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Crystalline protein profiling and *cry* gene detection in *Bacillus thuringiensis* strains isolated during epizootics in *Cydia pomonella* L.

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Abstract: The composition of *Bacillus thuringiensis* crystalline inclusions was characterized in 18 strains: 12 isolates were obtained from the intestinal tract of *Cydia pomonella* larvae during epizootics, 2 isolates were cultured from *Leucoma salicis* larvae taken from their natural populations, and 4 reference strains. The number and molecular mass of *B. thuringiensis* crystalline proteins (Cry and Cyt) was estimated by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The crystals contained 1–8 proteins with molecular masses of 36–155 kDa. The toxin profiles differed both quantatively and qualitatively. The *B. thuringiensis* MPU B9 isolate had the highest number and diversity of Cry toxins. The analysis of crystal composition by SDS-PAGE was insufficient to detect groups and subgroups of Cry proteins. We identified 20 groups and 3 subgroups of Cry and Cyt crystalline toxins. Only one epizootic strain harboured *cry25*. In single reference strains, the *cry1H, cry10* and *cry25* genes were found. We did not find any correlation between the occurrence of *cry* genes and electrophoretic protein profiles of crystalline toxins.

Keywords: *Bacillus thuringiensis, cry* gene, crystalline toxin

INTRODUCTION

Bacillus thuringiensis is a Gram-positive rod-shaped bacterium that produces spores. During sporulation, *B. thuringiensis* forms crystalline inclusions composed of 1–5 Cry and Cyt proteins that are active against some insects. The crystalline toxins are classified into groups with amino acid similarity of at least 45%. Within these groups, subgroups are distinguished within these groups, with 75% and 95% amino acid similarity (Crickmore et al. 1998). Toxins of Cry1, Cry3, Cry7, Cry8, Cry9, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry55, Cyt1, and Cyt2 groups show activity against some pests of the order Coleoptera. Crystalline

proteins belonging to groups Cry1, Cry2, Cry4, Cry10, Cry11, Cry16, Cry19, Cry20, Cry24, Cry27, Cry32, Cry39, Cry44, Cry47, Cry48, and Cry49 may be toxic to dipterans, while Cry23 and Cry11 are active against hemipterans insects. Lepidoptera pests may be susceptible to Cry1, Cry8, Cry9, Cry15,Cry22, Cry32, and Cry51. Proteins Cry2, Cry3 and Cry22 are toxic to hymenopterans (Van Frankenhuyzen 2009).

The insecticidal toxicity towards pests and the lack of pathogenicity to mammalian cells make Cry proteins an attractive tool for controlling harmful insects in agriculture, forestry, and horticulture (Schnepf et al. 1998). Preparations of *B. thuringiensis* crystals and spores are an alternative to chemical insecticides. Selective activity and high effectivness in reducing the number of plant pests make biopesticides more attractive than synthetic preparations (Sanahuya et al. 2011). These biopesticides are safe for the enviroment and non-target organisms. Moreover, the production cost of *B. thuringiensis* insecticides is lower than that of chemical pesticides (Schnepf et al. 1998).

B. thuringiensis is commonly isolated from soil (Martin & Travers 1989), plant surface (Bernhard et al. 1997), animal faeces, and other habitats (Meadows et al. 1992). New isolates can display higher crystal toxicity, with a wider spectrum of activity than the strains already used in pesticide production. However, searching for new isolates, as promising candidates for creating an effective insecticide, requires reliable methods for determination of crystal insecticide activity. The analysis of *B. thuringiensis* crystal composition is useful in determination of inclusion toxicity. The insecticidal toxicity of *B. thuringiensis* can also be predicted by characterization of the *cry* gene profile (Nazarian et al. 2009; Vidal-Quist et al. 2009). The analysis of *cry* gene and crystalline toxin patterns could lead to selection of effective strains that may be employed in production of a new insecticide and be a source of novel genes for bioengineered crop protection (Jouzani et al. 2008). Polymerase chain reaction (PCR) method can be used not only for detection of already known *cry* genes but also for identification of novel *cry* genes by applying the same initial set of primers (Nazarian et al. 2009), but some *cry* genes are not expressed (Masson et al. 1998; Porcar & Caballero 2000).

A massive infection of laboratory culture lines of *Cydia pomonella* (Lepidoptera) was caused by *B. thuringiensis* (in the absence of any other insect pathogens) in 1990–1999 in Poznań, Poland (Ziemnicka & Ziemnicki 2001). The lines were kept in the Department of Biological Control and Quarantine, Institute of Plant Protection, Poznań and the Department of Physiology and Animal Development Biology, Adam Mickiewicz University, Poznań. The mortality of *C. pomonella* ranged from 44% to 91%, and the epizootics occurred several times a year in 1990, 1995 and 1996.

The aim of the present work was to predict the insecticidal activity of *B. thuringiensis* isolates and to evaluate their potential usefulness for further research, leading to create a new biopesticide with a broader and higher spectrum of toxicity against insect pests than those already used. We used PCR to detect crystalline protein genes, while SDS-PAGE to estimate the composition of toxins in bacterial crystals.

MATERIALS AND METHODS

Bacterial strains

Eighteen *B. thuringiensis* strains were used in this study. Twelve isolates were cultured from the intestinal tracts of *C. pomonella* larvae reared in laboratory conditions during epizootics. Additionally, two *B. thuringiensis* strains were isolated from *L. salicis* larvae collected from their natural populations. All the isolates formed crystals inside cells during sporulation (Konecka et al. 2007b). Four reference strains were received from Dr D. R. Zeigler of The Ohio State University, Columbus, USA (*Bacillus* Genetic Stock Center – BGSC): *B. thuringiensis* serotype *thuringiensis* 4A3, *B. thuringiensis* serotype *aizawai* 4J3, *B. thuringiensis* serotype *israelensis* 4Q1, and *B. thuringiensis* serotype *thompsoni* 401.

Estimation of the number and molecular mass of B. thuringiensis crystalline proteins

B. thuringiensis strains were cultured until sporulation in modified M.B.Th medium composed of casein hydrolysate (7 g/l), KH_2PO_4 (6.8 g/l), $MgSO_4 \cdot 7H_2O$ (0.12 g/l), $MnSO_4 \cdot 4H_2O$ (0.0022 g/l), $ZnSO_4 \cdot 7H_2O$ (0.014 g/l), $Fe_2(SO_4)_3$ (0.02 g/l), and $CaCl_2 \cdot 4H_2O$ (0.18 g/l), with pH adjusted to 7.2 (Lecadet & Dedonder 1971). Spores and crystals were collected, suspended in 1 M NaCl and pipetted vigorously up and down until foam appeared. The foam containing spores was removed with a sterile spatula. After centrifugation (3000 rpm, 30 min), the pellet was washed with sterile distilled water (Zhu et al. 1998). The spore-toxin mixture was suspended in 50 mM Tris HCl pH 7.5 with 10 mM KCl, layered onto sucrose density gradient (67%, 72%, 79%, 87%) and centrifuged at 15 000rpm for 30 min. The crystal layer was harvested and washed three times with sterile distilled water (Guz et al. 2005). The crystals were solubilized in buffer containing 50 mM $NaCO_3$, 10 mM dithiothreitol, pH 10.0 (MOHAN et al. 2009). After 2-h incubation at 37°C with shaking, the proteins were analysed by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Costas (1992). Gels were fixed in 12% trichloroacetic acid (Costas 1992), stained with Brilliant Blue G-250 and with methanol/acetic acid mixture (Monnerat et al. 2007). The molecular masses of proteins were determined by using GelCompar II 3.5 software (Applied Maths, Belgium).

Identification of B. thuringiensis crystalline toxin genes

Bacterial DNA was extracted by heating the 24-h culture in brain heart infusion (BHI) broth (bioMérieux, France) at 100°C for 10 min (Brousseau et al. 1993). PCR identified 19 groups and 3 subgroups of Cry and Cyt crystalline toxins. To amplify the *cry* genes, 1 µg of DNA was used in 25 µl of a mixture containing also 2.5 µl of 10×PCR buffer with $MgCl_2$, 1 µl of 5 mM dNTP, 25 pmol of primers and 1 U of HiFi*Taq* DNA polymerase. The PCR reagents were purchased from Novazym (Poland) and Oligo.pl (Poland). Each amplification was carried out with a negative control. The reactions were done in MyCycler thermal cycler (Bio-Rad, USA). Primer sequences and annealing temperatures for *cry1H, cry1J, cry1K* gene determination were presented by Monnerat et al. (2006). Amplification of *cry15, cry16, cry18, cry20, cry22, cry25, cry26, cry28,* and *cyt2* genes was accomplished as described

by Ejiofor & Johnson (2002). PCR detection of *cry10, cry17*, *cry24, cry27, cry29, cry30, cry32,* and *cry40* genes was conducted according to Ibarra et al. (2003). Detection of *cry19* and *cry39* genes was done as it was proposed by Jouzani et al. (2008). The PCR products were separated in 1.5% Basica LE GQT agarose gel (Prona, Spain), visualised by staining with 1 µg/ml ethidium bromide and documented using Bio-Print V.99 System (Vilber Lourmat, France). The amplicon sizes were determined with GelCompar II 3.5 software (Konecka et al. 2007b).

RESULTS

The composition of crystals determined for 12 *B. thuringiensis* isolates and 4 reference strains by SDS-PAGE differed both quantitatively and qualitatively (Fig. 1). The inclusions contained 1–8 proteins, with molecular masses of 36–155 kDa. Identical patterns of proteins were found in strains MPU B3 and MPU B4. Three isolates (MPU B5, MPU B6, and MPU B14) had the same Cry toxins. The reference strains *B. thuringiensis* BGSC 4A3 and *B. thuringiensis* serotype *aizawai* 4J3 produced identical crystalline proteins. The largest number and high diversity of Cry proteins were observed in *B. thuringiensis* MPU B9.

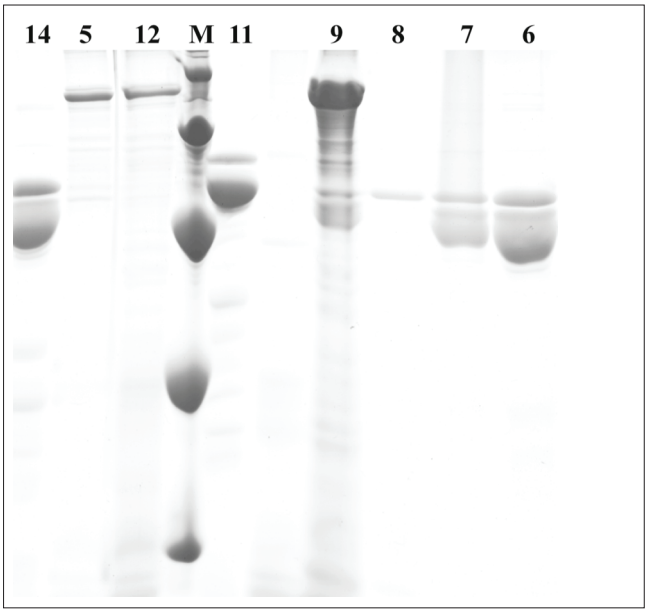

Fig. 1. Profiles of Cry proteins of *B. thuringiensis*. Lanes: 14 = *B. thuringiensis* MPU B14; 5 = *B. thuringiensis* MPU B5; 12 = *B. thuringiensis* MPU B12; M = Calibration proteins for sodium dodecyl sulphate gel electrophoresis (Boehringer Mannheim): 170000 Da, 97400 Da, 55400 Da, 3600 Da, 20100 Da; 11 = *B. thuringiensis* MPU B11; 9 = *B. thuringiensis* MPU B9; 8 = *B. thuringiensis* MPU B8; 7 = *B. thuringienis* MPU B7; 6 = *B. thuringiensis* MPU B6

PCR detected 19 groups and 3 subgroups of *cry* and *cyt* genes [15 groups and 19 subgroups of crystalline proteins were determined previously (KONECKA et al. 2007b)]. Only one epizootic strain MPU B7 harboured *cry25*. The strain was isolated from the intestinal tract of a dead *Cydia pomonella* larva from a laboratory culture line in the Department of Biological Control and Quarantine in Institute of Plant Protection, Poznań. Three *cry* genes (*cry1H, cry10, cry25*) were identified in the reference strains. *B. thuringiensis* serotype *israelensis* BGSC 4Q1 had the *cry10* gene. The *cry25* gene was found in *B. thuringiensis* serotype *aizawai* BGSC 4J3, whereas *cry1H* was observed in *B. thuringiensis* serotype *thompsoni* BGSC 4O1. The electropherograms of amplification products for *cry1H*, *cry10* and *cry25* are presented in Fig. 2. The results are listed in Table 1. None of the *B. thuringiensis* strains carried *cry1J, cry1K*, *cry15, cry16, cry17*, *cry18, cry19, cry20, cry22, cry24, cry26, cry27, cry28, cry29, cry30, cry32, cry 39, cry40*, and *cyt2*.

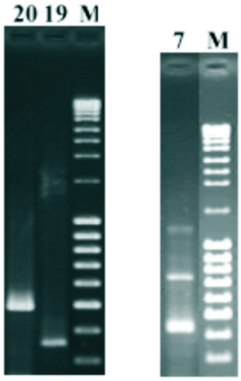

Fig. 2. PCR products of *Bacillus thuringiensis cry* genes. Lanes: 20 = amplicon of *cry1H* for *B. thuringiensis* serptype *thompsoni* BGSC 4O1; 19 = product of *cry10* for *B. thuringiensis* serotype *israelensis* BGSC 4Q1; 7 = amplicon of *cry25* for *B. thuringiensis* MPU B7; M = MassRuler™ DNA Ladder Mix (10000, 8000, 6000, 5000, 4000, 3000, 2500, 1500, 1031, 900, 800, 700, 600, 500, 400, 300 bp)

DISCUSSION

All the isolates used in this study were cultured from the intestinal tract of the same insect species and caused high level of larvae mortality, but the Cry protein profiles differed in a majority of the strains (Table 1). The highest number and diversity of toxins was observed for strains MPU B3, MPU B4, and MPU B9. These isolates had also a large variety of *cry* genes (KONECKA et al. 2007b). Single strains had identical or very similar protein profiles. Isolates MPU B3 and MPU B4 had 6 toxins with identical molecular mass. The strains had identical *cry* gene patterns and genotypes

Table 1. *Cry* genes and crystalline proteins in bacteria isolated from intestinal tracts of *Cydia pomonella* larvae, *Leucoma salicis* larvae, and reference strains

Strain designation	Source and year of isolation or reference	Size of crystalline proteins (kDa)	*cry* genes identified in this study	*cry* genes identyfied previously (Konecka et al. 2007b)
MPU B1	*C. pomonella* (IPP), 1990	155, 70, 64, 49	-	*cry1Ab, 1B*
MPU B2	*C. pomonella* (IPP), 1990	93, 70, 55, 42, 37	-	*cry1B, 2Aa, 2Ab*
MPU B3	*C. pomonella* (IPP), 1990	137, 96, 86, 70, 67, 63	-	*cry1Aa, 1C, 1D, 1I, 2Ab, 9B, 9E*
MPU B4	*C. pomonella* (IPP), 1992	137, 96, 86, 70, 67, 63	-	*cry1Aa, 1C, 1D, 1I, 2Ab, 9B, 9E*
MPU B5	*C. pomonella* (IPP), 1995	70, 67, 55	-	*cry1F, 2Ab*
MPU B6	*C. pomonella* (IPP), 1995	70, 67, 55	-	*cry1Aa, 1Ab, 1Ac, 1F, 2Ab*
MPU B7	*C. pomonella* (IPP), 1996	70, 67, 59	*cry25*	*cry1I, 2Ab*
MPU B8	*C. pomonella* (AMU), 1996	70	-	*cry1B, 1D, 2Ab, 9B, 9E*
MPU B9	*C. pomonella* (AMU), 1996	137, 96, 93, 86, 83, 70, 67, 63	-	*cry1Aa, 1B, 1C, 1D, 1I, 2Ab, 9B, 9E*
MPU B10	*C. pomonella* (AMU), 1996	70, 55	-	*cry2Aa, 2Ab, 2Ac*
MPU B11	*C. pomonella* (AMU), 1996	86, 70	-	*cry1Ab, 1Ac, 1I, 2Ab, 9B*
MPU B12	*C. pomonella* (IPP), 1999	140	-	*cry1Ab, 1Ac, 1I*
MPU B13	*L. salicis* (IPP), 1998	140	-	*cry1I*
MPU B14	*L. salicis* (IPP), 1999	70, 67, 55	-	*cry1I, 2Aa, 2Ab, 2Ac*
MPU B15	*B. thuringiensis* serotype *thuringiensis* BGSC 4A3	140, 137	-	*cry1Aa,1Ab,1IB, 1C, 1D, 1I, 9D*
MPU B18	*B. thuringiensis* serotype *aizawai* BGSC 4J3	140, 137	*cry25*	*cry1Aa, 1Ab, 1C, 1D, 1I, 2Ab, 2Ac, 9B, 9E*
MPU B19	*B. thuringiensis* serotype *israelensis* BGSC 4Q1	84, 67, 56, 49, 36	*cry10*	*cry4, 11, cyt*
MPU B20	*B. thuringiensis* serotype *thompsoni* BGSC 4O1	140, 96, 84, 70, 67, 55	*cry1H*	*cry1Ab, 1F, 1I, 2Aa, 2Ab, 2Ac*

AMU = Department of Physiology and Animal Development Biology, Adam Mickiewicz University, Poznań, Poland; BGSC = *Bacillus* Genetic Stock Center, Ohio State University, Columbia, USA; IPP = Department of Biological Control and Quarantine in Institute of Plant Protection, Poznań, Poland; MPU B = Collection of Department of Microbiology, Adam Mickiewicz University, Poznań, Poland.

as described previously (Konecka et al. 2007a; Konecka et al. 2007b). *B. thuringiensis* MPU B3 and MPU B4 were cultured from *C. pomonella* larvae in 1990 and 1992, respectively (Konecka et al. 2007a). The same patterns of Cry proteins were noted for isolates MPU B5, MPU B6 and MPU B14, but the strains harboured different *cry* genes (Konecka et al. 2007b). *B. thuringiensis* MPU B5 and MPU B6 were isolated from *C. pomonella* larvae in 1995, whereas *B. thuringiensis* MPU B14 was cultured from *L. salicis* in 1999 (Konecka et al. 2007a). Although reference strains *B. thuringiensis* BGSC 4A3 and *B. thuringiensis* serotype *aizawai* 4J3 expressed crystalline proteins of similar size, they had various genes coding for the toxins. *B. thuringiensis* isolates with identical protein profiles but different *cry* genes patterns have been also described by Armengol et al. (2007).

The SDS-PAGE of protein crystals is useful in estimating the inclusion composition, but only at the level of main protein groups. For example, protoxins of the Cry1 group have molecular masses of 130–140 kDa (Alzate et al. 2009). After enzymatic activation with trypsin, the mass of Cry1Ab is equal to 66–69 kDa (Alzate et al. 2009), Cry1C has a weight of 65 kDa (Park et al. 2000), and the mass of Cry1F is 60 kDa (Hua et al. 2001). Similar molecular masses of toxins excluded the possibility to distinguish between protein subgroups of the same group based on analysis of their weight. Moreover, different groups of toxins have similar masses. For example, the weight of Cry9 protoxins is equal to 130 kDa, but after activation it decreases to 50–70 kDa (Hua et al. 2001), like the Cry1 masses mentioned above. The same masses of Cry toxins from different groups did not allow distinguishing them in the SDS-PAGE analysis.

Some *B. thuringiensis* strains had crystalline proteins with molecular masses of 130 kDa or larger. This could indicate the presence of undigested proteins. Trypsin-like enzymes could participate in the activation of some proteins to a lesser extent than other insect proteolytic enzymes.

The analysis of *cry* profiles could be useful in estimating the toxicity of *B. thuringiensis* crystals (Nazarian et al. 2009; Vidal-Quist et al. 2009). The analysis of *cry* genes and Cry proteins could lead to detection of effective isolates that can be applied in a new pesticide production and as a source of novel genes for bioengineered crop protection (Jouzani et al. 2008).

The characterization of *cry* profiles performed previously revealed the presence of numerous subgroups of *cry1*, *cry2* and *cry9* genes in *B. thuringiensis* strains isolated from the intestinal tracts of *C. pomonella* caterpillars during epizootics in laboratory cultures of the insects (Konecka et al. 2007b). In this study, we extended the information about the crystalline toxin genes and identified 19 groups and 3 subgroups of other *cry* and *cyt* genes. Only one isolate *B. thuringiensis* MPU B7 and one reference strain *B. thuringiensis* serotype *aizawai* BGSC 4J3 had the *cry25* gene. The *cry10* gene was noted in the single reference strain *B. thuringiensis* serotype *israelensis* BGSC 4Q1. The harmful activity of Cry10 was noted against insects of the order Diptera. The *cry1H* gene was observed in *B. thuringiensis* serotype *thompsoni* BGSC 4O1. Cry1H protein is toxic to pests of the order Lepidoptera (Van Frankenhuyzen 2009). Our previous research (Konecka et al. 2007b) has revealed *cry1*, *cry2* and *cry9* genes in *B. thuringiensis* epizootic isolates. That pattern of genes showed potential toxicity against insects of the orders Coleoptera, Diptera, Hemiptera and Lepidoptera.

The genes detected in this work are not common among *B. thuringiensis* strains. Similar results have been described for isolates from the natural environment (Ejiofor & Johnson 2002; Ibarra et al. 2003). Ejiofor & Johnson (2002) analysed the *cry* profiles of *B. thuringiensis* strains isolated from uncultivated soil and ponds of South Central United States. They found *cry15*, *cry20*, *cry24*, *cry26,* and *cyt2* in single strains. Ibarra et al. (2003) searched for genes *cry10, cry17*, *cry24, cry27, cry29, cry30, cry32,* and *cry40* (detected also in our study) in strains cultured from soil in Latin America, but they found only *cry10, cry17, cry27*, and *cry30*.

We did not find any correlation between the profiles of *cry* genes and patterns of crystalline proteins. This is in agreement with the results described by Armengol et al. (2007) who did not find correlation between the profiles of Cry toxins and their genes among *B. thuringiensis* strains isolated from soil samples collected in Colombia. This could be explained by the possibility of production of Cry proteins encoded by unknown genes. Moreover, some *cry* genes could have low expression or even be inactive (Armengol et al. 2007; Jouzani et al. 2008). Additionally, some Cry proteins do not participate in creating the inclusions. Toxins, for example Cry1I, can also be secreted into the medium (Pocar & Caballero 2000). Difficulties in estimating the crystal composition in the SDS-PAGE preparation could be caused by the presence of protoxins, toxins and proteolysis intermediates in a sample (Vidal-Quist et al. 2009). Furthermore, problems with determining the mass of Cry toxins could be due to a more compact structure of protein profiles than the protein standard (Güereca & Bravo 1999).

Characterisation of *cry* gene profiles performed in this study and previously (Konecka et al. 2007b) initially allowed us to determine the potential activity of crystalline inclusions of *B. thuringiensis* isolates. The analysis of the *cry* genes and Cry proteins led to selection of *B. thuringiensis* isolate MPU B9, which seems to be a promising candidate for future research on a new efficient preparation against plant pests. The strain had the highest number and diversity of Cry toxins. Our results indicate that the analysis of crystal composition by SDS-PAGE is insufficient to detect groups and subgroups of Cry proteins.

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