Polish Journal of Microbiology
2009, Vol. 58, No 2, 155–161

ORIGINAL PAPER

The Occurrence, Biodiversity and Toxicity of *Bacillus thuringiensis* Strains Isolated from the Insect Pest *Lymantria dispar* (Poland)

KATARZYNA GUZ*, GABRIELA BUGLA-PŁOSKOŃSKA and WŁODZIMIERZ DOROSZKIEWICZ

Department of Microbiology, Institute Genetics and Microbiology
University of Wrocław, Wrocław, Poland

Received 20 February 2009, revised 15 April 2009, accepted 30 April 2009

Abstract

The aim of this investigation was to survey the occurrence, biodiversity, and toxicity of *Bacillus thuringiensis* strains originating from dead caterpillars of the forest pest, *Lymantria dispar* (Lepidoptera). Morphological, biochemical, and microscopic identification of isolates from the insects showed the presence of five different *Bacillus* species, including 2% of *B. thuringiensis*. Based on the biochemical profiles, the *B. thuringiensis* were determined to be *B. thuringiensis finitimus*-like and *B. thuringiensis alesti*-like bacilli. Both produced spherical inclusions composed of three or five protoxins. The molecular weights of these proteins varied from 20 to *ca.* 64 kDa. Mixtures of spores/inclusions of the *B. thuringiensis* were tested for their toxicity against larvae of *Drosophila melanogaster*. The mortality levels of the larvae caused by these spores and crystalline inclusions varied from 5 to 15%. The lethal doses (LD_{50}) of these isolates against *D. melanogaster* were 8.8×10^{12} spores/ml for *B. thuringiensis finitimus* and 1.3×10^{18} spores/ml for *B. thuringiensis alesti*.

Key words: *Bacillus thuringiensis*, *Lymantria dispar*, fruit fly assay, delta-endotoxin profiles

Introduction

The spore-forming, oxygenic Gram-positive bacilli *Bacillus thuringiensis* are among the best-known insect pathogens. For many years these strains have been used as bacterial insecticides for the specific control of some *Lepidoptera* and *Coleoptera* pests as well as dipteran vectors of infectious and invasive diseases (Glare and O'Callaghan, 2000; Lonc *et al*., 2006; Schnepf *et al*., 1998). The popularity of these microbes is due to their high insect toxicity, environmental safety, and lack of toxicity to humans and other vertebrates (Aronson and Yechiel, 2001; Lonc *et al.*, 2006; Siegel, 2001). During sporulation, *B. thuringiensis* strains produce insecticidal crystal proteins (ICPs) in the parasporal inclusions which exhibit a wide but specific range of toxicity against different insect orders (Aronson and Yechiel, 2001; Glare and O'Callaghan, 2000; Guz and Doroszkiewicz, 2005; Lonc *et al.*, 2006; Schnepf *et al*., 1998). The crystalline inclusions compounded with the Cry proteins (delta-endotoxins) of several *B. thuringiensis* strains have shown distinct specificity to the larvae or imago of different insect orders *Lepidoptera*, *Diptera*, *Coleoptera*, *Hymenoptera*, *Homoptera*, *Phthiraptera,* and *Mallophaga* (Aronson and Yechiel, 2001; Glare and O'Callaghan, 2000; Guz and Doroszkiewicz, 2005; Lonc *et al.*, 2006; Schnepf *et al*., 1998). More than 3,000 strains of *B. thuringiensis* producing crystalline inclusions have been isolated so far in 29 countries around the world. They have been categorized into 71 H serotypes based on flagellar antigens and registered at the International Entomophathogenic Bacillus Centere Collection of the Pasteur Institute in Paris, but only a few of these strains are used globally for the control of larval forms of agriculturally important insect pests (Guz and Doroszkiewicz, 2005; Hernandez *et al*., 2005; Ibara *et al*., 2003; Lecadet *et al*., 1999; Lonc *et al*., 2001).

Many European, Asian, African and North and South American countries as well as New Zealand and Mexico have joined the worldwide, environmental search program for natural isolates of *B. thuringiensis* with activity against economically important target insects (Al-Momani *et al*., 2004; Ibara *et al*., 2003; Ichimatsu *et al*., 2000; Karamanlidou *et al*., 1991;

* Corresponding author: K. Guz, Department of Microbiology, Institute Genetics and Microbiology, University of Wrocław, ul. Przybyszewskiego 66/77, 51-148 Wrocław, Poland; **e-mail: selene@microb.uni.wroc.pl**

Khyami-Horani *et al*., 2003; Quesach *et al.*, 2004; Uribe *et al*., 2003; Wang *et al.*, 2003). The first Polish collection of environmental *B. thuringiensis* strains was established by Lonc *et al.* (1997) and Doroszkiewicz *et al.* (1999). The new insecticidal *B. thuringiensis* isolates, among them the new strain *B. thuringiensis wratislaviensis* H47, were isolated from soil and phylloplane samples in the Wrocław area and the region of Lower Silesia. Further environmental research led to the isolation of *B. thuringiensis* strains in Upper Silesian fields (Guz *et al.*, 2005; Guz and Doroszkiewicz, 2005) and north-eastern Poland (Święcicka and Mahillon, 2005; Święcicka *et al*., 2002).

The natural epizootic of insect pests caused by different biological agents, for example bacteria, viruses and entomopathogenic fungi, is rare in the wild, but they could be important in the search for new entomopathogenic, *i.e. B. thuringiensis*, strains (Konecka *et al*., 2007; Porcar and Caballero, 2000). For example, dead mosquito larvae collected from the Negev Desert in Israel were the source of a new serotype of *B. thuringiensis israelensis* H14 (Porcar and Caballero, 2000). These bacilli are now commonly used in several biopreparations to control dipteran larvae, also in Europe and Poland (Simulin, Vectobac).

The aim of our investigation was to survey the occurrence, biodiversity, and toxicity of *B. thuringiensis* strains originating from dead caterpillars of the gypsy moth, *Lymantria dispar.*

Experimental

Materials and Methods

Bacterial strain isolation. Bacterial samples from dead gypsy moth (*Lymantria dispar*) caterpillars were collected and processed according to Alberola *et al.* (1999). Ten dead larvae were macerated in 20 ml of sterile distilled water and heated at 80°C for 10 min. The heat-treated suspension was plated on a nutrient agar (Biomed, Poland) and incubated for 72 to 96 h at 30 ± 2°C. Colonies with morphology similar to those of *B. cereus* or *B. thuringiensis* were re-isolated on nutrient agar plates (Biocorp, Poland) and examined for the presence of parasporal crystals on unstained slides using Nikon Eclipse E400 phase-contrast microscope.

Bacterial identification. Preliminary identification of the endospore-forming bacilli was done according to Sneath (1986). Glucose, lactose, arabinose, xylose and mannitol fermentation was used to define the bacilli. Standard biochemical tests were performed for *B. thuringiensis* subspecies according to Lecadet *et al.* (1999). The phenotype profile of the *B. thuringiensis* strains was determined by the presence of the activity of arginine dihydrolases (ADH), Tween 80 esterase, urease, lecithinase, the production of acetylmetyl-carbinol (AMC), the utilization of citrate and the fermentation of sucrose, cellobiose, trehalose, maltose and mannose. The ability of these bacteria to reduce nitrate and to hemolyse of sheep blood agar (BioMerieux, France) was also determined.

Separation of spores and parasporal crystals from *B. thuringiensis* cells. A mixture of spores/crystalline inclusions of *B. thuringiensis* was prepared according to Lonc *et al.* (1997). These isolates were grown overnight on a nutrient agar plate at 30 ± 2°C. The inoculum was suspended in 40 ml of sporulation medium (pH 7.5) containing glucose (POCh) (10 g/l), hydrolyzed casein (Difco) (7.5 g/l), KH_2PO_4 (POCh) (6.8 g/l), $Mg_2SO_4 \times 7H_2O$ (POCh) (123 mg/l), $MnSO_4 \times 4H_2O$ (POCh) (2.23 mg/l), $ZnSO_4 \times 7H_2O$ (POCh) (14 mg/l), and $Fe_2(SO_4)_3$ (POCh) (20 mg/l). The cultures of *B. thuringiensis* were incubated at 30±2°C for 5 days with shaking in orbital shakers at 130 rpm. Crystals and spores of each culture were harvested by centrifugation at 2800×g for 20 minutes at 4°C (MPW 370 centrifuge). The pellets of the spores and parasporal crystals of the *B. thuringiensis* strains were purified by three centrifugations at 2800×g for 20 minutes at 4°C (MPW 370 centrifuge) and washed in distilled water. The final pellets were resuspended in 8 ml of 0.85% NaCl or dissolved in carbonate buffer, [50 mM $NaHCO_3/Na_2CO_3$ (POCh) (pH 10.0)] at 37°C for 60 minutes. The material dissolved in carbonate buffer was centrifuged at 10 000×g for 5 minutes at 4°C (3 K30 Sigma ultracentrifuge). The obtained supernatant is referred to as parasporal inclusion solution throughout this study.

Protein concentration and SDS-PAGE electrophoretic patterns. The parasporal inclusion solutions were examined by discontinuous sodium dodecyl sulfate gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out on slabs with 3% acrylamide stacking gels and 12.5% acrylamide separating gels according to Laemmli (1970). The samples were applied to the slabs after heating at 100°C for 5 min. with sample buffer. Proteins were loaded in each of the wells in the same volume of 10 µl (protein concentrations: 400–500 µg/ml). The Bradford protein assay was used to measure the concentrations of proteins in the samples of parasporal inclusions (Bradford, 1976). The Wide-Molecular-Weight-Range Sigma Marker protein standard (twelve proteins for 6–205 kDa: myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase b 97 kDa, fructose-6-phosphate 84 kDa, albumin 66 kDa, glutamic dehydrogenase 55 kDa, ovalbumin 45 kDa, glyceraldehydes-3-phospate dehydrogenase 36 kDa, carbonic anhydrase 29 kDa, trypsinogen 24 kDa, trypsin inhibitor 20 kDa, α-lactalbumin 14.2 kDa, and aprotinin 6.5 kDa) was used for molecular mass calibration. Electrophoresis was

carried out at 50 V for 15 minutes and 100 V for 90 minutes. When electrophoresis was complete, the gels were kept for 1 h in a solution containing 25% (v/v) methanol, 10% (v/v) acetic acid, and 0.05% (v/v) Coomassie brilliant blue R-250 (BioRad) and destained in 10% (v/v) acetic acid for 3–5 h. The molecular masses of the proteins were analyzed with Bio-Profil Bio1D++ v.99 software. The molecular analysis of the proteins was done by the BIO-CAPT v.99 (Vilber Lourmat, France) computer program and the BIO-1D++ v.99 program. A similarity dendrogram for protein inclusions was constructed by the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm. This dendrogram is calculated from the similarity value in matrix and shows the degree of similarity between strains.

Insect bioassays. Toxicity tests were performed on 2 or 3-day-old larvae of the fruit fly (*Drosophila melanogaster*) according to Lonc *et al.* (1997). The fly lineage came from the collection of Institute Genetics and Microbiology, University of Wrocław. Twenty larvae were reared on the surface of Corn-Meal-Sugar-Yeast medium (CMSY, formula by the Institute of Genetics and Microbiology, University of Wrocław, Poland) with 1 ml and 0.05 ml of *B. thuringiensis* suspension containing 1.1×10^8 or 1.4×10^8 spores/ml at 25°C and 70–80% humidity for 12 days. Two replicates were used for each concentration of the mixture of *B. thuringiensis* spores and inclusions. Twenty larvae were fed on the surface of CMSY medium without the mixture of spores and crystals. The mortality of the fly larvae was recorded daily until 80% of the control larvae pupated. Unpupated larvae without a visible response as well as pupae from which no adults emerged were classified as dead. The Simstat Windows program was used to estimate the mean 50% lethal doses (LD_{50}) with regard to the death rate corrected according to Abbott's formula (1928).

Results

Isolation and identification of bacterial strains. One hundred endospore-forming bacilli were isolated from ten dead larvae of *L. dispar* originating from a forest near Warsaw. Five species of the genus *Bacillus* were identified based on morphological and biochemical features (Tab. I). The crystal-producing *B. thuringiensis* occurred together with the genetically closely related groups of bacilli *B. mycoides* and *B. cereus*, *i.e. Bacillus cereus*-like bacteria as well as *B. subtilis* and *Brevibacillus brevis.* These 100 isolates of bacilli had the ability to move and to ferment glucose but they did not produce indole. Most of them were positive for the production of acetyl-methyl-carbinol (AMC) in the Voges-Proskauer test and lecithinase (62.5% of the isolates). Less than half of the bacilli fermented mannitol (48% of the isolates), arabinose and xylose (12.5%).

The quantitative characteristics of the particular bacilli from the dead larvae are shown in Figure 1. Spore-forming bacilli other than *B. thuringiensis*, mainly *B. brevis*, *B. mycoides,* and *B. cereus,* were frequently detected (85% of the isolates). Only 2 of the 100 isolates of bacilli (2%) produced parasporal crystal inclusions of spherical morphology and were classified as *B. thuringiensis* (Fig. 2). In this study we found two subspecies of *B. thuringiensis*, *i.e. B. thuringiensis alesti* and *B. thuringiensis finitimus*. They fermented cellobiose, trehalose, and maltose and

Table I
Phenotypes and morphological features of the endospore-forming bacilli isolated from dead larvae of the gypsy moth, *Lymatria dispar*

Properties	*B. subtilis*	*B. brevis*	*B. cereus*	*B. mycoides*	*B. thuringiensis*
Glucose*	+	+	+	+	+
Xylose*	+	–	–	–	–
Arabinose*	+	–	–	–	–
Manitol*	+	+	–	–	–
Indole	–	–	–	–	–
Acetoine (AMC)	+	–	+	+	V
Lecithinase	–	–	+	+	+
Ability to move	+	+	+	+	+
Rysoidal growth of colony	–	–	–	+	–
Oval endospores	–	–	–	–	–
Size of cells >1mm	–	–	+	+	+
Distortion of cells	–	+	–	–	–
Parasporal bodies	–	–	–	–	+

Explanation: (*) – fermentation, (+) – test positive, (–) – test negative, (V) – test variable, AMC – Acetyl-Methyl-Carbinol in the test VP

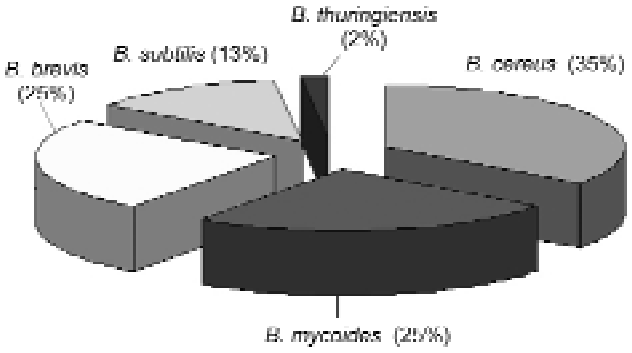

Fig. 1. The occurrence of endospore-forming bacilli (n = 100) isolated from dead larvae of L*ymatria. dispar*.

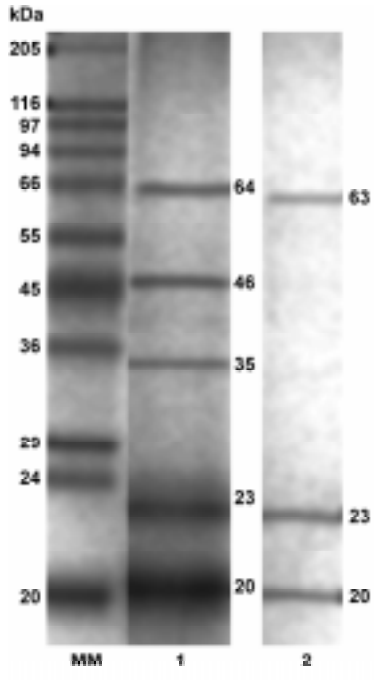

Fig. 3. Proteinogram of parasporal inclusions *Bacillus thuringiensis* isolated from larvae of gypsy moth. Line MN, marker of proteins Wide M 4038 (Sigma), line 1, *Bacillus thuringensis finitimus*-like strain, line 2, *Bacillus thuringensis alesti*-like strain

produced lecithinase, Tween 80 esterase, and acetoine from pyruvate. None of these isolates were positive for hydrolysis of the lactose, sucrose, mannose, citrate and urea. The ability to produce arginine dihydrolases, reduce nitrate, and hemolyse blood agar were adopted as characteristics for the differentiation of the *B. thuringiensis* strains into biochemical groups. The strains of the *B. thuringiensis alesti* group hydrolyzed arginine, and reduced nitrate but none of them produced hemolysies. The lysies of erythrocytes in sheep blood agar was shown only by isolates of the *B. thuringiensis finitimus* group.

Electrophoretic patterns of the crystal proteins. The crystal proteins of the *B. thuringiensis* isolates were analyzed using SDS-PAGE electrophoresis and the molecular weight of each band was calculated using the program BIO-1D++ v.99. The proteinogram is shown in Figure 3. Both strains produced spherical inclusions which were composed of three or five protoxins. The molecular weights of these proteins varied from 20 to *ca.* 64 kDa. The *B. thuringiensis finitimus*-like bacilli produced crystals which contained five proteins with molecular weights of 64, 46, 35, and two small 20 and 23 kDa proteins. The crystalline inclusions of the *B. thuringiensis alesti*-like strains were composed of three proteins of 63, 23, and 20 kDa.

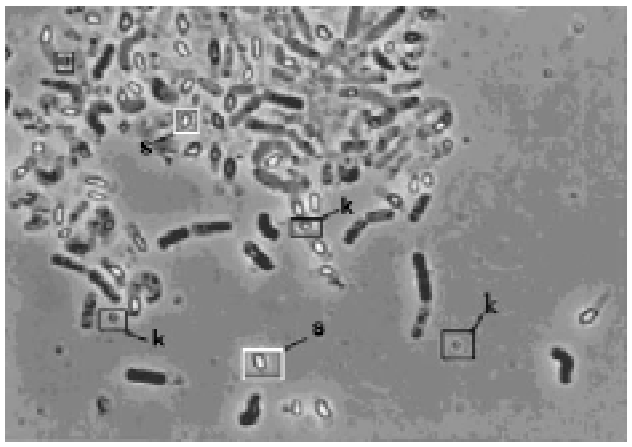

Fig. 2. Endospores and spherical crystalline inclusions of *Bacillus thuringiensis* isolated from dead larvae of the gypsy moth, *Lymatria dispar* (phase-contrast microscope Nikon Eclipse E400, magnification 100x).

Explanation: s – spores, k – crystalline inclusion of parasporal bodies

Insect bioassays. In the bioassays the toxicity of spores/crystal inclusions of the *B. thurigiensis* isolates against 2- or 3-day-old fruit fly larvae was rather low (Tab. II). The strains of the *B. thuringiensis alesti* group at concentrations of both 6.5×10^6 and 1.1×10^8 spores/ml caused 5% mortality of the fly larvae. The LD_{50} level of these isolates was 1.3×10^{18} spores/ml. However, the mortality of the larvae caused by the mixture of spores and crystalline inclusions of the *B. thuringiensis finitimus* isolates was 13.5% at concentration 7.0×10^6 spores/ml and 15% at concentration 1.4×10^8 spores/ml. The LD_{50} level for the *B. thuringiensis finitimus* isolates was considerably lower and amounted to 8.8×10^{12} spores/ml.

Table II
Levels of mortality of the fruit fly larvae, *Drosophila melanogaster*, and LD_{50} of the mixture of spores and crystalline inclusions of *Bacillus thuringiensis* isolates

B. thuringiensis isolate	LD_{50} [spores/ml]	% of mortality of fruit fly larvae *D. melanogaster*	spores/ml
B. thuringiensis finitimus	1.4×10^8 7.0×10^6	15% 13.5%	8.8×10^{12}
B. thuringiensis alesti	1.1×10^8 6.5×10^6	5% 5%	1.3×10^{18}

Discussion

Entomopathogenic microorganisms play an important environmental role for insect populations as etiological factors of many bacterioses, micoses and virioses. Pest plaques are often caused by endospore-forming bacilli of *Bacillus* spp., mainly *B. thuringiensis*, *B. sphearicus,* and *B. larvae* as well as *Paenibacillus lentimorbus*, and *P. popillae* (Snaeth, 1986; Lonc *et al*., 2006). Our present investigations confirmed the regulating role of many oxygenic spore-forming bacilli. The majority of the strains isolated from the dead insect samples were *Bacillus cereus*-like bacteria (62%), of which 60 of the 62 isolates produced no parasporal bodies. From the macerated insects we obtained only 2% *B. thuringiensis* isolates with spherical crystals. According to published data on the natural occurrence of *B. thuringiensis* strains (Bizzarri and Bishop, 2007; Doroszkiewicz and Lonc, 1999; Frederiksen *et al*., 2006; Hernandez *et al*., 2005; Ibara *et al*., 2003; Ichimatsu *et al*., 2000; Jensen *et al.*, 2002; Karamanlidou *et al*., 1991; Khyami-Horani *et al*., 2003; Konecka *et al*., 2007; Lonc *et al*., 1997; Mohammedi *et al*., 2006; Porcar and Caballero, 2000; Święcicka *et al*., 2002; Quesach *et al.*, 2004; Uribe *et al*., 2003; Wang *et al.*, 2003), these bacilli are commonly found in the environment. They have often been isolated from different ecological habitats, such as soil, plants, animal feces, dead and infected insects, stored food-products, aquatic environments, and wastewater sludge. *B. thuringiensis* bacteria closely related to *B. cereus* strains also appeared in fresh food, vegetables and fruits, as well as in the market's ready-to-eat food (Bea *et al.*, 2004; Frederiksen *et al*., 2006 Rosenquist *et al.*, 2005; Zahner *et al*., 2005).

In our environmental investigation of *L. dispar* material we found only two subspecies of *B. thuringiensis* strains, *i.e. B. thuringiensis finitimus* and *B. thuringiensis alesti*. In comparison with previously published data (Bizzarri and Bishop, 2007; Hernandez *et al*., 2005; Konecka *et al*., 2007; Mohammedi *et al*., 2006; Quesach *et al.*, 2004), this diversity of environmental *B. thuringiensis* isolates was rather poor. Many authors showed that the great diversity and abundance of *B. thuringiensis* depends on many ecological factors and geographical conditions (Al-Momani *et al*., 2004; Bizzarri and Bishop, 2007; Doroszkiewicz and Lonc, 1999; Hernandez *et al*., 2005; Ibara *et al*., 2003; Jensen *et al*., 2002; Karamanlidou *et al*., 1991; Mohammedi *et al*., 2006; Quesach *et al.*, 2004; Uribe *et al*., 2003; Wang *et al.*, 2003). Because the insects are a very good source of nutrients for bacterial growth, we expected to isolate many *B. thuringiensis* strains. However, the small percentage of isolated *B. thuringiensis* strains from the insects was probably caused by limiting factors such as the bactericidal properties of the hemocel fluids, the presence of other gut microorganisms, and their interaction. These factors in larvae insect samples could also influence the distribution of *B. thuringiensis* strains and limit their vegetative growth. To some extent it also explains the small differentiation of *B. thuringiensis* isolates. Two detected subspecies (*B. thuringiensis finitimus* and *B. turingiensis alesti*) produced visible spherical parasporal inclusions. This shape appears to be uncommon in the environment. These inclusions were formed by proteins with different molecular weights, ranging from 20 to approximately 64 kDa. In our investigation the crystals of the *B. thuringiensis* isolates possessed only medium (64 kDa) and small proteins (20–46 kDa). The molecular weights of these proteins could correspond to various classes of the known Cry proteins, for example Cry2, Cry3, Cry5, Cry10, Cry11, and Cry35 (>39 kDa) and Cyt proteins (<30 kDa) (Crickmore *et al*., 1998; Espinasse *et al.*, 2002; Guz *et al*., 2005; Saitoh *et al.*, 1998). Neither of the isolated *B. thuringiensis* subspecies produced large protoxins (>100 kDa), *i.e.* typical proteins with *Lepidoptera*- and/or *Diptera*- specific activity.

Many authors showed that the spherical inclusions of *B. thuringiensis* should be toxic mainly against dipteran larvae (Al-Momani *et al.*, 2005; Guz and Doroszkiewicz, 2005; Schnepf *et al.*, 1998). However, in our study we demonstrated that the entomopathogenicity of the isolated *B. thuringiensis* strains toward 2- or 3-day larvae of the fruit fly proved to be slight. The LD_{50} level of the *B. thuringiensis alesti* isolates amounted to 1.3×10^{18} spores/ml but LD_{50} for the *B. thuringiensis finitimus* isolates was considerably lower and amounted to 8.8×10^{12} spores/ml. The mortality levels of 5–15% caused by the mixtures of spores and crystal inclusions showed no strict correlation between the morphology of the crystals and the toxic-specificity of the *B. thuringiensis* protein toxins. Lack of such correlation was also noted in many other papers (Alberola *et al.*, 1999; Doroszkiewicz and Lonc, 1999; Guz *et al.*, 2005; Ibara *et al*., 2003; Lonc *et al*., 1997; Quesach *et al.*, 2004). The results of our bioassays may indicate that the isolated *B. thuringiensis* strains belong to *Lepidoptera*-specific *B. thuringiensis* strains and the entomotoxicity of their crystals is essential against insects of that order. Moreover, according to published data, different shapes of crystalline inclusions can be produced by the same serotype of *B. thuringiensis* strains (Quesach *et al.*, 2004; Uribe *et al*., 2003). Thus, this serotype of *B. thuringiensis* strains producing a variety of crystals can be specifically-toxic to the larvae of insects of different orders or families. Some authors suggest that this change in the morphology of parasporal

bodies could result from either a disturbance in the expression *cry* genes or from loss of the plasmid carrying *cry* genes and/or part of the chromosomal sequence encoding Cry proteins (Bizzarri and Bishop, 2007; Mohammedi *et al*., 2006).

Finally, many studies have demonstrated that almost all *B. thuringiensis* strains found in natural environments are effective against *Lepidoptera*. *B. thuringiensis* active against *Diptera* or *Coleoptera* are also rather difficult to isolate (Bizzarri and Bishop, 2007; Hernandez *et al*., 2005; Ibara *et al*., 2003; Lonc *et al*., 1997; Mohammedi *et al*., 2006; Quesach *et al.*, 2004; Uribe *et al*., 2003; Wang *et al.*, 2003). Many authors suggest that the isolation of *B. thuringiensis* strains active against *Diptera* could be found more frequently in dipteran larvae. Nevertheless, the detected *B. thuringiensis* strains with spherical parasporal bodies in insects of *L. dispar* during our investigation may indicate a new toxic attribute of these bacterial isolates.

Acknowledgments

We are very grateful to Professor Mieczysława Boguś of the Institute of Parasitology of the Polish Academy of Sciences in Warsaw (Poland) for making the biological materials accessible to the investigation and to Ewelina Lech for her help in the laboratory tests.

Literature

Abbott S.W. 1928. A method of computing the effectiveness of an insecticide. *J. Econom. Entomol.* 18: 265–267.

Alberola T.M., S. Aptosoglou, M. Arsenakis, Y. Bel, G. Delrio, D.J. Ellar, J. Ferre, F. Granero, D.M. Guttmann, S. Koliais and others. 1999. Insecticidal activity of strains of *Bacillus thuringiensis* on larvae and adults of *Bactrocera oleae* Gmelin (*Dipt. Tephritidae*). *J. Invert. Pathol.* 74: 127–136.

Al-Momani F., M. Obeidat, I. Saadoun and M. Meqdam. 2004. Serotyping of *Bacillus thuringiensis* isolates, their distribution in different Jordanian habitats and patogenicity in *Drosophila melanogaster. World J. Microbiol. Biotech.* 20: 749–753.

Aronson A.I. and S. Yechiel. 2001. Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *FEMS Microbiol. Lett.* 195: 1–8.

Bea S., G.H. Fleet and G.M. Heard. 2004. Occurrence and significance of *Bacillus thuringiensis* on wine grapes. *Intern. J. Food Microbiol.* 94: 301–312.

Bizzarri M.F. and A.H. Bishop. 2007. Recovery of *Bacillus thuringiensis* in vegetative form from the phylloplane of clover (*Trifolium hybridum*) during a growing season. *J. Invert. Pathol.* 94: 38–47.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochem.* 72: 248–254.

Crickmore N., D.R. Zeigler, J. Feitelson, E. Schnepf, J. van Rie, D. Lereclus, J. Baum and D.H. Dean. 1998. Revision of the Nomenclature for the *Bacillus thuringiensis* Pesticidal Crystal Proteins. *Microb. Mol. Biol. Rev*. 62: 807–821.

Doroszkiewicz W. and E. Lonc. 1999. Biodiversity of *Bacillus thuringiensis* strains in the phylloplane and soil of Lower Silesia region (Poland). *Acta Microbiol. Pol.* 48: 355–361.

Espinasse S., M. Gohar, J. Chafaux, C. Buisson, S. Perchat and V. Sanchis. 2002. Correspondence of high levels of beta-exotoxin I and the presence of *cry*1B in *Bacillus thuringiensis*. *Appl. Environ. Microbiol*. 68: 4182–4186.

Frederiksen K., H. Rosenquist, K. Jorgensen and A. Wilcks. 2006. Occurrence of natural *Bacillus thuringiensis* contaminants and residues of *Bacillus thuringiensis*-based insecticides on fresh fruits and vegetables. *Appl. Environ. Microbiol.* 72: 3435–3440.

Glare T.R. and M. O'Callaghan. 2000. *Bacillus thuringiensis*: Biology, Ecology and Safety. John Wiley and Sons, Chichester.

Guz K., J. Kucińska, E. Lonc and W. Doroszkiewicz W. 2005. Differentiated pattern of protein composition of crystalline inclusions of newly isolated *Bacillus thuringiensis* strains from Silesia in Poland. *Pol. J. Microbiol.* 54: 263–269.

Guz K. and W. Doroszkiewicz. 2005. Environmental *Bacillus thuringiensis* strains and their practical application (in Polish). *Forum Mikrobiologów Wrocławskich.* PAN, Wrocław, 1: 93–105.

Hernandez C.S., R. Andrew, Y. Bel and J. Ferre. 2005. Isolation and toxicity of *Bacillus thuringiensis* from potato-growing areas in Bolivia. *J. Invert. Pathol.* 88: 8–16.

Ibara J.E., C. del Rincon, S. Orduz, D. Noriego, G. Bentintede, R. Movinerat, L. Regis, C. de Oliveire, H. Lanz, M.H. Rodriguez and others. 2003. Diversity of *Bacillus thuringiensis* strains from Latin America with insecticidal activity against different mosquito species. *Appl. Environ. Microbiol.* 69: 5269–5274.

Ichimatsu T., E. Mizuki, H. Nishimura, T. Akao, H. Saitoh, K. Higuchi and M. Ohba. 2000. Occurrence of *Bacillus thuringiensis* in fresh water of Japan. *Curr. Microbiol.* 4: 217–220.

Jensen G.B., P. Larsen, B.L. Jacobsen, B. Madsen, L. Smidt and L. Andruo. 2002. *Bacillus thuringiensis* in fecal samples from greenhouse worker after exposure to *B. thuringiensis*-based pesticides. *Appl. Environ. Microbiol.* 68: 4900–4905.

Karamanlidou G., A.F. Lambropoulos, S.I. Koliais, T. Manousis, D. Ellar and C. Kastritsis. 1991. Toxicity of *Bacillus thuringiensis* to laboratory populations of the olive fruit fly (*Dacus oleae*). *Appl. Environ. Microbiol.* 57: 2277–2282.

Khyami-Horani H., M. Hajaij and J.F. Charles. 2003. Characterization of *Bacillus thuringiensis ser. jordanica* (serotype H71), a novel serovariety isolated in Jordan. *Curr. Microbiol.* 47: 26–31.

Konecka E., A. Kaznowski, J. Zimnicka and K. Zimnicki. 2007. Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated during epizootics in *Cydia pomonella* L. *J. Invert. Pathol.* 94: 56–63.

Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–686.

Lecadet M.M., E. Frachon, V.C. Dumanoir, H. Ripouteau, S. Hamon, P. Laurent and I. Thiery. 1999. Updating the H-antigen classification of *Bacillus thuringiensis*. *J. Appl. Microbiol.* 86: 660–672.

Lonc E., K. Guz and W. Doroszkiewicz. 2006. Microbiological insecticides and organic agriculture (in Polish). *Ogólnopolski Miesięcznik Ekologiczny*, *Ekonatura* 8: 12–15.

Lonc E., W. Doroszkiewicz, M.J. Klowden, K. Rydzanicz and K. Gałgan. 2001. Entomopathogenic activities of environmental isolates of *Bacillus thuringiensis* against dipteran larvae. *J. Vec. Ecol.* 26: 15–26.

Lonc E., M.M. Lacadet, T.M. Lachowicz and E. Panek. 1997. Description of *Bacillus thuringiensis wratislaviensis* (H-47), a new serotype originating from Wrocław (Poland), and other *Bt* soil isolates from the same area. *Lett. Appl. Microbiol.* 24: 474–478.

Mohammedi S., S.B. Subramanian, S. Yan, R.D. Tyagi and J.R. Valero. 2006. Molecular screening of *Bacillus thuringiensis* strains from wastewater sludge for biopesticide production. *Process Biochem.* 41: 829–835.

Quesach M.E., E. Gracia-Tovar, O. Valvada-Gracia and C. Skutiago-Alvarez. 2004. Isolation, geographical diversity and insecticidal activity of *Bacillus thuringiensis* from soils in Spain. *Microbiol. Rev.* 156: 59–71.

Porcar M. and P. Caballero. 2000. Molecular and insecticidal characterization of a *Bacillus thuringiensis* strains isolated during a natural epizootic. *J. Appl. Microbiol.* 89: 309–316.

Rosenquist H., L. Schmidt, S.R. Andersen, G.B. Jensen and A. Wilcks. 2005. Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat food. *FEMS Microbiol. Lett.* 250: 129–136.

Saitoh H., K. Higuchi, E. Mizuki, S.H. Hwang and M. Ohba. 1998. Characterization of mosquito larvacidal parasporal inclusions of a *Bacillus thuringiensis serovar higo* strain. *J. Appl. Microbiol*. 84: 883–888.

Schnepf E., N. Crickmore, J. Van Rie, D.L. Baum, J. Feitelson, D.R. Zeigler and D.H. Dean. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* 62: 775–806.

Siegel J. P. 2001. Mammalian safety of *Bacillus thuringiensis*-based insecticides. *J. Invert. Pathol.* 77: 13–21.

Sneath P.H.A. 1986. The genus *Bacillus. In Bergey's Mannual of Systematic Bacteriology*, Baltimore, MD: Wiliams and Wilkins.

Święcicka I. and J. Mahillon. 2005 The clonal structure of *Bacillus thuringiensis* isolates from north-east Poland does not correlate with their *cry* gene diversity. *Appl. Environ. Microbiol.* 7: 34–39.

Święcicka I., K. Fiedoruk and G. Bednarz. 2002. The occurrence and properties of *Bacillus thuringiensis* isolated from free-living animals*. Lett. Appl. Microbiol.* 34: 194–198.

Uribe D., W. Martinez and J. Ceron. 2003. Distribution and diversity of *cry* genes in native strains of *Bacillus thuringiensis* obtained from different ecosystems from Columbia. *J. Inter. Pathol.* 82: 119–127.

Wang J., A. Boets, J. Van Rie and G. Ren. 2003. Characterization of *cry*1, *cry*2 and *cry*9 genes in *Bacillus thuringiensis* isolates from China. *J. Invert. Pathol.* 82: 63–71.

Zahner V., D.A. Cabra, A.H. Reguq-Mangia, L. Rabinovitch, G. Moreau and D. McIntosh. 2005. Distribution of genes encoding putative factors and fragment length polymorphisms in the *vrrA* gene among Brazilian isolates of *Bacillus cereus* and *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* 71: 8107–8114.