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Molecular and phenotypic characterisation of *Bacillus thuringiensis* isolated during epizootics in *Cydia pomonella* L.

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Abstract

Twelve *Bacillus thuringiensis* strains were isolated from intestinal tracts of *Cydia pomonella* larvae during epizootics in different laboratory insect culture lines. Phenotypic and genetic similarity of these isolates, together with two cultured from *Leucoma salicis* larvae and 14 reference *B. thuringiensis* strains were determined. The epizootic bacteria did not form a single group based on numerical analysis of biochemical properties. Simple RAPD method with only one primer does not allow estimating the genetic similarity of *B. thuringiensis* strains. We propose a novel strategy based on combining several DNA patterns obtained by RAPD technique with different primers for *B. thuringiensis* typing. Majority of infections in the *C. pomonella* culture lines were caused by bacteria with different genotypes. However, two isolates cultured from infected insects at different time (one strain was isolated in 1990 and the other in 1992) had identical DNA fingerprint that suggested a possibility of these bacteria to survive in the laboratory and to cause infections in different years. The results of SDS–PAGE of whole-cell proteins revealed a possibility to apply protein profile analysis in epidemiological investigations of infections caused by *B. thuringiensis*. Strains with identical DNA patterns had very similar whole-cell protein profiles.

Keywords: *Bacillus thuringiensis*; *Cydia pomonella*; Phenotypic properties; RAPD; Whole-cell protein profiles

1. Introduction

Bacillus thuringiensis is a Gram-positive, rod-shaped, and sporulating bacterium closely related to *Bacillus cereus*, *Bacillus mycoides*, and *Bacillus anthracis*. The ability to produce crystal proteins Cry with insecticide activity is a feature discriminating *Bacillus thuringiensis* from bacterial species mentioned above. The Cry proteins are toxic to certain insects of *Lepidoptera* (Jalali et al., 2004; Peyronmet et al., 2001; Zeigler, 1999; Zhong et al., 2000), *Diptera* (Hughes et al., 2005; Ibarra et al., 2003; Zeigler, 1999; Zhong et al., 2000), *Coleoptera* (Ellis et al., 2002; Zeigler, 1999; Zhong et al., 2000), *Hymenoptera* (Garcia-Robles et al., 2001), *Homoptera*, *Orthoptera*, and *Mallophaga* (de Maagd et al., 2003; Schnepf et al., 1998). Some of the Cry toxins are active against other organisms as nematodes (Wie et al., 2003), mites, and protozoa (de Maagd et al., 2003; Schnepf et al., 1998).

The selective toxicity of Cry proteins against insects and the lack of pathogenicity for mammalian cells make these molecules a biological control agent used against insect pests on a commercial scale. They are an alternative for synthetic chemical insecticides employed in forestry and agriculture (Schnepf et al., 1998). *B. thuringiensis* Cry toxins and spores have been used as insecticides against, for example, *Choristoneura fumiferana*, *Galleria mellonella*, *Leptinotarsa decemlineata*, and *Lymantria dispar* (Schnepf et al., 1998; Weinzierl et al., 1998). Cry proteins are also effective in controlling *Diptera* transmitting etiological agents of some human diseases (Regis et al., 2001; Schnepf et al., 1998). A good example of that is the use of *B. thuringiensis*

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subsp. *israelensis* toxins to reduce the number of larvae and adult insects of *Anopheles* that spread *Plasmodium vivax*, the causative agent of malaria (Kroeger et al., 1995).

The epizootics in insects caused by *B. thuringiensis* are not common. An example was the epizootic in *Ephestia cautella* in Kenyan maize stores (Burges and Hurst, 1977). Another case of massive infection caused by *B. thuringiensis* serovar *aizawai* was noted in *Mythimna loreyi* collected in a corn crop in Spain in 1989 (Porcar and Caballero, 2000). One case of epizootic in *Cydia pomonella* occurred in orchard in Czechoslovakia (Zimmermann and Weiser, 1991). However, clonal relationship of *B. thuringiensis* isolated during massive insect infections has not been determined.

In 1990–1999, a massive death of laboratory culture lines of *C. pomonella* caused by *B. thuringiensis* was observed. Bacteria isolated from the intestinal tracts of *C. pomonella*, were cultured on nutrient medium and we obtained homogeneous growths of the microorganisms (monoculture). No other microorganisms were observed in the cultures. The culture lines of *C. pomonella* were held in Department of Biological Control and Quarantine, Institute of Plant Protection and Department of Physiology and Animal Development Biology, Adam Mickiewicz University in Poznań, Poland. The mortality of insects in cultures reached 44–91%. In 1990, 1995, and 1996, the epizootics repeated several times a year. In view of the high mortality of infected insects and frequency of epizootics, the aim of the present work was to determine clonal relationship of strains by the random amplified polymorphic DNA (RAPD) method and analysis of whole-cell proteins.

2. Materials and methods

2.1. Bacterial strains

Twenty-eight *B. thuringiensis* strains were used in this study (Table 1). Eight isolates were cultured from intestinal tracts of *C. pomonella* larvae cultured in Department of Biological Control and Quarantine in Institute of Plant Protection (IPP) during epizootics. Four isolates were taken from similar insect line culture of Department of Animal Physiology, Adam Mickiewicz University (AMU) in Poznań. Two isolates taken from IPP were cultured from *Leucoma salicis* larvae collected from their natural populations. The dead *C. pomonella* larvae were swabbed with 70% ethanol, the intestinal tracts were taken out aseptically with the help of a binocular. Bacteria from the intestinal tracts were grown in nutrient medium—brain heart infusion BHI (Difco) at 27 ± 1 °C. All isolates produced crystalline inclusions during sporulation. Fourteen *B. thuringiensis* reference strains were included in this study as comparative material. Seven reference strains were purchased from Dr D.R. Zeigler of The Ohio State University in Columbus USA (*Bacillus* Genetic Stock Center—BGSC), two strains obtained from polish collection of microorganisms (PCM) of Polish Academy of Sciences, one strain was cultured from commercial bacterial preparation Biobit (Novo Nordisk). *B. thuringiensis* subsp. *kurstaki* HD-1-S-1980 was obtained from Dr A. Sierpińska (Department of Forest Research, Warsaw). Spores and crystal proteins suspension of *B. thuringiensis* subsp. *kurstaki* HD-1-S-1980 are routinely used as the international standard to assess the potency of pesticides. In this study, *B. subtilis* ATCC 11778 and *B. cereus* ATCC 6633 were also included. Bacteria were stored frozen at −80 °C in BHI broth (Difco) containing 50% glycerol.

Table 1
Bacillus thuringiensis strains isolated from intestine tracts of *Cydia pomonella*, *Leucoma salicis*, and *Bacillus* spp. reference strains used in the study

Strain destination	Source and year of isolation or reference
MPU[a] B1	*C. pomonella* (IPP)[b], 1990
MPU B2	*C. pomonella* (IPP), 1990
MPU B3	*C. pomonella* (IPP), 1990
MPU B4	*C. pomonella* (IPP), 1992
MPU B5	*C. pomonella* (IPP), 1995
MPU B6	*C. pomonella* (IPP), 1995
MPU B7	*C. pomonella* (IPP), 1996
MPU B8	*C. pomonella* (AMU)[c], 1996
MPU B9	*C. pomonella* (AMU), 1996
MPU B10	*C. pomonella* (AMU), 1996
MPU B11	*C. pomonella* (AMU), 1996
MPU B12	*C. pomonella* (IPP), 1999
MPU B13	*L. salicis* (IPP), 1998
MPU B14	*L. salicis* (IPP), 1999
MPU B15	*B. thuringiensis* serotype *thuringiensis* BGSC[d] 4A3
MPU B16	*B. thuringiensis* serotype *kurstaki* BGSC 4D1
MPU B17	*B. thuringiensis* serotype *morrisoni* BGSC 4AA1
MPU B18	*B. thuringiensis* serotype *aizawai* BGSC 4J3
MPU B19	*B. thuringiensis* serotype *israelensis* BGSC 4Q1
MPU B20	*B. thuringiensis* serotype *thompsoni* BGSC 4O1
MPU B21	*B. thuringiensis* serotype *higo* BGSC 4AU1
MPU B22	*B. thuringiensis* serotype *israelensis* PCM[e] 2516
MPU B23	*B. thuringiensis* serotype *thuringiensis* PCM 2517
MPU B24	*B. thuringiensis* subsp. *kurstaki* (Biobit)
MPU B25	*B. thuringiensis* subsp. *kurstaki* HD-1-S-1980
MPU B26	*B. thuringiensis* subsp. *morrisoni* (Novodor)
MPU B27	*B. cereus* ATCC[f] 11778
MPU B28	*B. subtilis* ATCC 6633

[a] Bacteria Collection of Department of Microbiology, Adam Mickiewicz University, Poznań, Poland.
[b] *Bacillus thuringiensis* strain obtained from Department of Biological Control and Quarantine in Institute of Plant Protection, Poznań (IPP), Poland.
[c] *Bacillus thuringiensis* strain taken from Department of Physiology and Animal Development Biology, Adam Mickiewicz University (AMU), Poznań, Poland.
[d] BGSC—*Bacillus* Genetic Stock Center, The Ohio State University, Columbus, USA.
[e] PCM—Polish Collection of Microorganisms of Polish Academy of Sciences, Wroclaw, Poland.
[f] ATCC—American Type Culture Collection, Manassas, VA, USA.

2.2. Biochemical characterisation

Biochemical properties of *B. thuringiensis* were determined by API 20 NE and API 50 CHB tests according to manufacturer's instructions (bioMérieux, France). Numerical