *cry1* or *cry2* genes (Wang et al., 2003). A *vip3* gene was also detected in the DNA of S725, confirming the previously reported existence of the linkage between the presence of *cry1I* and *vip3* in Bt strains (Espinasse et al., 2003).

The novel *cry9Bb* gene clearly differs from the cryptic *cryX* gene, currently classified as *cry9Ba*, isolated from a strain of Bt subsp. *galleriae*, which is not expressed *in vivo* (Shevelev et al., 1993). Analysis of the phylogenetic tree of protoxin sequences of the Cry9 family, including the novel Cry9 from S725 (Cry9Bb), showed

Table 2
LC_{50} values for Cry9Bb, Cry9BbA84P and Cry1Ac crystals against neonate larvae of *Manduca sexta*

Toxin	Average LC_{50}^{*} (μg/cm^2)	95% Confidence limits (μg/cm^2)
Cry9Bb	6.84	3.76–17.30
Cry9BbA84P	0.78	0.41–1.53
Cry1Ac	0.0039	0.0026–0.0061

LC_{50}^{*} = toxin concentration lethal to 50% of the insects tested.

that this sequence is closer to Cry9B, while Cry9A is further away from the other Cry9 proteins. Analysing Cry protein phylogenetic relationships, Bravo (Bravo, 1997) also found that the Cry9Aa toxin was located far away from the Cry9Ba and Cry9Ca toxins, suggesting that the Cry9Aa toxin evolved independently from the other Cry9 toxins.

The spherical crystals formed by the Cry9Bb protein expressed in SVBt725 were solubilized at varying conditions, yielding a 130 kDa protein that upon activation with trypsin produced two main fragments around 69 and 58 kDa. Immunoblotting of the Cry9Bb crystals, solubilized and activated toxin against anti-Cry9 polyclonal antibody resulted in strong positive reactions for crystals and soluble toxin, but it was not detected in the activated toxin fragments, suggesting that the protein epitope for Cry9 is not localised on the main activated toxin fragments.

Bioassays of the Cry9Bb protein from the Bt strain S725 and the recombinant strain SVBt725 were performed against a range of insects of three orders. The toxin showed activity against the lepidopterans *M. sexta* (tobacco hornworm), *A. gemmatalis* (velvetbean caterpillar) and *S. frugiperda* (fall armyworm), although at low level for the latter. On the other hand, binding assays using biotin-labeled Cry9Bb activated protein and BBMV from the coleopteran *A. grandis* and the lepidopteran *P. brassicae* showed no affinity of the toxin to receptors on BBMV of these insects (data not shown), which is in agreement with the observed lack of toxicity.

The LC_{50} of Cry9Bb crystals against neonate *M. sexta* larvae (6.84 μg/cm^2) was much higher than the LC_{50} for the reference Cry1Ac crystals (3.93 ng/cm^2). The high level of susceptibility of *M. sexta* to Cry1Ac toxin has been reported by several research groups (Van Rie et al., 1989; Lee et al., 2001; Gilliland et al., 2002; Vilchez et al., 2004). Gilliland and co-workers (Gilliland et al., 2002), for example, obtained an LC_{50} of 0.033 μg/ml for Cry1Ac crystals against neonate *M. sexta* larvae and of 1.24 μg/ml against third instar larvae. However, a much lower activity was reported for Cry1Ba1 toxin against *M. sexta*. Zhong et al. (2000) obtained an LC_{50} of 0.19 mg/ml for Cry1Ba1 crystals against neonate *M. sexta* larvae.

Cytotoxicity assays of activated Cry9Bb against cells of *Choristoneura fumiferana* CF1 (originated from trypsinized larval tissue), *A. gemmatalis* UFL-Ag-286 (from embryonic tissue), and *M. sexta* GV1 (from embryonic tissue) were performed. The toxin did not show visible cytological effects against any of these insect cells at concentrations up to 200 μg/ml, after up to 3 h (data not shown). This might be due to the absence of receptors. It has been reported that cultured insect cells lack normal midgut receptors and do not respond as specifically to toxins as does the whole insect (Schnepf et al., 1998).

To determine whether a substitution in a predicted conserved amino acid in domain I resulted in biological effects, Ala84 in Cry9Bb was replaced by Pro84. The properties of the mutant protein were assessed through analysis of solubility, trypsin activation, and toxicity against *M. sexta*. Cry9Bb and Cry9BbA84P showed a similar protein profile upon solubilization and activation, indicating that the mutant toxin was stable and the protein structure was not altered significantly. However, a slight increase in crystal protein solubility and almost 9-fold increase in toxicity of the mutant to *M. sexta* was observed. The domain I in Cry toxins is involved in membrane insertion, toxin oligomerisation and pore formation (Bravo, 1997; Gerber and Shai, 2000; de Maagd et al., 2001). Mutations in different residues in this domain have shown variable effects in toxicity, with either increase or reduction (Schnepf et al., 1998). As a reason for the enhanced activity of Cry9BbA84P, it is suggested that the replacement of the more flexible Ala side chain by the more hydrophobic and less flexible Pro in domain I may have contributed to a higher thermodynamic stability of the protein, enhancing the process of pore formation and, consequently, its toxicity.

Expression analysis by RT-PCR of the *cry1I*-type gene in S725 revealed that the *cry1I* gene is transcribed during sporulation (T_2 and T_5 stages). Transcription of *cry1I* genes at these stages has been reported for some Bt strains, such as Bt subsp. *aizawai* HD-133 (Masson et al., 1998; Tounsi and Jaoua, 2002; Tounsi et al., 2003).

Thus, the Bt subsp. *japonensis* strain S725 produces crystals comprised of a novel and unique Cry9Bb δ-endotoxin, which possesses moderate activity against some lepidopteran insects. To further investigate the activity spectrum of the novel toxin, it should be assayed against other lepidopteran species, against insects of other orders, like Orthoptera and Hemiptera, and even against other invertebrates like nematodes.

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