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Activity of spore-crystal mixtures of new *Bacillus thuringiensis* strains against *Dendrolimus pini* (Lepidoptera: Lasiocampidae) and *Spodoptera exigua* (Lepidoptera: Noctuidae)

Edyta Konecka[1] ✉, *Adam Kaznowski*[1], *Małgorzata Stachowiak*[2], *Mirosław Maciąg*[2]

[1] Adam Mickiewicz University in Poznań, Faculty of Biology, Department of Microbiology, Umultowska 89, 61-614 Poznań, Poland, phone: +48 61 8295935, fax: 061-829-5636, e-mail: edkon@amu.edu.pl

[2] General Directorate of State Forest, Forest Protecting Unit Łopuchówko, Łopuchówko 1, 62-095 Murowana Goślina, Poland

Abstract

We estimated the usefulness of spore-crystals preparations of the two *B. thuringiensis* isolates, MPU B9 and MPU B54, for reducing the number of pests. The potential insecticidal toxicities of *B. thuringiensis* isolates were assessed by the analysis of the genes coding for crystalline proteins. The activities of spore-crystals preparations were determined against *Dendrolimus pini* L. (Lepidoptera: Lasiocampidae) and compared with the toxicity of spores and crystals of *B. thuringiensis* subsp. *kurstaki* HD-1 from commercial biopesticide Foray. Although the analysis of crystalline toxin gene profiles indicated potentially higher activities of MPU B9 and MPU B54 crystals against the pests than that of HD-1, the toxicities of isolate and HD-1 preparations against *D. pini* caterpillars were similar. The LC_{50} amounted to 3.42×10^4 spores and crystals for HD-1, 3.36×10^4 for MPU B9 and 3.5×10^4 for MPU B54. Additionally, the toxicity of the MPU B54 preparation was evaluated against *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae). The LC_{50} was 4.5×10^5 spores and crystals of MPU B54, and 2.69×10^6 spores and crystals of HD-1. The LC_{50} of the MPU B54 preparation against *S. exigua* was approximately six-fold higher than that of HD-1. However, due to the very wide fiducidal limits for LC_{50} values, which for both preparations overlap to a large extent, the toxicity of the preparations should be considered the same. The varied profiles of crystalline toxin genes and important toxicity of spore-crystal mixtures of isolates against *S. exigua* and *D. pini* indicate the effectiveness of the mixtures against pests and make the strains an alternative for HD-1 for reducing the number of insects.

Key words

Bacillus thuringiensis, bacterial toxins, insecticidal toxicity, Lepidoptera, plant protection

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Introduction

Insect pests devastate approximately 18% of crops every year and contribute to a loss of 20% of stored food grains causing US$ 100 billion damage per year all over the world. Lepidopteran caterpillars are considered as the most harmful pests (Nicholson 2007). Chemical insecticides are effective in reducing the pest number, but their usage contributes to the occurrence of resistance in insects. Furthermore, they have a wide spectrum of toxicity and act against non-target organisms, including natural enemies of pests. Preparations based on *Bacillus thuringiensis* are an attractive alternative due to the lack of harmful impact on human and other vertebrates, and low costs of production. There is still an urgent need to isolate a new, effective insecticidal factor that would be useful in plant protection and safe to non-target organisms (Nicholson 2007).

One of the most important pests from Lepidoptera is *Dendrolimus pini* L. (Lasiocampidae) that damages the Scots pine *Pinus sylvestris* L. and causes severe economic losses. The insect's larvae eat a part or whole pine needles. They defoliate from spring to autumn (Davis et al. 2008). The pest larvae feed mainly on leaves (Albrecht and Davis 2012). Frequent and repeated defoliations caused by *D. pini* may result in tree death. The pest prefers *Pinus sylvestris* but feeds also on many other species of *Pinus*, *Picea*, *Abies*, *Cedrus*, *Juniperus*, *Larix* and *Pseudotsuga* (Davis et al. 2008). *D. pini* is a widely distributed pest of *P. sylvestris* in Central and Eastern Europe: in Germany (Le Mellec and Michalzik 2008), Poland (Sierpińska 1998), Lithuania (Gedminas and Žiogas 2008), Russia (Molet 2012), and Ukraine (Meshkova 2003; Hardin and Suazo 2012). There is also a growing concern about *D. pini* as a forest pest in Great Britain. The insect prefers sites with well-drained soil, warm summers, cold and dry winters. Due to climatic changes, the weather becomes warmer and drier, and as a result, the climate will be milder with colder winters. This suggests that future outbreaks of *D. pini* will be more serious and damaging in Great Britain, where there are only occasional, rare sightings of the insects (Ray et al. 2011).

Another lepidopteran pest is *Spodoptera exigua* (Hubner) (Lasiocampidae). It is a polyphagous insect that can develop in different vegetables, grasses, weeds and flowering plants in field and greenhouse throughout the world (Huffman et al. 1996; Capinera 1999). The pest is distributed in 101 countries of Africa, Asia, Europe, Australia, South and North America (Zheng et al. 2011). The insect larvae consume plants completely, leaving only roots and leaf veins. It is regarded as one of the most problematic agricultural pests worldwide that causes economic losses for the industry, because it can contribute to the reduction of crop production, especially commercial crops such as cotton and tobacco. The crop yields can be decreased not only by eating the whole plants by *S. exigua* larvae but also by leaving faeces in crops or causing significant scarring of fruit by the pest (Hua et al. 2013). The insecticide resistance of *S. exigua* is a serious problem in the management of this pest, probably because it attacks crops that are treated frequently with chemical pesticides (Capinera 1999).

The use of microbial insecticides is available in the market against lepidopteran pests. Preparations based on spore-crystal mixtures of *B. thuringiensis* comprise a large group of biological insecticides intended for plant protection against lepidopteran pests. *B. thuringiensis* crystals that are produced during sporulation include proteins toxic for insects. Various *B. thuringiensis* strains synthesize crystals that differ in protein compositions and thereby in the spectrum and amount of the crystal activity (Sarker and Mahbub 2012). The contents of crystalline inclusions and their potential toxicity can be estimated indirectly by the determination of the profiles of genes coding for the synthesis of crystal proteins (Nazarian et al. 2009). Despite formulations based on the bacterial spores and crystals available in the market against Lepidoptera, a growing concern prompted us to seek for new bacterial strains that could be applied in effective plant protection in both agriculture and forestry and be an alternative to both chemical pesticides and biological insecticides currently recommended for plant protection.

We attempted to find *B. thuringiensis* isolates that could be a valuable alternative to the HD-1 strain for reducing the number of lepidopteran pests in plants protection. We searched for isolates with diverse gene profiles of crystalline toxins and significant insecticidal toxicity of spore-crystal preparations against pests. We decided to determine the activity of spore-crystal preparation of a new *B. thuringiensis* isolate MPU B54 against *D. pini* and *S. exigua* and compare its toxicity

with the activity of spore-crystal mixture of *B. thuringiensis* subsp. *kurstaki* HD-1 strain from Foray biopesticide that is recommended against insects of the order Lepidoptera. In order to predict the crystal toxicity of the isolate, identification of 39 groups and 22 subgroups of *cry* and *cyt* genes was conducted by using PCR assays. Additionally, we included *B. thuringiensis* MPU B9 isolate cultured from *Cydia pomonella* larva and determined the activity of its spore-crystal preparation against *D. pini*. Identification of *cry* genes of *B. thuringiensis* MPU B9 has been done previously (Konecka et al. 2007b) and the toxicity of its crystals for *S. exigua* has been determined (Konecka et al. 2012).

Material and methods

Bacterial strains

Three *Bacillus thuringiensis* strains were used in this study. *B. thuringiensis* MPU B54 was cultured from a soil sample collected in Wielkopolski National Park, Poland, 52º15'N 16º48'E. It was identified by using API 50 CHB kit according to the manufacturer's instructions (bioMérieux, France). The presence of crystalline inclusions of MPU B54 strain was evaluated according to Smirnoff (1962). *B. thuringiensis* MPU B9 was isolated from the intestinal track of a dead *C. pomonella* larva (Konecka et al. 2007a). *B. thuringiensis* HD-1 was isolated from the commercial insecticide Foray (Valent BioSciences Corporation, Libertyville, USA).

Insects

D. pini larvae were collected from pine trees from their natural populations in central and western regions of Poland. As the analysis was a comparative study, the insects used in the experiments were in the same instar. The larvae collected from the natural populations were in the fourth instar and they were used in the experiment of determination of the activity of *B. thuringiensis* spores and crystals against insects on the following day after their delivery to the laboratory. All the larvae used were healthy, as was confirmed in the control sample in which 30 larvae were reared in the same conditions as those used in the experiment but with sterile distilled water applied instead of bacterial spore-crystal suspension on pine needles for insect feeding.

S. exigua larvae were from a standardized laboratory culture maintained in the Department of Microbiology, Adam Mickiewicz University, Poznań originated from insect culture of Institute of Plant Protection – National Research Institute, Poznań. The insects were reared in stable, standardized parameters of 26°C with 40–60% humidity and at 16:8 (day:night) period, with the use of a chamber simulating controlled cyclical environment conditions. The insects used in the experiments were seven-day-old when they reached the third instar.

Identification of the genes of crystalline toxins of *B. thuringiensis*

Identification of *cry* genes of *B. thuringiensis* MPU B9 has been done previously (Konecka et al. 2007). Thirty-nine groups and 22 subgroups of *cry* and *cyt* genes were analysed for *B. thuringiensis* MPU B54 isolate by using PCR method. The identification of the genes of MPU B9 and MPU B54 strains was conducted based on the same method. Amplifications were performed in a PCR mixture containing: 1 µg bacterial DNA, 2.5 µl of 10×PCR buffer (Novazym, Poland), 1 µl of 5 mM dNTPs (Novazym, Poland), 50 pmol of forward and reverse primers (Oligo.pl, Poland), 0.2 µl of 5U/µl DNA Allegro Taq polymerase (Novazym, Poland) and sterile distilled water to a total volume of 25 µl. PCR reactions were conducted in a MyCycler thermal cycler (Bio-Rad, USA) according to the amplification conditions described by other authors. Genes *cry1*, *cry2* with their subgroups, as well as *cry3*, *cry4*, and *cry7*/8 were identified as proposed by Ben-Dov et al. (1997). Subgroups of *cry1* gene were detected according to Juárez-Pérez et al. (1997), Masson et al. (1997), and Monnerat et al. (2007). Bravo et al. (1998) and Jouzani et al. (2008a) have proposed amplification conditions for identification of *cry5*, *cry12*, *cry13*, *cry14*, and *cry21* genes. Detection of *cry6*, *cry15*, *cry16*, *cry18*, *cry20*, *cry22*, *cry25*, *cry26*, *cry28*, *cyt1*, and *cyt2* genes was done according to Ejiofor and Johnson (2002). Identification of the *cry9* gene and its subgroups was accomplished as described by Ben-Dov et al. (1999). Presence of *cry10*, *cry17/27*, *cry24/40*, *cry29*, and *cry32* genes was evaluated as presented by Ibarra et al. (2003). Genes *cry19* and *cry39* were identified according to Jouzani et al. (2008b). The condition for *cry11* and *cry13* gene amplification has been proposed by Bravo et al. (1998). Identifica-

tion of *cry30/44* genes was conducted as depicted by Ito et al. (2006). Detection of *cry34* and *cry35* genes was proposed by Schnepf et al. (2005). PCR products were mixed with 6×DNA loading dye (MBI Fermentas, USA) and electrophoresed in 1.5% Nova Mini agarose (Novazym, Poland). DNA was stained with ethidium bromide and electropherograms were documented by a Bio-Print V.99 system (Vilber-Lourmat, France). Molecular weights of amplicons were estimated with GelCompar II 3.5 software (Applied Maths, Belgium).

Preparation of *B. thuringiensis* spore-crystals mixtures

B. thuringiensis MPU B9, MPU B54 and HD-1 strains were cultured on sporulation medium (Lecadet and Dedonder 1971) for five days at 30°C. The spore-toxin mixtures of each strain were collected and weighted. Three mg of spores and crystals were suspended in 20 ml of sterile distilled water and their number in 1 ml was calculated in a Bűrker cell. Four dilutions of the mixture (10^{-4}–10^{-7} in 10 µl) were prepared.

Activity of *B. thuringiensis* spores and crystals against *D. pini*

Pinus sylvestris needles of equal length were immersed in the spore-toxin mixture for 5 minutes. One pine needle and one *D. pini* larvae of the fourth instar were placed in a separate Petri dish. For each dilution of the spore and crystals mixture, thirty larvae were used (three pseudoreplications with 10 insects each). Additional pine needles were supplemented to avoid insects' death from starvation – after eating the infected needle, the larvae were provided with other uninfected needles. As a negative control, thirty larvae were reared at the same conditions on needles without bacterial spores and crystals (the needles were dipped in sterile distilled water).

The number of spores and crystals on selected needles were determined for each crystal-spore dilution. The spores and crystals were rinsed from a needle and their number was determined in a Bűrker cell under a light microscope. The pine needles dipped in spore-crystal suspensions of the lowest dilution had 7×10^6 spores and crystals on their surface. Each tenfold dilution of spore-crystal suspension resulted in tenfold lower number of spores and crystals on the surface of pine needles.

Activity of *B. thuringiensis* spores and crystals against *S. exigua*

The activity of *B. thuringiensis* MPU B9 crystal suspension against *S. exigua* has been published previously (Konecka et al. 2012). The toxicity of *B. thuringiensis* MPU B54 and HD-1 spores and crystals against *S. exigua* was determined in the same manner as described for MPU B9 (Konecka et al. 2012). Each dilution of spore-crystals suspension was spread on the surface of medium for insects rearing formed in rollers 3 mm high and 5 mm in diameter. The composition of the medium has been described by Poitout and Bues (1970). One *S. exigua* caterpillar was placed on each roller of the medium. Thirty larvae were used for each dilution (three pseudoreplications with 10 insects each). As a negative control, thirty larvae were reared in the same conditions on the medium in which sterile water was applied instead of bacterial spores and crystals.

Calculation of LC50 *B. thuringiensis* spores and crystals against insects

After a 14-day incubation for *D. pini* and seven days for *S. exigua* at 26°C with 40–60% humidity and at 16:8 (day:night) period, the number of dead insects was determined and the value of 50% lethal concentration (LC_{50}) was evaluated by probit analysis with BioStat 2009 Professional 5.8.4 software (AnalystSoft, Canada).

Results

B. thuringiensis MPU B54 harboured *cry1Aa*, *cry1Ab*, *cry1C*, *cry1D*, *cry1I*, *cry2Ab*, *cry9B* and *cry9E*. We found *cry1*, *cry2* and *cry9* genes in both isolates. The strains differed in two genes only. The *cry1B* gene was identified in MPU B9 but not in MPU B54, whereas the *cry1Ab* gene was detected only in MPU B54 strain.

The LC_{50} values of the *B. thuringiensis* preparations were calculated with consideration of the percent of mortality in control sample. All the control insects survived the experiments. The LC_{50} values of spore-crystal mixture of all *B. thuringiensis* strains against *D. pini* were similar and amounted to 3.42×10^4 spores and crystals of HD-1, 3.36×10^4 spores and crystals of MPU B9 and 3.5×10^4 spores and crystals of MPU B54 (Tab. 1).

Table 1. LC_{50} of *B. thuringiensis* spore-crystal mixtures against *D. pini*

Strain	LC_{50} of preparation against *D. pini**	95% fiducidal limits
HD-1	3.42×10^4	8.21×10^3–8.56×10^4
MPU B9	3.36×10^4	2.66×10^3–1.21×10^5
MPU B54	3.5×10^4	1.48×10^4–6.78×10^4

* The number of spores and crystals on the surface of pine needles per one larva.

The LC_{50} values against *S. exigua* were 4.5×10^5 spores and crystals of MPU B54, and 2.69×10^6 spores and crystals of HD-1 (Tab. 2). The LC_{50} of the MPU B54 preparation against *S. exigua* was approximately six-fold higher than that of HD-1 from Foray. However, due to the very wide fiducidal limits for LC_{50} values, which for both preparations overlap to a large extent, the toxicity of the preparations should be considered the same.

Table 2. LC_{50} of *B. thuringiensis* spore-crystal mixtures against *S. exigua*

Strain	LC_{50} of preparation against *S. exigua**	95% fiducidal limits
HD-1	2.69×10^6	1.28×10^6–1.35×10^7
MPU B54	4.5×10^5	3.11×10^5–7.13×10^6

* The number of spores and crystals on the surface of medium per one larva.

Discussion

New *B. thuringiensis* strains synthesizing crystals with a wide variety of insecticidal toxins that show high activity against target insect pests are an issue of growing interest. In our research, we focused on searching for bacterial strains that harbour toxins active against the Lepidoptera pests and might be an alternative for *B. thuringiensis* strains used in the production of commercial biopesticides. In our paper, two isolates of different origins were investigated: *B. thuringiensis* MPU B9 was isolated from infected *Cydia pomonella* (Konecka et al. 2007a), whereas *B. thuringiensis* MPU B54 was recovered from a soil sample. For *B. thuringiensis* MPU B9, nine groups of crystalline protein genes and some of their subgroups have been identified previously (Konecka et al. 2007b) and additional subgroups of the detected genes and twenty one other crystalline toxins genes were determined. *B. thuringiensis* MPU B9 strain had *cry1Aa*, *1B*, *1C*, *1D*, *1I*, *2Ab*, *9B*, and *9E* genes (Konecka et al. 2007b).

The profile of *cry* genes suggests the insect specificity of crystalline inclusions of *B. thuringiensis*. Cry1, Cry2, and Cry9 proteins, which genes were detected in MPU B9 and MPU B54, have been found to be toxic to pests from the order Lepidoptera (van Frankenhuyzen 2009). *B. thuringiensis* HD-1 from Foray carries *cry1Aa*, *1Ab*, *1Ac*, *2Aa*, *2Ab*, and *2Ac,* but none of *cry9* genes and no other *cry1* besides *cry1A* (Konecka et al. 2007b).

MPU B9 and MPU B54 strains seemed to have a higher activity of crystals against lepidopteran pests than *B. thuringienis* HD-1 from Foray because they had a higher number and variability of *cry* genes. Therefore, these two bacterial isolates were selected for the determination of the insecticidal activity of their spore-crystal preparations. The level of pesticidal toxicity of spores and crystals of MPU B9 and MPU B54 indicated the same effectiveness of the isolates' preparations against *D. pini* as that of HD-1 strain. The results may suggest that the Cry9 toxin, whose gene was found in the genomes of MPU B9 and MPU B54 but not in HD-1, is not active against *D. pini*.

The LC_{50} of the MPU B54 preparation against *S. exigua* was approximately six-fold higher than that of HD-1 from Foray. The reason of the high activity of MPU B54 spore-crystal preparation can be the contribution of Cry1C, Cry1D, Cry9B and Cry9E proteins in the crystals toxicity against the pest. The genes of these four insecticidal toxins were found in that isolate but not in HD-1 strain from Foray. The activity of Cry1C against *S. exigua* has been confirmed by other researchers (Porcar et al. 2000; Lu et al. 2012; Ren et al. 2013). The toxicity of Cry1D has been reported as well (Porcar *et al*. 2000). Moreover, the toxins Cry1C and Cry1A, whose genes were found in MPU B54, can act synergistically against *S. exigua* (Xue et al. 2005) and this could also explain higher toxicity of spore-crystal preparations of the isolate than that of HD-1 against the insect.

The LC_{50} of crystals of *B. thuringiensis* MPU B9 exhibited high level of activity against *S. exigua* (Konecka et al. 2012). Moreover, the MPU B9 synthesizes spores and crystalline inclusions active also against *C.*

pomonella (Konecka et al. 2007b), and crystals toxic for *Leucoma salicis* (Konecka et al. 2010; Konecka et al. 2011). The high value of the activity of spores and crystals of MPU B9 against *D. pini* determined in this study revealed the usefulness of the spore-crystal mixture of the isolate in plant protection.

In conclusion, the activity of spores and crystals of the two *B. thuringiensis* isolates studied exhibited important mortality levels against lepidopteran larvae. The diverse gene profiles of crystalline toxins and significant insecticidal toxicity of spore-crystal preparations of *B. thuringiensis* isolates against *S. exigua* and *D. pini* larvae make the strains a valuable alternative of HD-1 strain for reducing the number of lepidopteran pest.

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