Folia Microbiol. **52** (2), 146–152 (2007) **http://www.biomed.cas.cz/mbu/folia/**

# Characterization of a Highly Pathogenic *Bacillus thuringiensis* Strain Isolated from Common Cockchafer, *Melolontha melolontha*

H. KATI[a], K. SEZEN[b], Z. DEMIRBAĞ[b]

[a]*Department of Biology, Faculty of Arts and Sciences, Giresun University, 28049 Giresun, Turkey*
[b]*Department of Biology, Faculty of Arts and Sciences, Karadeniz Technical University, 61080, Trabzon, Turkey*
fax +90 454 216 4518
e-mail hkati@ktu.edu.tr

*Received 3 May 2006*
*Revised version 20 September 2006*

**ABSTRACT.** A bacterial isolate (Mm2) of *Melolontha melolontha* was identified and characterized. Based on various morphological, physiological, biochemical and molecular characteristics, it was identified as *Bacillus thuringiensis* subsp. *tenebrionis*. This isolate was compared to the reference strains by electron microscopy, SDS-PAGE analysis, plasmid pattern, *cry* gene content and insecticidal activity. Cells of the isolate harbored flat square inclusions containing a protein component of ≈65 kDa. After trypsin digestion of solubilized crystals, SDS-PAGE resolved a unique proteinase-resistant peptide of ≈50 kDa. Plasmid pattern showed similar bands to those of the reference strain, PCR analysis showed that the isolate has *cry3* gene. Toxicity tests (against 5 coleopteran species) showed 80 % insecticidal activity against the larvae of *M. melolontha*. The isolate Mm2 may be valuable as biological control agent for *M. melolontha* and other coleopteran insects.

*Abbreviations*

| | | | |
|---|---|---|---|
| PCR | polymerase chain reaction | SCM | spore–crystal mixture |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis | | |

Strains of *Bacillus thuringiensis*, a $G^+$, spore-forming bacterium, are strongly recommended against agricultural pests in biological control. The organism was first isolated from diseased silkworm (*Bombyx mori*) by Ishiwata (1901) and is naturally present in both live and dead insects (Damgaard *et al.* 1997).

*B. thuringiensis* is characterized by the production of parasporal crystals composed of protein molecules known as δ-endotoxins, insecticidal crystal proteins (ICP) or crystal proteins (Cry proteins) that are toxic for the larvae of various insects (Höfte and Whiteley 1989). ICPs produced during the sporulation of *B. thuringiensis* strains show specific insecticidal activity against insect species of different orders, such as Coleoptera, Diptera, Homoptera, Hymoneptera, Lepidoptera, Mallophaga and Orthoptera (Höfte and Whiteley 1989; Feitelson *et al.* 1992; Becker and Margalit 1993). Crystal proteins from numerous strains have been classified based on the similarity of their amino acid sequences and their insecticidal specificity (Höfte and Whiteley 1989).

Investigations for new *cry* genes are an on-going effort worldwide. So far 321 *cry* genes have been reported and classified into *cry1–cry51* according to the degree of amino acid sequence homology (a full list is at **http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/**). *B. thuringiensis* crystalline inclusions ranging in molar mass from 27 to 140 kDa are protoxins that are proteolytically converted into smaller toxic polypeptides in the larval midgut (Peferoen 1997). Midgut proteinases process the protoxin to form a stable 55–70 kDa toxin core (Bietlot *et al.* 1990). Crystal protein genes are located as satellite chromosome and/or chromosomal DNA (Kronstad *et al.* 1983).

Isolation and characterization of *B. thuringiensis* from insects is important for evaluating relevant biological control agents against target insects. *Melolontha melolontha* (common cockchafer) is one of the most important agricultural pests (Froschle 1994; Luisa and Mauro 1996; Vlug 1996; Sezen 2004). It causes various types of damage on hazelnut, forest trees and vegetables. At present 2 pesticides (endosulphan and chlorpyrifos-ethyl) are used in Turkey. Increasing interest in developing environmentally safe pest control methods has inspired us to study the potential of bacteria for controlling *M. melolontha*. Recently, the use of crystal proteins which are effective and safer than chemical control insecticides, is finding an ever increasing application worldwide. Here a bacterial isolate (Mm2) isolated from *M. melolontha* was identified in detail and tested for its insecticidal activity.

## MATERIAL AND METHODS

Bacillus thuringiensis *strains*. Mm2 strain was isolated from *Melolontha melolontha* (*Coleoptera*:*Scarabaeidae*) in the *Microbiology Laboratory, Department of Biology, Karadeniz Technical University*, Trabzon (Turkey). Reference strains were

*B. thuringiensis* subsp. *tenebrionis* (*Plant Genetic Systems*, 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).

*Isolation and identification of Mm2 from* M. melolontha. Second third-stage collected larvae of *M. melolontha* were surface sterilized with 70 % ethanol. In some cases, attempts were made to isolate the bacteria from hemolymph; in other cases the insect body was triturated. The hemolymph and the larval suspension were heated (10 min, 80 °C) in a water bath to eliminate the nonspore-forming bacteria (Ohba and Aizawa 1986; Lee *et al.* 1992; Thiery and Frachon 1997). One-hundred μL volumes of the heat-treated samples were spread on nutrient agar plates and incubated (2–4 d, 28 °C) (Lee *et al.* 1995), then examined and bacterial colonies were selected based on color and morphology. Pure cultures of bacterial colonies were then prepared and identified by tests (for the isolates) for utilization of organic compounds, spore formation, NaCl tolerance, optimum pH, optimum temperature, catalase test, oxidase test and gelatine hydrolysis.

The identification procedure of isolated bacteria was done according to *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt 1986; Sneath *et al.* 1986).

*Microscopic examination.* (*a*) Light microscopy: In order to obtain SCM, Mm2 was grown in nutrient agar medium (5 d, 30 °C) until lysis. SCM were suspended in 1 mL of ice-cold 1 mol/L NaCl and centrifuged (13 000 ***g***, 5 min). The pellet was suspended in distilled $H_2O$. The presence and morphology of crystals was recorded during sporulation, by direct examination of the smear of this culture under light microscope (100×), and confirmed by staining with Coomassie brilliant blue (0.25 % solution in 50 % ethanol and 7 % acetic acid) (Sharif and Alaeddinoğlu 1988).

(*b*) Scanning electron microscopy: SCM were air-dried on cover slips and coated with gold. Spores and crystals were examined with a JSM 6400 scanning electron microscope operated at 15 kV.

*Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.* SCM was resuspended in sample buffer (60 mmol/L Tris-HCl (pH 6.8), 25 % glycerol, 5 % 2-sulfanylethanol, 0.1 % bromophenol blue), boiled for 10 min, and electrophoresed in 10 % SDS-PAGE (Laemmli 1970). The gel was then stained with Coomassie brilliant blue.

*Solubilization and proteolytic digestion of crystal proteins.* Solubilization of SCM of the Mm2 isolate was performed according to Iriarte *et al.* (2000) with minor modifications. SCM was incubated in solubilizing buffer (in mmol/L: NaCl 100, $Na_2CO_3$ 50, 2,3-dithiothreitol 10; pH 11.3) for 2 h under continuous shaking (*Hoefer*). Solubilized protein was separated from the spores by centrifugation (13 000 ***g***, 5 min). Trypsin was added in a ratio of 1 : 20 (trypsin-to-protein, *M*/*M*), and the suspension was inoculated at 37 °C for 4 h. The same ratio of trypsin was added after 1 and 2 h. The proteolytic products were analyzed by 10 % SDS-PAGE.

*Plasmid isolation.* Cells were grown overnight in 5 mL Luria–Bertani (LB) medium at 30 °C with continuous shaking. Two mL of the culture was pelleted and resuspended in 100 μL TE buffer (40 mmol/L Tris-HCl, 2 mmol/L EDTA, pH 7.9). Cells were then lysed by the addition of 200 μL lysing solution (3 % (*M*/*V*) SDS, 15 % (*M*/*V*) sucrose, 50 mmol/L Tris-hydroxide, pH 12.5). The mixture was incubated (30 min, 60 °C), followed by the addition of 5 U of proteinase K. Tubes containing the reaction mixture were gently mixed and incubated (90 min, 41 °C). One mL of phenol–chloroform–3-methylbutan-1-ol (25 : 24 : 1) was added to the solution and the tubes were carefully inverted several times. The samples were then centrifuged (6000 ***g***, 7 min) and 60 μL of the supernatant fluid containing plasmid DNA was directly loaded on a 0.5 % agarose gel prepared in a 1× TBE (Tris-borate, EDTA). Electrophoresis was carried out at 62 V and 4 °C. Gels were routinely run for 5–7 h, stained overnight with diluted ethidium bromide and then examined in BioDoc Analyse System (*Biometra*).

*Oligonucleotide primers.* Universal primers used for the detection of subgroups of *cry* genes were as follows:

*cry1* F, 5′-CAT GAT TCA TGC GGC AGA TAA AC-3′
R, 5′-TTG TGA CAC TTC TGC TTC CCA TT-3′

*cry2* F, 5′-GTT ATT CTT AAT GCA GAT GAA TGG G-3′
R, 5′-CGG ATA AAA TAA TCT GGG AAA TAG T-3′

*cry3* F, 5′-CGT TAT CGC AGA GAG ATG ACA TTA AC-3′
R, 5′-CAT CTG TTG TTT CTG GAG GCA AT-3′
*cry4* F, 5′-GCA TAT GAT GTA GCG AAA CAA GCC-3′
R, 5′-GCG TGA CAT ACC CAT TTC CAG GTC C-3′ (Ben-Dov *et al.* 1997).

*DNA templates and PCR analysis.* DNA templates were prepared according to Sambrook *et al.* (1989). Reactions were routinely carried out in 25 μL: 1 μL of template DNA was mixed with reaction buffer, 150 μmol/L (each) dNTP, 0.5 μmol/L (each) primer, and 0.5 U of *Taq* DNA polymerase. Amplification was carried out in a DNA thermal cycler (*Hybaid*) using the step-cycle program described (Ben-Dov *et al.* 1997) for identification of *cry1–cry4* genes. The program included 30 cycles, each cycle consisting of denaturation (60 s, 94 °C), annealing (50 s, 55 °C), extension (90 s, 72 °C), and final extension (10 min, 72 °C). Each experiment was associated with negative (without DNA template) and positive (with *B. thuringiensis* subsp. *tenebrionis*) controls. PCR products were analyzed by 1.3 % agarose gel electrophoresis. Then gel was examined in BioDoc Analyse System (*Biometra*).

*Insect toxicity assays*. Toxicity assays were carried out with the SCM of Mm2 isolate and standard strain (*B. thuringiensis* subsp. *tenebrionis*). Ten insects were transferred to dishes. Mm2 suspension contained ≈$10^9$ SCM/mL. Bioassays with coleopteran larvae of *Agelastica alni* L. (*Chrysomelidae*), *Amphimallon solstitiale* L. (*Scarabaeidae*), *Leptinotarsa decemlineata* SAY (*Chrysomelidae*) and *Melolontha melolontha* L. (*Scarabaeidae*), and adults of *Anoplus roboris* SUFR. (*Curculionidae*), were performed with the SCM applied on the diet. Mortality was recorded 3 d after initiation of the treatment. Infectivity test was carried out, with positive and negative controls, at least 30 larvae being assayed for each isolate. All bioassays were repeated 3× on different occasions. The data were analyzed using one-way analysis of variance (ANOVA) and compared by the least significant difference (LSD) test (Minitab 1997).

## RESULTS AND DISCUSSION

Finding an effective and safer biological control agent against hazardous insects is a matter of increasing interest. Our *B. thuringiensis* collection comprises several strains isolated from soil and dead insect larvae. Some of these strains have been characterized and tested against some members of the *Coleoptera*, *Diptera* and *Lepidoptera* orders (*unpublished data*).

Here we report on identification and the results of insecticidal activity of a local isolate of *B. thuringiensis* (Mm2) isolated from *M. melolontha*. Morphological, nutritional, physiological and biochemical characteristics of Mm2 are given in Table I. These tests showed that this bacterium is *B. thuringiensis* (Bucher 1981; Sneath *et al.* 1986; Thiery and Frachon 1997). Serological studies indicated that H antigenic

**Table I.** The morphological, physiological and biochemical features of Mm2 isolate

| Test | Feature | Test | Feature | Test | Feature |
|---|---|---|---|---|---|
| Colony color | cream | hydrolysis of urea | – | tyrosinase production | – |
| Shape of bacteria | bacillus | utilization of citrate | + | hydrolysis of phenylalanine | – |
| Gram staining | + | propionate | – | casein | + |
| Spore staining | + | test, indole | – | egg-yolk lecithinase | + |
| Shape of spores | ellipsoid | Methyl red | + | growth at pH > 7 MVRP | + |
| Location of spores | central | Voges–Proskauer | – | pH < 6 MVRP | + |
| Length, μm | 3.13–4.75 | growth with lysozyme at °C | + | fermentation of glucose | + |
| Width, μm | 0.85–1.04 | 4 | –[a] | arabinose | – |
| Motility | + | 40 | + | xylose | – |
| Nitrate reduction | + | 41 | –[a] | mannitol | – |
| Hydrolysis of starch | + | 50 | – | sucrose | + |
| Oxidase | – | utilization of L-arginine | + | optimum pH | 6–8 |
| Hydrolysis of gelatine | + | hydrolysis of Tween-80 | + | | |

[a]Not determined.

structure of this isolate is identical with *B. thuringiensis* subsp. *tenebrionis* (H8ab, *morrisoni*). *B. thuringiensis* has been also isolated from other hazelnut pests, *Balaninus nucum* (*Coleoptera*:*Curculionidae*) (Sezen and Demirbag 1999) and *Malacosoma neustria* (*Lepidoptera*:*Lasiocampidae*) (Katı *et al.* 2005).

The crystal morphology of Mm2 was observed in light (*data not shown*) and scanning electron microscope (Fig. 1). The isolate contained both spores and crystals that were released from the rod-shaped vegetative cells. The crystals are the most distinguishing characteristics of *B. thuringiensis* strains (Gonzales *et al.* 1981). Therefore, Mm2 isolate was identified as *B. thuringiensis* strain on the basis of the crystal formation produced when cultured on nutrient agar medium. Examination by a scanning electron microscope showed that the isolate had a flat square crystals, morphological characteristics being similar to those of other *B. thuringiensis* strains (Höfte and Whiteley 1989).

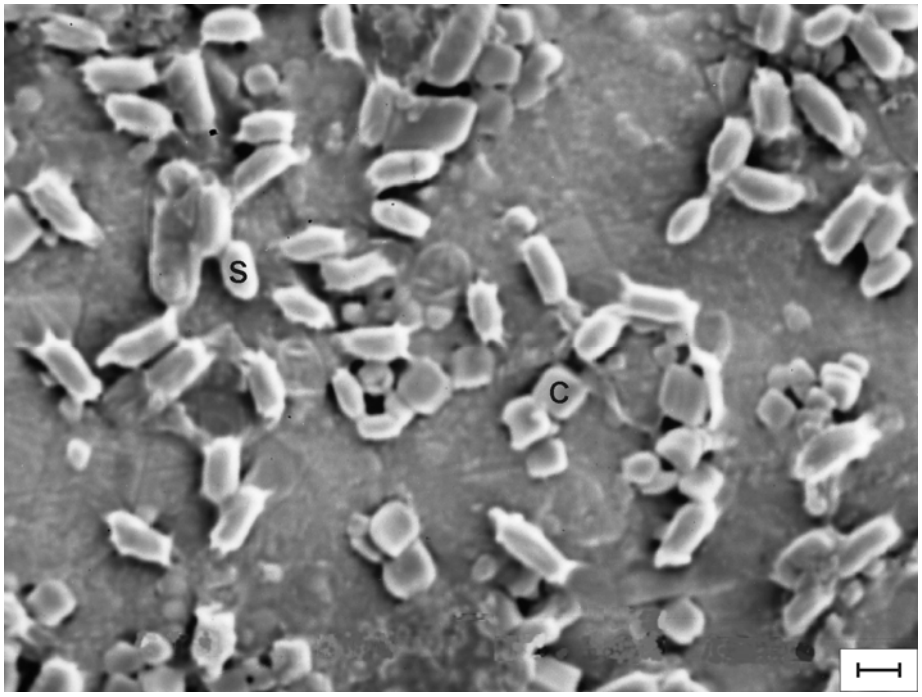


**Fig. 1.** Scanning electron micrograph of *B. thuringiensis* Mm2 isolate; S – spore, C – flat square crystal; *bar* = 1 μm.

The SDS-PAGE protein components of *B. thuringiensis* subsp. *tenebrionis*, *B. thuringiensis* subsp. *kurstaki* HD-1, *B. thuringiensis* subsp. *israelensis* and Mm2 isolate were examined using SDS-PAGE (Fig. 2). The results of analysis of Mm2 crystals gave ≈65-kDa protein bands similar to those of *B. thuringiensis* subsp. *tenebrionis*. This protein of Mm2 is similar in size to those reported for Cry3 protein (Honigman *et al.* 1986). Crystals of Mm2 isolate were completely solubilized after 2-h incubation followed by trypsinization. This crystal protein yielded a trypsin-resistant peptide of ≈50 kDa (Fig. 3). Mm2 was found to have insecticidal activity against all of the coleopteran species included here.

The plasmid DNA patterns of Mm2 were compared with the profile arrays of the reference strains *B. thuringiensis* subsp. *tenebrionis*, *B. thuringiensis* subsp. *kurstaki* HD-1 and *B. thuringiensis* subsp. *israelensis*, and Mm2 isolate gave patterns identical to those of *B. thuringiensis* subsp. *tenebrionis* (Fig. 4). Plasmids with molar mass >16.2 kbp were detected. The *cry* genes so far described have been located on large plasmids (Lereclus *et al.* 1993).

We used a PCR-based method to allow rapid and highly sensitive determination of the *cry* gene content of Mm2 isolate. DNA amplification was carried out using universal primers (*cry1–cry4*). PCR showed that Mm2 isolate had one *cry* gene, *viz. cry3* (Fig. 5). Fragment with the expected sizes of ≈589 bp corresponding to *cry3* gene was amplified with DNA from the Mm2 isolate (Ben-Dov *et al.* 1997). The Mm2 isolate contained the *cry3* gene similarly to the reference strain, *B. thuringiensis* subsp. *tenebrionis* (Höfte and Whiteley 1989).

Our *B. thuringiensis* strain has a potential use for insect pest control. The SCM of Mm2 showed toxicity against *A. alni*, *A. solstitiale*, *A. roboris*, *L. decemlineata* and *M. melolontha* (Table II). The recorded mortality was 76 % for *A. alni* larvae, 75 % for *A. solstitiale* larvae, 60 % for *A. roboris* adults, 90 % for *L. decemlineata* larvae and 80 % for *M. melolontha* larvae (ANOVA LSD, $p < 0.05$). These values were 16, 15, 20, 20 and 10 % higher, respectively, than those of the SCM of a reference strain (*B. thuringiensis* subsp. *tenebrionis*). The capacity of this strain to control several coleopteran pests is clear, and the strain is δ-endo-

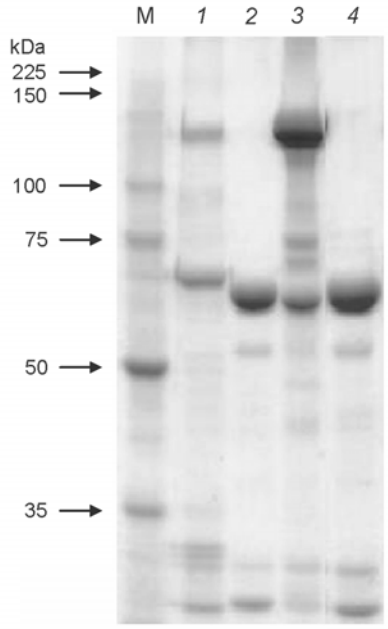


**Fig. 2.** SDS-PAGE analysis of crystals of Mm2 isolate; M – molar mass markers, *1* – *B. thuringiensis* subsp. *israelensis*, *2* – *B. thuringiensis* subsp. *tenebrionis*, *3* – *B. thuringiensis* subsp. *kurstaki* HD-1, *4* – Mm2 isolate.

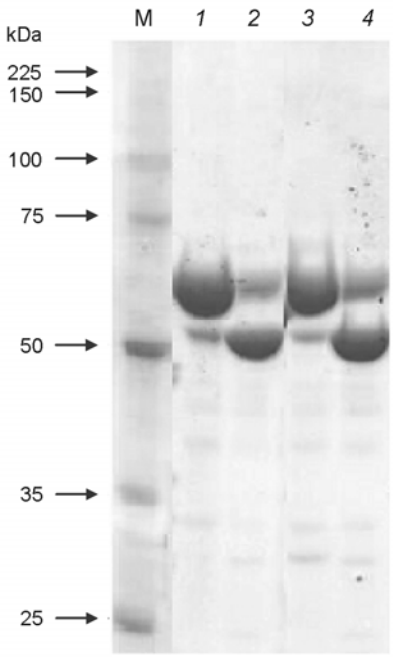


**Fig. 3.** SDS-PAGE analysis of undigested and trypsin-digested crystal proteins of Mm2; M – molar mass markers, *1* and *2* – undigested and digested *B. thuringiensis* subsp. *tenebrionis*, *3* and *4* – undigested and digested Mm2 isolate.

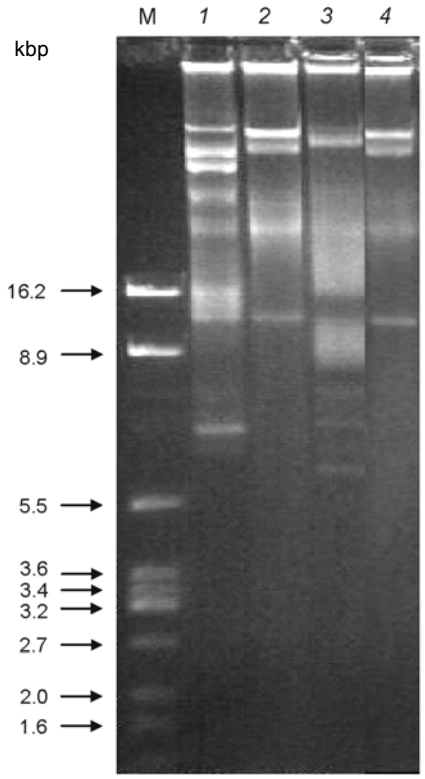


**Fig. 4.** Plasmid pattern of Mm2 isolate; M – marker (DNA digested with *Hin*dIII, *Bam*HI, and *Eco*RI), *1* – *B. thuringiensis* subsp. *kurstaki* HD-1, *2* – *B. thuringiensis* subsp. *tenebrionis*, *3* – *B. thuringiensis* subsp. *israelensis*, *4* – Mm2 isolate.

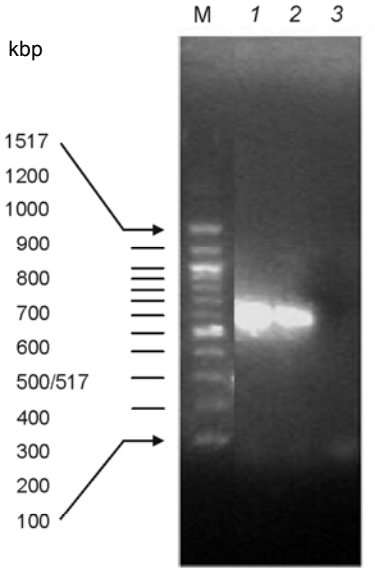


**Fig. 5.** Agarose gel electrophoretic analysis of PCR products obtained by using the *cry3* general primers; M – marker (100-bp DNA ladder), *1* – *B. thuringiensis* subsp. *tenebrionis* (*cry3*), *2* – Mm2 isolate (*cry3*), *3* – negative control (without DNA).

toxin producer specific for coleopteran pests including insects from the *Chrysomelidae*, *Curculionidae* and *Scarabaeidae* families. Tamez-Guerra *et al.* (1998) reported that it can be grown using inexpensive ingredients and simple fermentation technology.

**Table II.** The insecticidal effects of spore–crystal mixture ($10^9$/mL) of isolate Mm2 on coleopteran insects[a] (mortality, %)

| Isolate | *A. alni* | *A. solstitiale* | *A. roboris* | *L. decemlineata* | *M. melolontha* |
|---|---|---|---|---|---|
| Mm2 | 96 | 85 | 70 | 90 | 90 |
| + control[b] | 80 | 70 | 50 | 70 | 80 |
| – control[c] | 20 | 10 | 10 | – | 10 |

[a]ANOVA LSD test, $p < 0.05$. [b]*B. thuringiensis* subsp. *tenebrionis*. [c]PBS.

Our results indicate that Mm2 isolated from *M. melolontha* in Turkey is a strain of *B. thuringiensis* subsp. *tenebrionis*. It has almost identical features with other strains of *B. thuringiensis* subsp. *tenebrionis*. However, the insecticidal activity of this new isolate is significantly higher than that of the reference strain.

This work was supported by the *Karadeniz Technical University* research foundation (KTU 21.111.004.4) and *Turkish Republic Prime Ministry State Planning Organization* (21.111.004.1). The authors would like to thank M. Ohba and K. Kagoshima for performing the H-serotyping, Dr. D. Zeigler for providing the reference *B. thuringiensis* subsp. *kurstaki* HD-1 and Dr. S. Jansens for providing *B. thuringiensis* subsp. *tenebrionis*.

**REFERENCES**

BECKER N., MARGALIT J.: Use of *Bacillus thuringiensis israelensis* against mosquitoes and blackflies, pp. 145–170 in P.F. Entwistle, P.F. Cory, M.J. Bailey, S. Higgs (Eds): Bacillus thuringiensis*, an Environmental Pesticide: Theory and Practice*. John Wiley & Sons, New York 1993.

BEN-DOV E., ZARITSKY A., DAHAN E., BARAK Z., SINAI R., MANASHEROB R., KHAMERAEV A., TROITSKAYA E., DUBITSKY A., BEREZINA N., MARGALITH Y.: Extended screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. *Appl.Environ.Microbiol.* **63**, 4883–4890 (1997).

BIETLOT H.P.L., VISHNUBHATLA I., CAREY P.R., POZSGAY M., KAPLAN H.: Characterization of the cysteine residues and disulphide linkages in the protein crystal of *B. thuringiensis*. *J.Biochem*. **267**, 309–315 (1990).

BUCHER G.E.: Identification of bacteria found in insect, pp. 10–30 in H.D. Burges (Ed.): *Microbial Control of Pest and Plant Diseases*. Academic Press, London 1981.

DAMGAARD P.H., HANSEN B.M., PEDERSEN J.C., EILENBERG J.: Natural occurrence of *Bacillus thuringiensis* on cabbage foliage and in insects associated with cabbage crops. *J.Appl.Microbiol.* **82**, 253–258 (1997).

DEAN D.H., ZEIGLER D.R.: Bacillus *Genetic Stock Center Strains and Data*, 6th ed. Ohio State University Press, Columbus 1994.

FEITELSON J.S., PAYNE J., KIM L.: *Bacillus thuringiensis*: insects and beyond. *Biotechnology* **10**, 271–275 (1992).

FROSCHLE M.V.: The common cockchafer (*Melolontha melolontha* L.) has to be taken seriously again in Baden-Württemberg. *Pflanzenschutzdienstes* **46**, 6–9 (1994).

GONZALES J.M.J., DULMAGE H.T., CARLTON B.C.: Correlation between specific plasmids and δ-endotoxin production in *Bacillus thuringiensis*. *Plasmid* **5**, 351–365 (1981).

HÖFTE H., WHITELEY H.R.: Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol.Rev.* **53**, 242–255 (1989).

HONIGMAN A., NEDJAR-PAZERIN G., YAWETZ A., ORON U., SCHUSTER S., BROZA M., SNEK B.: Cloning and expression of the lepidopteran toxin produced by *Bacillus thuringiensis* in *Escherichia coli*. *Gene* **42**, 69–77 (1986).

IRIARTE J., DUMANOIR V.C., BEL Y., PORCAR M., FERRANDIS M.D., LECADET M.M., FERRE J., CABALLERO P.: Characterization of *Bacillus thuringiensis* ser. *balearica* (serotype H48) and ser. *navarrensis* (serotype 50): two novel serovars isolated in Spain. *Curr.Microbiol.* **40**, 17–22 (2000).

ISHIWATA S.: On a kind of severe flacherie (sotto disease). *Dainihon Sanshi Kaiho* **114**, 1–5 (1901).

KATI H., SEZEN K., BELDÜZ A.O., DEMIRBAĞ Z.: Characterization of a *Bacillus thuringiensis* subsp. *kurstaki* strain isolated from *Malacosoma neustria* L. (*Lepidoptera*: *Lasiocampidae*). *Biologia* **60**, 301–305 (2005).

KRIEG N.R., HOLT J.G.: Gram-negative aerobic rods and cocci, pp. 140–218 in N.J. Palleroni (Ed.): *Bergey's Manual of Systematic Bacteriology*. Williams & Wilkins, Baltimore 1986.

KRONSTAD J.W., SCHNEPF H.E., WHITELEY H.R.: Diversity of locations for *Bacillus thuringiensis* crystal protein genes. *J.Bacteriol*. **154**, 419–428 (1983).

LAEMMLI U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685 (1970).

LEE H.H., JOO D.G., KANG S.C., LIM H.G.: Characterization of *B. thuringiensis* seven isolates from soil (I). *Korean J.Appl.Microbiol. Biotechnol.* **20**, 377–388 (1992).

LEE H.H., JUNG J.D., YOON M.S., LEE K.K., LECADET M.M., CHARLES J.F., DUMANOIR V.C., FRANCHON E., SHIM J.C.: Distribution of *Bacillus thuringiensis* in Korea. B.thuringiensis *Biotechnol.Environ.Benefits* **1**, 201–215 (1995).

LERECLUS D., DELECLUSE A., LECADET M.M.: Diversity of *Bacillus thuringiensis* toxins and genes, pp. 37–70 in P.F. Entwistle, J.S. Cory, M. Bailey, S. Higgs (Eds): Bacillus thuringiensis*, an Environmental Biopesticide: Theory and Practice*. Chichester–John Wiley, New York 1993.

LUISA M., MAURO V.: Presence and diffusion of the common cockchafer (*Melolontha melolontha* L.) in the areas of Mezzocorona and San Michele a/A in Trento Province. *Bulletin OILB SROP* **19**, 15–20 (1996).

*Minitab: User's Guide, Release 11*. Minitab–State College Pennsylvania (USA) 1997; **http://www.minitab.com**.

OHBA M., AIZAWA K.: Distribution of *Bacillus thuringiensis* in soil of Japan. *J.Invertebr.Pathol*. **47**, 277–286 (1986).

PEFEROEN M.: Insect control with transgenic plants expressing *Bacillus thuringiensis* crystal proteins, pp. 21–48 in N. Carozzi, M. Koziel (Eds): *Advances in Insect Control, the Role of Transgenic Plants*. Taylor and Francis, Bristol (USA) 1997.

SAMBROOK J., FRITSCH E.F., MANIATIS T.: *Molecular Cloning*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989.

SEZEN K.: Determination of viruses from coleopteran pests of hazelnut and the potential usage as biological control agent. *PhD Thesis*. Department of Biology, Karadeniz Technical University, Graduate School of Natural and Applied Sciences, Trabzon (Turkey) 2004.

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

SHARIF F.A., ALAEDDINOĞLU N.G.: A rapid and simple method for staining of the crystal protein of *Bacillus thuringiensis*. *J.Industr. Microbiol*. **3**, 227–229 (1988).

SNEATH P.H.A., MAIR N.S., SHARPE M.E., HOLT J.G.: Regular, nonsporing Gram-positive rods, pp. 1208–1260 in O. Kandler, N. Weiss (Eds): *Bergey's Manual of Systematic Bacteriology*. Williams & Wilkins, Baltimore 1986.

TAMEZ-GUERRA P., CASTRO-FRANCO R., MEDRANO-ROLDAN H., MCGUIRE M.R., GALAN-WONG L.J., LUNA-OLVERA H.A.: Laboratory and field comparisons of strains of *Bacillus thuringiensis* for activity against noctuid larvae using granular formulations (*Lepidoptera*). *J.Econ.Entomol.* **91**, 86–93 (1998).

THIERY I., FRACHON E.: Identification, isolation, culture and preservation of entomopathogenic bacteria, pp. 55–73 in A.L. Lacey (Ed.): *Manual of Techniques in Insect Pathology*. Academic Press, London 1997.

VLUG H.J.: Occurrence and biocontrol of grass grubs, especially of *M. melolontha*. *Bull.OILB SROP* **19**, 35–36 (1996).