The Journal of Microbiology, December 2007, p. 553-557
Vol. 45, No. 6

A Highly Pathogenic Strain of *Bacillus thuringiensis* serovar *kurstaki* in Lepidopteran Pests

Hatice Kati[1]*, Kazim Sezen[2], Remziye Nalcacioglu[2], and Zihni Demirbag[2]

[1]*Department of Biology, Faculty of Arts and Sciences, Giresun University, Giresun 28049, Turkey*
[2]*Department of Biology, Faculty of Arts and Sciences, Karadeniz Technical University, Trabzon 61080, Turkey*

(Received December 12, 2006 / Accepted November 9, 2007)

In order to detect and identify the most toxic *Bacillus thuringiensis* strains against pests, we isolated a *B. thuringiensis* strain (Bn1) from *Balaninus nucum* (Coleoptera: Curculionidae), the most damaging hazelnut pest. Bn1 was characterized via morphological, biochemical, and molecular techniques. The isolate was serotyped, and the results showed that Bn1 was the *B. thuringiensis* serovar, *kurstaki* (H3abc). The scanning electron microscopy indicated that Bn1 has crystals with cubic and bipyramidal shapes. The Polymerase Chain Reactions (PCRs) revealed the presence of the *cry1* and *cry2* genes. The presence of Cry1 and Cry2 proteins in the Bn1 isolate was confirmed via SDS-PAGE, at approximately 130 kDa and 65 kDa, respectively. The bioassays conducted to determine the insecticidal activity of the Bn1 isolate were conducted with four distinct insects, using spore-crystal mixtures. We noted that Bn1 has higher toxicity as compared with the standard *B. thuringiensis* subsp. *kurstaki* (HD-1). The highest observed mortality was 90% against *Malacosoma neustria* and *Lymantria dispar* larvae. Our results show that the *B. thuringiensis* isolate (Bn1) may prove valuable as a significant microbial control agent against lepidopteran pests.

Keywords: *Bacillus thuringiensis*, *Balaninus nucum*, *cry* gene, *Lymantria dispar*, *Malacosoma neustria*

The microbiological control of agricultural and forest-damaging insect pests has been often considered as a favorable alternative to the use of chemical insecticides. *Bacillus thuringiensis* is one of the most effectively utilized microbial pesticides thus far. Preparations of *B. thuringiensis* are extensively utilized as a safe and effective pesticide in horticulture and forestry (Kellar and Langenfruch, 1993; Teakle, 1994) and in the control of mosquitoes and black flies (Ritchie, 1993; Becker, 1997). The control of a host of pests of hazelnut and some fruits, relies heavily on chemical insecticides. However, the continued use of chemical pesticides over several decades has resulted in the emergence of resistance. In addition, problems of insecticide residues in meat and environmental concerns continue to complicate the issue. Therefore, interest in using alternative pest management strategies has been growing for some time. We have been exploring the development of a biopesticide for lepidopterans predicated on the bacterium, *B. thuringiensis*.

The most widely used *B. thuringiensis* insecticides are known as crystal toxins (Cry and Cyt proteins; also called δ-endotoxins). During sporulation, the bacterium synthesizes and packages one or more proteins into parasporal crystals. These proteins exert specific toxicity effects on a variety of insect species (Schnepf *et al.*, 1998; Kati *et al.*, 2005; Kurt *et al.*, 2005). When susceptible insect larvae ingest *B. thuringiensis* spore-crystals, the crystal δ-endotoxins are solubilized within the alkaline environment of the midgut, and these protoxins are then proteolytically cleaved by midgut proteases into active toxic peptides. The active toxin binds to the receptors on the surfaces of midgut cells, and is inserted into the membrane to form pores which disrupt transmembrane potential, thereby inducing an osmotic lysis of the cells lining the midgut (Schnepf *et al.*, 1998). Commercial preparations of *B. thuringiensis* (spore-crystal mixtures) have been registered as insecticides since 1961, and a significant quantity of toxicological data has been collected over recent years (Baum *et al.*, 1999).

This is the first study regarding the molecular characterization and pathogenicity of a strain of *B. thuringiensis* isolated from *B. nucum*. In this study, we have focused on the light and electron microscopy of spores and crystals, the SDS-PAGE analysis of the proteolytic products of crystals, plasmid content, *cry* gene profiles, and insecticidal activity of the *B. thuringiensis* strain isolated from *B. nucum*.

Materials and Methods

B. thuringiensis isolate and strains

The Bn1 strain of *B. thuringiensis* was isolated from *Balaninus* (*Curculio*) *nucum* L. (Coleoptera: Curculionidae) at the Microbiology Laboratory, Department of Biology at Karadeniz Technical University, Trabzon, Turkey (Sezen and Demirbağ, 1999). *B. thuringiensis* subsp. *tenebrionis* (Plant Genetic Systems J. Plateaustroat 22, 9000 Gent, Belgium), *B. thuringiensis* subsp. *kurstaki* HD-1 (Bacillus Genetic Stock Center, Columbus, Ohio) (Dean and Zeigler, 1994) and *B. thuringiensis* subsp. *israelensis* (DSM-5724) were utilized as reference strains. The serotyping of Bn1 was kindly conducted by M. Ohba

* To whom correspondence should be addressed.
(Tel) 90-454-216-1255; (Fax) 90-454-216-4518
(E-mail) hkati@ktu.edu.tr

and K. Kagoshima at the Kyushu University in Japan.

Microscopic examination

(a) *Light microscopy*: In order to obtain the spore-crystal mixture, the Bn1 isolate was grown in nutrient agar medium for 5 days at 30°C, until lysis. The spore-crystal mixtures were suspended in 1 ml of ice-cold 1 M NaCl and centrifuged for 5 min at 13,000×g. The pellets were suspended in distilled H_2O. The presence and morphology of crystals were recorded during the sporulation, via the direct examination of smears of this culture under light microscopy (100×), and verified via staining with Coomassie brilliant blue (0.25% solution in 50% ethanol and 7% acetic acid), as described by Sharif and Alaeddinoglu (1988).

(b) *Scanning electron microscopy*: The spore-crystal mixtures were air-dried on cover-glasses and then coated with gold. The spores and crystals were examined using a JSM 6400 scanning electron microscopy operated at 15 kV (JEOL, Japan).

16S rDNA sequencing

For 16S rDNA sequencing, DNA was extracted as previously described (Sambrook *et al.*, 1989). The amplification and sequencing of the nearly complete 16S rDNA gene was conducted in accordance with the methods described previously (Ben-Dov *et al.*, 1997). The PCR amplification conducted using the following UNI Primers: forward; 5'-ATT CTA GAG TTT GAT CAT GGC TCA-3', reverse; 5'-ATG GTA CCG TGT GAC GGG CGG TGT GTA-3'. The PCR product was cloned into pGEM-T and then the 16S rRNA gene sequnce was determined with an Applied Biosystems model 373A DNA sequencer, using the ABI PRISM cycle-sequencing kit. A sequence consisting of about 1,400 nt of the 16S rRNA gene of the isolates was determined. The sequence obtained was compared with those acquired from GenBank using the BLAST program (Altschul *et al.*, 1990).

SDS-PAGE

The spore-crystal mixture was re-suspended in sample buffer (60 mM Tris-HCl; pH 6.8, 25% glycerol, 5% 2-mercaptoethanol, 0.1% bromophenol blue), boiled for 10 min, and electrophoresed in 10% SDS-PAGE as described by Laemmli (1970). The gel was then stained with Coomassie brilliant blue. The solubilization of the spore-crystal mixture of the Bn1 isolate was conducted as previously described with minor modifications (Iriarte *et al.*, 2000). The spore-crystal mixture was incubated in solubilizing buffer [Na_2CO_3 50 mM, 0.1 M NaCl, 10 mM dithiothreitol (DTT), pH 11.3] for 2 h with continuous shaking (Hoefer). The solubilized protein was separated from the spores via 5 min of centrifugation at 13,000×g. Trypsin was then added at a ratio of 1:20 (trypsin/protein, w/w), and the suspension was inoculated for 4 h at 37°C. An identical ratio of trypsin was added after 1 and 2 h. The proteolytic products were then analyzed via 10% SDS-PAGE.

Plasmid isolation

The plasmid DNA of bacterial cells was extracted via a modification of the alkaline lysis technique (Jensen *et al.*, 1995; Porcar *et al.*, 1999). The cells were grown overnight in 5 ml of LB medium at 30°C with continuous shaking. Two milliliters of the culture were pelleted and re-suspended in 100 µl of TE buffer (40 mM Tris-HCl, 2 mM EDTA, pH 7.9). The cells were then lysed via the addition of 200 µl of lysing solution [3% (w/v) SDS, 15% (w/v) sucrose, 50 mM Tris-hydroxide, pH 12.5]. The mixture was then incubated for 30 min at 60°C, followed by the addition of five units of proteinase K. The tubes containing the reaction mixture were gently mixed and incubated for 90 min at 41°C. One ml of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the solution, and the tubes were inverted carefully several times. The samples were then centrifuged for 7 min at 6,000×g and 60 µl of the supernatant fluid harboring plasmid DNA were loaded directly on a 0.5% agarose gel prepared in 1× Tris Borate EDTA (TBE). Electrophoresis was conducted at 62 V and 4°C. The gels were run routinely for 5-7 h, stained overnight in diluted ethidium bromide, and then examined using a BioDoc Analysis System (Biometra GmbH, Germany).

Identification of cry genes

The DNA templates were prepared as previously described by Sambrook *et al.* (1989). The PCR mixtures were prepared using 0.5 µM of each specific primer, 0.5 U of *Taq* DNA polymerase, 150 µM (each) of deoxynucleoside triphosphate, 1× Tris buffer and 0.6 mM $MgCl_2$. The following *cry*-genes primers were used: (forward; 5'-CAT GAT TCA TGC GGC AGA TAA AC-3', reverse; 5'-TTG TGA CAC TTC TGC TTC CCA TT-3' for *cry1*-gene), (forward; 5'-GTT ATT CTT AAT GCA GAT GAA TGG G-3', reverse; 5'-CGG ATA AAA TAA TCT GGG AAA TAG T-3' for *cry2*-gene), (forward; 5'-CGT TAT CGC AGA GAG ATG ACA TTA AC-3', reverse; 5'-CAT CTG TTG TTT CTG GAG GCA AT-3' for *cry3*-gene) and (forward, 5'-GCA TAT GAT GTA GCG AAA CAA GCC-3'; reverse, 5'-GCG TGA CAT ACC CAT TTC CAG GTC C-3' for *cry4*-gene) (Ben-Dov *et al.*, 1997). Amplification was conducted in a thermal cycler using a single denaturation step (2 min at 95°C), followed by a 30 cycles program, each cycle of which consisted of a denaturation step of 94°C for 60 sec, an annealing step of 55°C for 55 sec, and an extension step of 72°C for 90 sec. A final 10 min extension step at 72°C was also included (Ben-Dov *et al.*, 1997). Each experiment was associated with negative (without DNA template) and positive (with *B. thuringiensis* subsp. *kurstaki* HD-1) controls. The PCR products were analyzed via 1.3% agarose gel electrophoresis. The gels were then assessed with a BioDoc Analysis System (Biometra GmbH, Germany).

Insects and bioassay

The insect toxicity tests were conducted with Lepidoptera, Diptera, and Coleoptera larvae. Additionally, it was tested against its hosts (Coleoptera: Curculionidae: *Balaninus nucum* L.). The tested insects were as follows: *Galleria mellonella* L. (Lepidoptera: Pyralidae), *Hyphantria cunea* Drury. (Lepidoptera: Arctiidae), *Lymantria dispar* L. (Lepidoptera Lymantridae), *Malacosoma neustria* L. (Lepidoptera: Lasicampidae), *Leptinotarsa decemlineata* L. (Coleoptera: Chrysomelidae) second and third stadium larvae and *Drosophila melanogaster* Meig adults (Diptera: Drosophilidae). The diets were prepared from fresh

hazelnut leaves for the lepidopteran and *B. nucum* larvae, fresh potato leaves for *L. decemlineata* larvae and an artificial diet for dipteran adults. The diets were placed in individual sterilized glass containers (80 mm in diameter). The spore-crystals mixtures (10^9 spores-crystals/ml) were applied to the surface of the experimental diets. Ten second and third stadium larvae and adults were placed on the diet in containers. The containers were maintained at 26±2°C and 60% RH on a 12:12 h photocycle, with the diet changed after eating. The mortalities of larvae were recorded every 24 h, and all dead larvae were removed from the containers. The *B. thuringiensis* subsp. *kurstaki* HD-1 strain was utilized as a positive control and sterilized dH_2O was employed as a negative control. Mortality was assessed 3 days after the initiation of treatment. At least 30 larvae were assayed for each of the isolates. All bioassays were repeated three times on different occasions. The means were assessed via one-way analysis of variance (ANOVA) and compared by the least significant difference (LSD) test (Minitab, 1997).

Results and Discussion

The Bn1 isolate of *B. nucum* L. was analyzed via light microscopy and revealed the presence of crystals. The ultra-structural analysis of the crystals and spores of the Bn1 isolate was obtained via scanning electron microscopy. As is shown in Fig. 1, the bacterial cells evidenced a bipyramidal and cubic crystal shape. According to Bernhard *et al.* (1997), initial studies of *B. thuringiensis* isolates recorded in the literature reported that the bipyramidal crystals are more commonly obtained, and are more toxic to lepidopteran larvae. However, the correlation between the crystal morphology and the level of insecticidal activity remains to be clearly elucidated (Bohorova *et al.*, 1996).

The nearly complete 16S rDNA sequence of the Bn1 strain evidenced 99% similarity with those of *B. thuringiensis* (Stackebrandt and Goebel, 1994). A total of 1,413 nucleotides of the 16S rDNA from this isolate were deposited in the GenBank sequence database under the accession number EU009208.

In addition to the results of SEM and molecular analyses, the serotyping of this isolate identified it as the *B. thuringiensis* subsp. *kurstaki* serotype H3abc (data not shown).

The presence of *cry* genes in this isolate was assessed via PCR using general *cry1*, *cry2*, *cry3*, and *cry4* primers and DNA from the Bn1 isolates. We observed two fragments, with sizes of approximately 272 and 725 bp (Fig. 2). These fragments corresponded to the expected sizes of the *cry1* and *cry2* genes (Ben-Dov *et al.*, 1997). The *cry3* and *cry4*

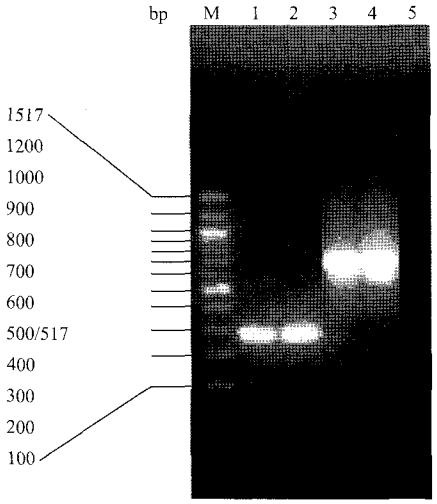

Fig. 2. Agorose gel electrophoresis analysis of PCR products obtained by using the *cry1* and *cry2* general primers pairs. Lanes M, Marker (100 bp DNA Ladder); 1, *B. thuringiensis* subsp. *kurstaki* HD-1 (*cry1*); 2, Bn1 (*cry1*); 3, *B. thuringiensis* subsp. *kurstaki* HD-1 (*cry2*); 4, Bn1 (*cry2*); 5, Negative control (without DNA).

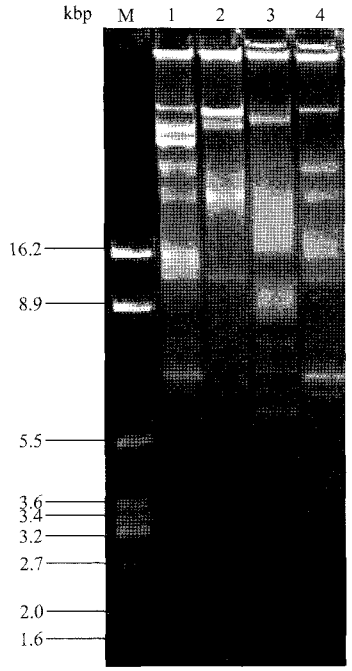

Fig. 3. Plasmid pattern of *B. thuringiensis* subsp. *kurstaki* HD-1 (lane 1), *B. thuringiensis* subsp. *israelensis* (lane 2), *B. thuringiensis* subsp. *tenebrionis* (lane 3), and Bn1 isolate (lane 4). M, Marker (DNA digested with *Hind*III, *Bam*HI and *Eco*RI).

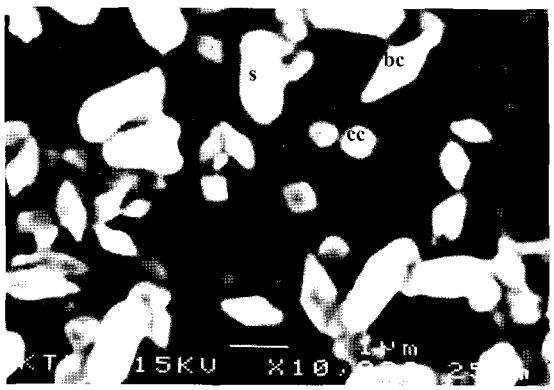

Fig. 1. Scanning electron micrograph of *B. thuringiensis* (Bn1) isolate. s, spore; bc, bipyramidal crystal; cc, cubic crystal. Bar=1 μm.

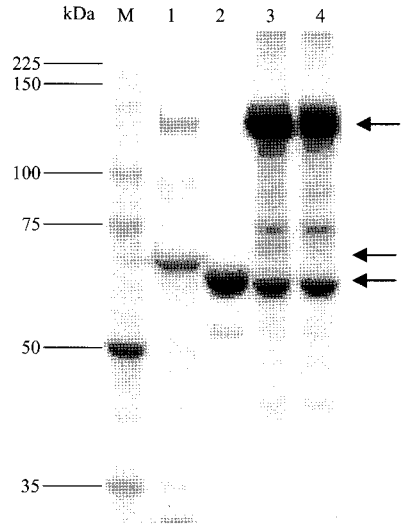

Fig. 4. SDS-PAGE analysis of crystals of Bn1 isolate. Lanes M, Molecular weight markers; 1, *B. thuringiensis* subsp. *israelensis*; 2, *B. thuringiensis* subsp. *tenebrionis*; 3, *B. thuringiensis* subsp. *kurstaki* HD-1; 4, Isolate Bn1.

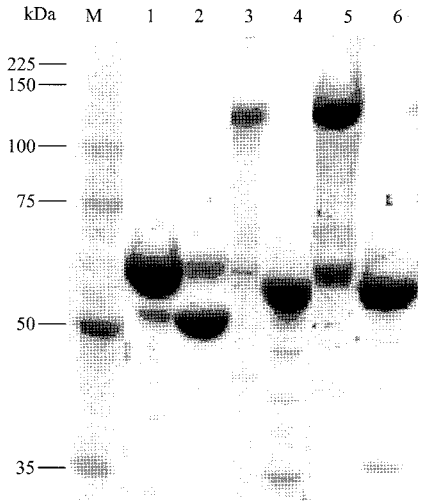

Fig. 5. SDS-PAGE of undigested (lane 1, 3, and 5) and trypsin digested (lane 2, 4, and 6) crystal proteins of *B. thuringiensis* subsp. *tenebrionis* (lane 1 and 2); *B. thuringiensis* subsp. *kurstaki* HD-1 (lane 3 and 4); Isolate Bn1 (lane 5 and 6).

genes were not detected in this isolate. Cry1 is toxic to Lepidoptera, Coleoptera, and Diptera (Zhong *et al.*, 2000); and Cry2 is active against Lepidoptera and/or Diptera (Donovan *et al.*, 1988).

The plasmid DNA patterns of the Bn1 isolate were compared with the profile arrays of the reference strains, *B. thuringiensis* subsp. *kurstaki* HD-1, *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *tenebrionis* (Fig. 3). The Bn1 strain was determined to have several relatively large plasmids which may harbor *cry* genes. The *cry* genes described thus far are located on large plasmids (Lereclus *et al.*, 1993). The plasmid profile of the Bn1 isolate was different than that of the reference strain, *B. thuringiensis* subsp. *kurstaki* HD-1. The Bn1 isolate harbors one larger plasmid, but the *B. thuringiensis* subsp. *kurstaki* harbors three larger plasmids. The *B. thuringiensis* isolates belonging to the same strain may have different plasmid profiles, increasing even more apparently the heterogeneity of the species (Aptosoglou *et al.*, 1997).

Polyacrylamide gel electrophoresis was conducted in order to determine the presence of Cry proteins in the total protein extracts of the Bn1 isolate. The result shown in Fig. 4 clearly indicated the presence of proteins with molecular masses of approximately 65 and 130 kDa in the Bn1 strain. The detection of protein bands of approximately 65 and 130 kDa was consistent with the PCR tests, as this is the approximate mass of the Cry1 toxins (Honigman *et al.*, 1986). The crystals of the Bn1 isolate were solubilized completely after 2 h of incubation and trypsinization. These crystal proteins yielded a trypsin-resistant peptide of approximately 60 kDa (Fig. 5). The size of the digested product of Bn1 was similar to that of the Cry1 toxins which range between 60 and 70 kDa (Adang, 1991). Therefore, Bn1 was determined to exhibit insecticidal activity against 3 of the 4 species tested of the lepidopteran species included in this study. Other dipteran and lepidopteran insects were determined to be non-susceptible to Bn1.

The bioassays were conducted with Lepidoptera larvae; *Galleria mellonella* L., *Hyphantria cunea* Drury., *Lymantria dispar* L., *Malacosoma neustria* L., and Coleoptera larvae; *Balaninus nucum* L. and *Leptinotarsa decemlineata* L., and Diptera adults; *Drosophila melanogaster* Meig using 10^9 spores-crystals/ml mixture. The data results, which are presented in Table 1, indicate that the crystal-spore mixture from the Bn1 isolate was highly toxic against *Malacosoma neustria* and *Lymantria dispar* larvae ($p<0.05$). However, the 90%

Table 1. The insecticidal effects of spore-crystal mixture (10^9 spore-crystal/ml) of Bn1 isolate on lepidopteran and coleopteran larvae. ANOVA LSD test, $p<0.05$

Isolates	Mortality (%) (Mean±SD)				
	M. neustria	*L. dispar*	*H. cunea*	*B. nucum*	*L. decemlineata*
Bn1	90±5	90±3	30±7	45±5	25±5
+ Control[a,b]	83±3[a]	90±5[a]	43±5[a]	25±5[a]	15±4[a]
				50±4[b]	30±5[b]
- Control[c]	-	-	-	-	-

[a] *B. thuringiensis* subsp. *kurstaki* HD-1
[b] *B. thuringiensis* subsp. *tenebrionis*
[c] steril ddH_2O

value observed against *M. neustria* was 7% higher than that of the crystal-spore mixture of the reference strain, *B. thuringiensis* subsp. *kurstaki* HD-1. However, the toxicity of the Bn1 isolate against the tested lepidopteran *G. mellonella* larvae and dipteran *D. melanogaster* adults was not determined.

Our results show that Bn1 from *B. nucum* is a strain of *B. thuringiensis* subsp. *kurstaki*. It evidences features almost identical to *B. thuringiensis* subsp. *kurstaki* HD-1. However, the insecticidal activity of this new isolate is higher than that of the reference strain. Further study will involve the characterization of novel *B. thuringiensis* strains and provide good sources for the development of microbial pesticides against *M. neustria* and other lepidopteran pests.

Acknowledgments

This work was supported by the Karadeniz Technical University Research Foundation (KTU 21.111.004.4) and the Turkish Republic Prime Ministry State Planning Organization (21.111.004.1). The authors would like to thank Dr. Michio Ohba and Dr. Kumiko Kagoshima for providing the H-Serotyping, to Dr. Dan Zeigler for providing the reference *B. thuringiensis* subsp. *kurstaki* HD-1, and to Dr. Stefan Jansens for providing *B. thuringiensis* subsp. *tenebrionis*.

References

Adang, M.J. 1991. *Bacillus thuringiensis* insecticidal crystal proteins: gene structure, action and utilization, p. 3-24. *In* K. Maramorosch (ed.), Biotechnology for biological control of pests and vectors. CRC Press, Boston, USA.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215, 403-410.

Aptosoglou, S.G., A. Sivropoulou, and S.I. Koliais. 1997. Plasmid patterns of *Bacillus thuringiensis* strains and isolates. *Microbios* 91, 203-214.

Baum, J.A., T.B. Johnson, and B.C. Carlton. 1999. *Bacillus thuringiensis*: natural and recombinant bioinsecticide products, p. 189-210. *In* F.R. Hall, N.J. Totowa, and J.J. Menn (eds.), Biopesticides: use and delivery, Humana Press, Totowa, NJ, USA.

Becker, N. 1997. Microbial control of mosquitoes: management of the upper rhine mosquito population as a model programme. *Parasitol. Today* 13, 485-487.

Ben-Dov, E., A. Zaritsky, E. Dahan, Z. Barak, R. Sinai, R. Manasherob, A. Khameraev, E. Troitskaya, A. Dubitsky, N. Berezina, and Y. Margalith. 1997. Extended screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* 63, 4883-4890.

Bernhard, K., P. Jarret, M. Meadows, D.J. Ellis, G.M. Roberts, S. Pauli, P. Rodgers, and H.D. Burges. 1997. Natural isolates of *Bacillus thuringiensis*: worldwide distribution, characterization, and activity against insects pests. *J. Invertebr. Pathol.* 70, 59-68.

Bohorova, N., A.M. Maciel, R.M. Brito, L. Aguilat, J.E. Ibarra, and D. Hoisington. 1996. Selection and characterization of Mexican strains of *Bacillus thuringiensis* active against from major lepidopteran maize pests. *Entomophaga* 41, 153-165.

Dean, D.H. and D.R. Zeigler. 1994. *Bacillus* genetic stock centers and data, 6th ed. Ohio State University Press, Colombus, USA.

Donovan, W.P., C.C. Dankocsik, M.P. Gilbert, M.C. Gawron-Burke, R.G. Groat, and B.C. Carlton. 1988. Amino acid sequence and entomocidal activity of the P2 crystal protein. An insect toxin from *Bacillus thuringiensis* var. *kurstaki*. *J. Biol. Chem.* 263, 561-567.

Honigman, A., G. Nedjar-Pazerin, A. Yawetz, U. Oron, S. Schuster, M. Broza, and B. Snek. 1986. Cloning and expression of the Lepidopteran toxin produced by *Bacillus thuringiensis* in *Escherichia coli*. *Gene* 42, 69-77.

Iriarte, J., V.C. Dumanoir, Y. Bel, M. Porcar, M.D. Ferrandis, M.M. Lecadet, J. Ferre, and P. Caballero. 2000. Characterization of *Bacillus thuringiensis* ser. *balearica* (Serotype H48) and ser. *navarrensis* (Serotype 50): Two novel serovars isolated in Spain. *Curr. Microbiol.* 40, 17-22.

Jensen, G.B., A. Wilcks, S.S. Petersen, J. Damgaard, J.A. Baum, and L. Andrup. 1995. The genetic basis of the aggregation system in *Bacillus thuringiensis* subsp. *israelensis* is located on the large conjugative plasmid pXO16. *J. Bacteriol.* 177, 2914-2917.

Kati, H., K. Sezen, A.O. Beldüz, and Z. Demirbağ. 2005. Characterization of a *Bacillus thuringiensis* subsp. *kurstaki* strain isolated from *Malacosoma neustria* L. (Lepidoptera: Lasiocampidae). *Biologia* 60, 301-305.

Kellar, B. and G.A. Langenfruch. 1993. Control of coleopteran pests by *Bacillus thuringiensis*, p. 171-191. *In* P.F. Entwhistle, J.S. Cory, M.J. Bailey, and S. Higgs (eds.), *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. Wiley, New York, USA.

Kurt, A., M. Özkan, K. Sezen, Z. Demirbağ, and G. Özcengiz. 2005. Cry3Aa11: A new Cry3Aa δ-endotoxin from a local isolate of *Bacillus thuringiensis*. *Biotechnol. Lett.* 27, 1117-1121.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lereclus, D., A. Delecluse, and M.M. Lecadet. 1993. Diversity of *Bacillus thuringiensis* toxins and genes, p. 7-70. *In* P.F. Entwistle, J.S. Cory, M. Bailey, and S. Higgs (eds.), *Bacillus thuringiensis* an environmental biopesticide: theory and practice. Wiley, New York, USA.

Minitab, 1997. User's Guide, Release 11. Minitab, State College, PA, USA.

Porcar, M., J. Iriarte, V.C. Dumanoir, M.D. Ferrandis, M.M. Lecadet, J. Ferre, and P. Caballero. 1999. Identification and characterization of the new *Bacillus thuringiensis* serovars *pirenaica* (serotype H57) and *iberica* (serotype H59). *J. Appl. Microbiol.* 87, 640-648.

Ritchie, S. 1993. *Bacillus thuringiensis* subsp. *israelensis* use in Australia: the opportune moment, p. 111-115. *In* R.J. Akhurst (ed.), Proceedings of the second canberra *Bacillus thuringiensis* meeting. CSIRO, Canberra, USA.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning, 2nd ed. Cold Spring, Harbor Laboratory Press, New York, USA.

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

Sezen, K. and Z. Demirbağ. 1999. Isolation and insecticidal activity of some bacteria from the hazelnut beetle (*Balaninus nucum* L.). *Appl. Entomol. Zool.* 34, 85-89.

Sharif, F.A. and N.G. Alaeddinoglu. 1988. A rapid and simple method for staining of the crystal protein of *Bacillus thuringiensis*. *J. Ind. Microbiol.* 3, 227-229.

Stackebrandt, E. and B.M. Goebel. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* 44, 846-849.

Teakle, R.E. 1994. Present use of, and problems with, *Bacillus thuringiensis* in Australia. *Agric. Ecosyst. Environ.* 49, 39-44.

Zhong, C., D.J. Ellar, A. Bishop, C. Johnson, S. Lin, and E.R Hart. 2000. Characterization of a *Bacillus thuringiensis* δ-endotoxin which is toxic to insects in three orders. *J. Invertebr. Pathol.* 76, 131-139.