World Journal of Microbiology & Biotechnology (2005) **21**: 285–292
DOI 10.1007/s11274-004-3633-y

Isolation and characterization of *Bacillus thuringiensis* strains from different grain habitats in Turkey

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Received 8 March 2004; accepted 17 July 2004

Keywords: *Bacillus thuringiensis*, *cry* genes, grain habitats, isolation, plasmid, PFGE profiles

Summary

Bacillus thuringiensis (Bt) is a gram-positive, spore-forming bacterium and it produces insecticidal crystal (cry) proteins during sporulation. Because the genetic diversity and toxic potential of Bt strains differ from region to region, strains have been collected and characterized all over the world. The aim of this study is to isolate Bt strains in grain-related habitats in Turkey and to characterize them on the basis of crystal morphology, *cry* gene content, and chromosomal and plasmid DNA profiles. Four approaches were taken: analysis with phase contrast (PC) microscopy, polymerase chain reaction (PCR), pulsed field gel electrophoresis (PFGE) and plasmid isolation. Ninety-six samples were collected from Central Anatolia and the Aegean region. Bt was isolated from 61 of 96 samples (63.5%) and 500 Bt-like colonies were obtained. One hundred and sixty three of the colonies were identified as Bt based on cry protein formation using PC microscopy. Among the examined colonies, the overall proportion identified (as Bt index) was 0.33. We found that 103 isolates were positive for the five different *cry* genes (*cry*1, *cry*2, *cry*3, *cry*4 and *cry*9) examined with PCR. In addition, plasmid profiling of 37 *cry* gene-positive isolates indicated that the 15 kb plasmid band was present in all isolates; however, 11 of 37 isolates had more than one plasmid band at different sizes. Finally, chromosomal DNA profiling by PFGE gave rise to different DNA patterns for isolates containing the same *cry* gene which suggests a high level of diversity among the Bt strains isolated.

Introduction

Bacillus thuringiensis (Bt) is a gram-positive, facultative anaerobe and spore-forming bacterium. It produces different insecticidal toxic proteins in parasporal crystals during the stationary phase of its growth cycle (Rowe *et al.* 1987). The genes coding for cry proteins are mostly carried on plasmids ranging from 3–4 to 150 Mda (Gonzales & Carlton 1980; Aronson 2002). Up to now, many cry protein genes have been cloned, sequenced and named *cry* genes. Over 100 *cry* gene sequences are organized into 32 groups and different subgroups based on nucleotide similarities and range of host specificity (Crickmore *et al.* 1998; Bravo *et al.* 1998).

Insecticidal activity of Bt depends mostly on cry proteins and varies with insect type. Natural isolates of Bt have been used as biological pesticides against different insect orders such as Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera and Acari (Cannon 1993; Fieltson *et al.* 1992). In addition, some strains of Bt have been found to be toxic to nematodes and protozoa (Feitelson *et al.* 1992; Edwards *et al.* 1988). The lack of mammalian toxicity of cry proteins has resulted in an increase in the use of Bt as an insecticide and intensified the search for new strains with different toxic activities.

It has been reported that Bt can be present in many different habitats such as soil, stored product dust, insect cadavers, grains, agricultural soils, olive tree related-habitats, different plant and aquatic environments (Martin & Travers 1989; Meadows *et al.* 1992; Ben-Dov *et al.* 1997; Theunis *et al.* 1998; Bravo *et al.* 1998; Bel *et al.* 1997; Mizuki *et al.* 1999; Iriarte *et al.* 2000). Bt strains show genetic diversity with different toxic potential mostly due to plasmid exchange between strains (Thomas *et al.* 2001). In fact, each habitat may contain a novel Bt strain awaiting discovery which has a toxic effect on a target insect group. Therefore, Bt strains have been collected from different environments and characterized to evaluate their toxic potential against various insect orders (Chak *et al.* 1994; Theunis *et al.* 1998; Bravo *et al.* 1998; Forsyth & Logan 2000; Uribe *et al.* 2003). Different methods are used for the

characterization of Bt isolates such as polymerase chain reaction (PCR), southern blotting, serotyping and bioassay; however, PCR is the most widely used, efficient and rapid technique for screening of a large number of isolates (Juarez-Perez *et al*. 1997; Porcar & Juarez-Perez 2002).

Because the use of Bt products as an alternative to chemical insecticides is increasing rapidly, many research centres have focused on isolation of the native strains in order to establish Bt strain collection worldwide. Therefore, the purpose of this study is to initiate the establishment of a native Bt strain collection from different regions of Turkey and to determine its diversity. We isolated and characterized 103 Bt isolates from grain-related habitats of Central Anatolia and the Aegean regions of Turkey based on crystal formation, *cry* gene content, and plasmid and chromosomal DNA profiles.

Materials and methods

Sample collection

Soil, grain, stored product dust, straw, insect cadavers and various residues were collected from grain silos, crop fields, farms, caves, haylofts where Bt preparation have not been applied, in central Anatolia (Ereğli/Konya, Takale/Karaman) and the Aegean region (Nikfer/Denizli, Bozbük/Söke) as shown in Table 1. Samples were taken from the places not exposed to sunlight or at 5 cm depth from the surface and were placed into plastic

Table 1. Distribution of *Bacillus thuringiensis* based on sample types and location[a].

Location	Type of sample	No. of sample	No. of sample yielding Bt	No. of isolates obtained	No. of isolates producing crystals	No. of isolates positive for *cry* genes	Bt Index
Ayranli/Ereğli-Konya (CA)	Soil	9	7	70	42	33	0.60
Eregli/Konya (CA)	Grain	3	1	4	1	0	0.25
	Soil	3	3	19	13	8	0.68
	Stored product dust	2	1	6	3	3	0.50
		8	5	29	17	11	0.59[b]
Ivriz/Ereğli-Konya (CA)	Soil	7	7	57	15	14	0.26
Üçharman/Ereğli-Konya (CA)	Soil	7	6	52	17	7	0.33
	Various residues	1	0	1	0	0	0.00
		8	6	53	17	7	0.32[b]
Manazan Caves (MC)	Animal faeces	1	0	0	0	0	-
Taşkale-Karaman (CA)	Soil	9	5	37	8	3	0.22
	Stored product dust	5	2	22	4	1	0.18
	Various residues	5	1	23	1	0	0.04
		20	8	82	13	4	0.16[b]
Natural Grain Silos (NGS)	Animal faeces	1	1	6	2	1	0.33
Taşkale-Karaman (CA)	Grain	9	2	11	2	1	0.18
	Stored product dust	16	13	101	28	18	0.28
		26	16	118	32	20	0.27[b]
Bozbük/Söke (AR)	Animal faeces	1	0	4	0	0	-
	Dead insect	1	0	0	0	0	-
	Grain	1	0	0	0	0	-
	Soil	5	4	25	11	6	-
	Straw	1	0	0	0	0	0.44
		9	4	29	11	6	0.38[b]
Nikfer/Denizli (AR)	Soil	3	3	26	10	3	0.38
	Stored product dust	4	3	20	3	3	0.15
	Straw	2	2	16	3	2	0.19
		9	8	62	16	8	0.26[b]
	Total	96	61	500	163	103	0.33

[a]Isolates were examined with PC microscope for crystal formation and *cry* gene content of crystal positive isolates was screened by PCR. CA: Central Anatolia, AR: Aegean Region. Bt index is the ratio of Bt isolates producing crystal to all isolates in each sample group.
[b]Indicates the total Bt index in each geographical location.

bags aseptically. All samples were stored at 4 °C until processed.

Bacterial strains

B. thuringiensis subsp. *kurstaki* (HD1), *B. thuringiensis* subsp. *Aizawai* (HD133), *B. thuringiensis* subsp. *kumamotoensis* (HD867), *B. thuringiensis* biovar. *tenebrionis* (tenebrionis), *B. thuringiensis* biovar. *israelensis* (HD500) were kindly supplied by Dr Daniel R. Zeigler from the Bacillus Genetic Stock Center (Ohio, USA) and used as reference strains.

Bacillus thuringiensis *isolation and crystal morphology analysis*

Bt strains were isolated based on the acetate selection method described by Travers *et al.* (1987). Briefly, 0.25 g of the environmental sample were suspended in 10 ml nutrient broth (Applichem) medium containing 0.25 M sodium acetate and left for microbial growth at 37 °C overnight. Heat treatment was then applied for 5 min at 80 °C to kill vegetative cells. After that, they were plated on nutrient agar plates and allowed to grow overnight at 37 °C. Bt-like colonies which are usually described as cream-coloured and have the appearance of a fried egg on the plates (Travers *et al.* 1987) were labelled and subcultured. Subculturing from an individual colony was repeated three times to obtain a pure culture. Finally, each pure culture was grown on T3 agar plates and colonies dispersed in sterile distilled water were examined with a PC microscope for crystal production and morphology. Duplicate stock samples were prepared from each of the isolates in 25% glycerol and kept at −80 °C.

Cry gene identification

Polymerase chain reaction (PCR) was used to identify *cry* gene content. All isolates producing crystal proteins were screened by five pairs of universal primers for the *cry1, cry2, cry3, cry4* and *cry9* genes described by Ben-Dov *et al.* (1997, 1999). DNA isolation was performed by the method of Bravo *et al.* (1998). Briefly, a loopful of cells from overnight Bt cultures was transferred into 0.2 ml of water and suspended. After freezing at −80 °C for 20 min, they were transferred into boiling water for 10 min. The cell lysate was centrifuged (Henttich, Micro 12-24 Eppendorf Model) for 10 s at 11,000 × *g* and 15 μl of supernatant were used as DNA template. PCR reactions were carried out in 50 μl reaction volumes. DNA template was mixed with reaction buffer containing 200 μM deoxynucleotide triphosphate mix, 0.5 μM each primer (synthesized by Integrated DNA Technologies), 3 mM MgCl and 2 U of Taq DNA polymerase (Fermentas). Amplifications were carried out in a DNA thermal cycler (Techne Progen, England). For all *cry* genes, an initial denaturation step was applied at 94 °C for 1 min and followed by denaturation at 94 °C for 1 min, annealing at 54 °C (for cry1) and 60 °C (for cry2, cry3, cry4 and cry9) for 1 min, then extension at 72 °C for 1 min. Thirty-five cycles were carried out for the amplification of *cry* gene fragments. Finally, an extra extension step was applied at 72 °C for 10 min. After amplifications, 10 μl of each PCR product were electrophoresed on 1% agarose–ethidium bromide (Sigma) gel in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA [pH 8]) at 95 V for 40 min. Gels were visualized in a gel documentation system (Vilber Lourmat, France).

Plasmid profiling

Plasmid isolation was performed with minor modifications of the method described by O'Sullivan & Klaenhammer (1993). Bacterial cultures were grown on nutrient agar plates overnight and transferred into eppendorf tubes by scraping gently with the help of sterile distilled water. After pelleting the cells, they were resuspended in 200 μl of a solution containing 25% sucrose and 30 mg lysozyme/ml (Applichem) and incubated at 37 °C for 15 min. The sample was mixed with 400 μl alkaline SDS solution (3% SDS, 0.2 N NaOH) and incubated for 7 min at room temperature. Then 300 μl ice-cold 3 M sodium acetate (pH 4.8) was added, mixed and centrifuged at 11,000 × *g* for 20 min (4 °C). Supernatants were transferred into new eppendorf tubes, mixed with 650 μl of isopropanol (Sigma) and centrifuged again at 11,000 × *g* for 20 min (4 °C). After discarding all liquid, pellets were resuspended in 320 μl sterile distilled water. They were mixed with 200 μl 7.5 M ammonium acetate containing 0.5 mg/ml ethidium bromide and 400 μl phenol/chloroform, then centrifuged at 11,000 × *g* for 10 min, at room temperature. Upper phases were transferred into new eppendorf tubes and mixed with 1 ml ethanol at −20 °C. After centrifugation at 11,000 × *g* for 20 min (4 °C), pellets were washed with 70% ethanol. All liquid was discarded and the pellets were dissolved in 25 μl TER (TE, pH 7.8 and RNase, 0.1 mg/ml). After incubation at 37 °C for 20 min, plasmid samples were electrophoresed in 0.8% agarose–ethidium bromide gel at 80 V for 3 h and visualized with the gel documentation system.

PFGE analysis

PFGE analysis was carried out according to Rivera & Priest (2003) with some modifications. Bacterial strains were grown in 10 ml nutrient broth overnight and cells were harvested by centrifugation at 4 °C for 2 min at 4500 rev/min. Cells were washed with 500 μl TE (50 mM Tris, 1 mM EDTA, pH 8.0) and SE (10 mM NaCl, 30 mM EDTA, pH 7.5) buffer, respectively. The cells were then resuspended in 50 μl SE buffer, mixed with 50 μl 2% agarose (Low Melt) at 50 °C, and dispensed into the slots of plug mould. The plugs were

allowed to set at room temperature. The cells embedded into agarose were allowed to lyse in lysis buffer (30 mM Tris, 50 mM NaCl, 5 mM EDTA, pH 8.0) containing 2 mg/ml lysozyme at 37 °C for 18 h. Bacterial plugs were then washed three times with 5 ml of buffer containing 20 mM Tris, 50 mM EDTA, pH 8.0. Proteins were digested with 2 ml proteinase K solution (0.5 mg proteinase K/ml and 0.1% *N*-lauroylsarcosine–EDTA, 50 mM, pH 8.0) at 50 °C overnight. Then plugs were washed twice with 5 ml of buffer containing 20 mM Tris, 50 mM EDTA, 1 mM NaCl, pH 8.0; once with buffer containing Tris, 50 mM EDTA, 1 mM PMSF, pH 8.0, and once with buffer containing 20 mM Tris, 50 mM EDTA, pH 8.0. After the plugs were equilibrated with 1 ml restriction enzyme buffer, they were digested with 40 U of SmaI (Fermentas) at 30 °C for 20 h. Then the plugs were electrophoresed in 1% agarose in TBE buffer in a CHEF-DRII system at 14 °C for 40 h at 4 V/cm with pulse times of 15 s rising to 60 s. After staining the gel in ethidium bromide (1 μl/ml) for 45 min and destaining in distilled water for 1 h, DNA profiles were recorded in a gel documentation system (Vilber Lourmat, France).

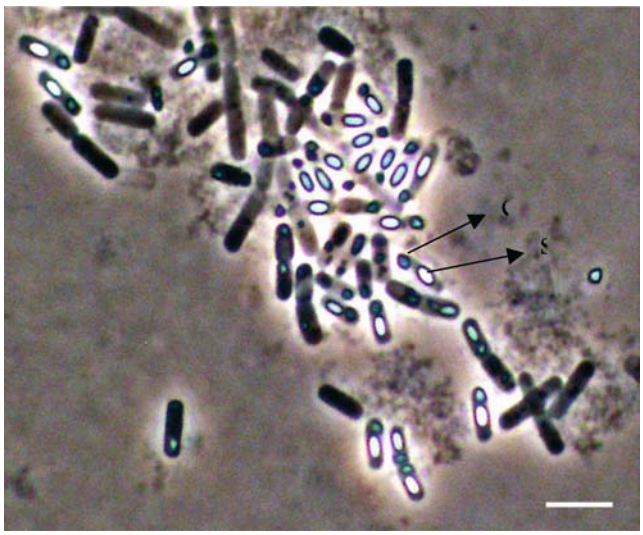

Figure 1. Photomicrography of a Bt isolate, 39Ya. The Bt isolate was grown for 48 h and examined with the PC microscope for spore formation and crystal protein production. Some cells were lysed and spores and crystals released into the medium whereas the others were intact. Arrow C and S indicate crystal protein and spore, respectively. Bar represents 2.5 μm.

Results

Bacillus thuringiensis *distribution shown by sample types and locations*

In total 96 samples, 78 from Central Anatolia region and 18 from the Aegean region were examined in this study (Table 1). Sample types consist of 43 soil, 27 stored product dust, 13 grain and 13 other samples including straw, animal faeces, various residues and an insect cadaver. After acetate selection, no microbial growth was observed in six grain samples and six other samples in different groups. According to colony morphology and PC microscopy analysis, Bt was isolated from 61 of the 96 samples which corresponds to 63.5% of the whole number of samples (Table 1). Five hundred isolates were obtained from these 61 samples. Bt index, reflecting the ratio of Bt colonies in total colonies isolated, was found to vary between 0.00 and 0.68 through origins with the average value of 0.33 (Table 1). Compared to all locations, Ereğli/Konya was the richest area for Bt occurrence with an 0.59 Bt index.

Crystal composition of the isolates

Five hundred isolates were examined with the PC microscope for spore formation and crystal production (Figure 1). Among them, 163 isolates produced crystals (Table 1). Even though 99 other isolates had Bt-like spore and colony morphology, they did not show crystal formation. The remaining 238 isolates did not exhibit any morphological similarities to Bt nor did they produce crystals.

Crystal morphology of Bt can give information about target insect spectra (Maeda *et al.* 2000). Therefore, in order to determine the crystal morphology of each Bt isolate, all isolates were grown for 48 h and examined with the PC microscope. Five different crystal shapes were observed in 163 isolates. Although only one type of crystal morphology was observed in some isolates, more than one type of crystal morphology was present in others (Figure 2). Distribution of crystal shapes in 163 isolates was 36% spherical (S), 5% cubic (C), 9% irregular pointed (IP), 2% bipyramidal (B), 19% cubic and spherical (C&S), 22% spherical and irregular pointed (S&IP), 1% cubic and irregular pointed (C&IP), 2% irregular shaped (IS), and 6% not defined (Figure 2).

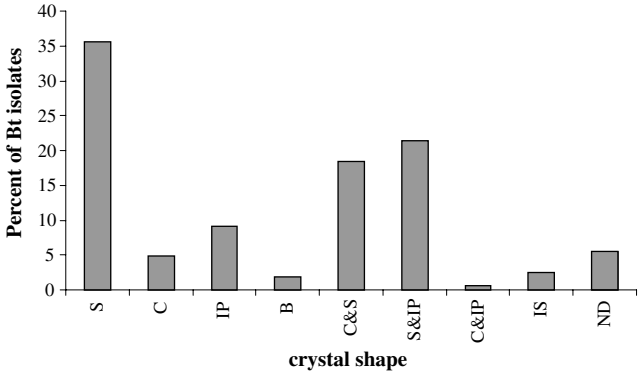

Figure 2. Crystal shape distribution of Bt isolates. After growing the isolates for 48 h, crystal protein formation was observed using a PC microscope. Description of crystal shapes is: C: cubic, B: bipyramidal, S: spherical, IP: irregular pointed, IS: irregular shaped, ND: not defined.

Characterization of cry gene content of the isolates

Because crystal proteins are encoded by *cry* genes and one Bt strain can contain more than one cry protein, the *cry* gene content of each isolate had to be determined. PCR reactions for each isolate were carried out with universal primers specific for *cry1*, *cry2*, *cry3*, *cry4* and *cry9* genes. One hundred and three of 163 isolates were positive for the *cry* genes examined. Some examples of PCR products amplified with different *cry* gene primers are shown in Figure 3. Even though most of the isolates gave only one DNA band with a specific *cry* gene primer, some of them showed two or three DNA bands with the same *cry* gene primer (Figure 3).

PCR analysis of each isolate with five different *cry* gene primers indicated that 63 of the isolates had only one type of *cry* gene; however, 40 of them contained more than one type of *cry* gene (Figure 4). The number of isolates carrying one type of *cry* gene are 17 for *cry1*, 6 for *cry2*, 10 for *cry3*, 7 for *cry4* and 21 for *cry9*. On the other hand, 28 isolates contained two different *cry* genes. In addition, 8 isolates were positive for 3 different *cry* genes and 4 isolates for 4 different *cry* genes (Figure 4).

No amplification of DNA template was observed for 60 isolates producing crystal protein indicating that they have *cry* genes different from the genes examined in this study.

Plasmid and PFGE profiles of the isolates

Bt has been known to have several circular/linear plasmids, and *cry* genes are generally found in these plasmids (Carson *et al*. 1996). Therefore, in the present study plasmids were isolated from 33 *cry* gene-positive isolates as well as four different Bt reference strains. A major plasmid band at 15 kb in size was obtained in all isolates (Figure 5). In addition, plasmid bands varying in length between 15 and 22 kb were observed in some of the isolates: *cry2*-positive (lanes 11, 12, 13, 14, 16), *cry3*-positive (lanes 18, 19), and *cry9*-positive (lanes 30, 31).

Pulsed field gel electrophoresis (PFGE) of chromosomal DNA digested with a restriction enzyme is an

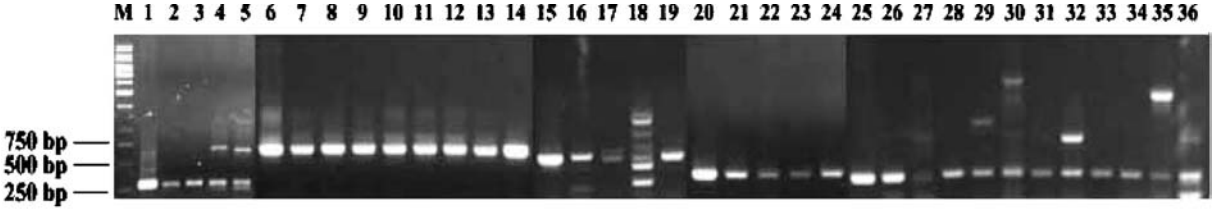

Figure 3. PCR analysis of crystal protein positive isolates. DNA template from each isolate was amplified with PCR in the presence of each *cry* gene primer. PCR products of some of the isolates are for *cry1*, lanes 1–5; *cry2*, lanes 6–14; *cry3*, lanes 15–19; *cry4*, lanes 20–24; *cry9*, lanes 25–36. Identity of isolates in each lane is LaneM: 1kb DNA MW marker; Lane1: *Bacillus thuringiensis* subsp. *aizawai*; Lane2: 39Yb; Lane3: 43Db; Lane4: 48Ra; Lane5: 71Na; Lane6: *Bacillus thuringiensis* subsp. *kurstaki*; Lane7: 18Fa; Lane8: 93Ha; Lane9: 93Da; Lane10: 93FFa; Lane11: 27Fb; Lane12: 19Rb; Lane13: 19Hb; Lane14: 85PPb; Lane15: *Bacillus thuringiensis* biovar. *tenebrionis*; Lane16: 21KB; Lane17: 71Lb; Lane18: 98Lb; Lane19: 86Db; Lane20: *Bacillus thuringiensis* biovar. *israelensis*; Lane21: 19Pb; Lane22: 2Ja; Lane23: 28Da; Lane24: 113Ya; Lane25: *Bacillus thuringiensis* subsp. *aizawai*; Lane26: 82YYb; Lane27: 36Ba; Lane28: 24Ca; Lane29: 25Ca; Lane30: 94YYb; Lane31: 24Nb; Lane32: 93Da; Lane33: 25Aa; Lane34: 29Fa; Lane35: 53Yb; Lane36: 62PPa.

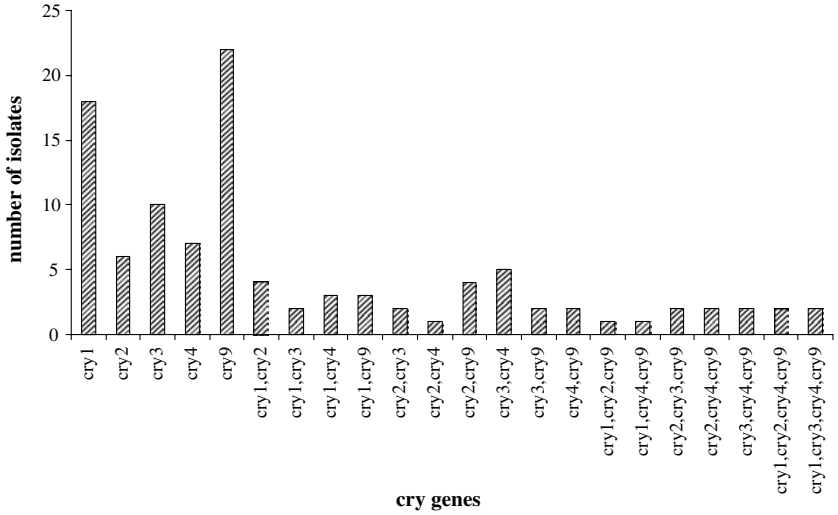

Figure 4. *cry* gene distribution of Bt isolates. Crystal protein-producing isolates were screened by PCR to find out their *cry* gene contents by using five primer pairs for cry1, cry2, cry3, cry4 and cry9. Figure shows number of isolates and their *cry* gene profiles.

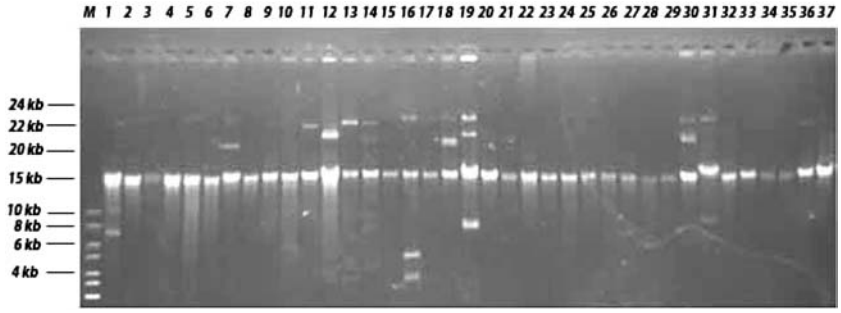

Figure 5. Plasmid patterns of Bt isolates. Plasmid DNA was prepared from 33 *cry* gene-positive isolates and subjected to electrophoresis in 0.8% agarose gel with ethidium bromide. Name of the isolate or reference strain in each lane is as follows: Lane M: 1 kb DNA Ladder; Lane1: *Bacillus thuringiensis* subsp. *aizawai*; Lane2: 48Ra; Lane3: 39Ya; Lane4: 39Yb; Lane5: 43Db; Lane6: 71Na; Lane7: 55Ka; Lane8: *Bacillus thuringiensis* subsp. *kurstaki*; Lane9: 18Fa; Lane10: 93FFa; Lane11: 93Ha; Lane12: 19Rb; Lane13: 27Fb; Lane14: 93Da; Lane15: 19Hb; Lane16: 85PPb; Lane17: *Bacillus thuringiensis* biovar. *tenebrionis*; Lane18: 71Lb; Lane19: 2Ja; Lane20: 98Lb; Lane21: 86Db; Lane22: *Bacillus thuringiensis* biovar. *israelensis*; Lane23: 19Pb; Lane24: 28Da; Lane25: 113Ya; Lane26: *Bacillus thuringiensis* subsp. *aizawai*; Lane27: 82Yb; Lane28: 24Nb; Lane29: 25Aa; Lane30: 25Ca; Lane31: 36Ba; Lane32: 29Fa; Lane33: 93Da; Lane34: 94YYb; Lane35: 53Yb; Lane36: 24Ca; Lane37: 62PPa.

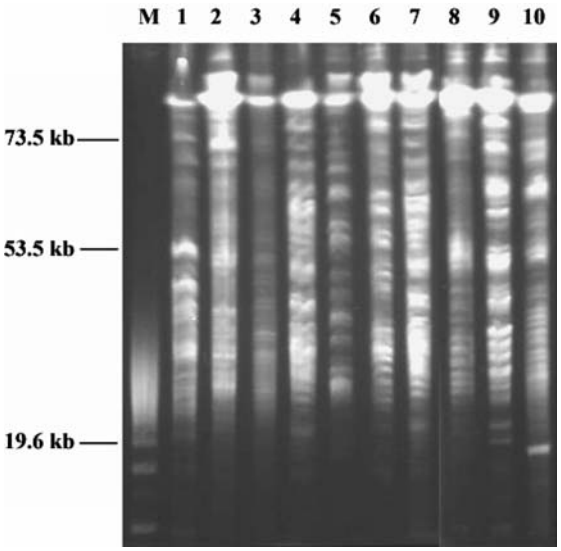

Figure 6. PFGE profiles of *cry 9*-positive isolates. PFGE analysis was carried out as described in Materials and Methods section. Identity of the isolate in each lane is as follows: LaneM: 5 kb DNA ladder; Lane1: 62PPa; Lane2: 24Ca; Lane3: 28Aa; Lane4: 53Yb; Lane5: 94YYb; Lane6: 93Da; Lane7: 29Fa; Lane8: 25Aa; Lane9: 82YYb; Lane10: *Bacillus thuringiensis* subsp. a*izawai*.

accurate typing method for bacteria (Tenover *et al.* 1995). In order to see if Bt isolates carrying the same *cry* gene show similar PFGE patterns, chromosomal DNA from 6 *cry1*, 8 *cry2*, 5 *cry3*, 4 *cry4* and 11 *cry9* positive isolates was subjected to PFGE analysis. Even though there are some similarities among the PFGE patterns of the isolates (data not shown except for *cry*9), no identical patterns were obtained within each of the *cry* gene groups (Figure 6).

Discussion

In this study, Bt occurrence was examined in grain-related habitats of Central Anatolia and in the Aegean region where no Bt products have been applied before. Bt occurence in all soil samples collected from Konya was found to be relatively high compared to other soil samples (Table 1). Especially in İvriz, Bt was isolated from all of the samples. These places are crop fields and this suggests the abundant presence of Bt in agricultural lands. The percentage of samples yielding Bt from Nikfer was also high, 89%. This is because the sampled haylofts had been used for 65 years. In addition, natural grain silos (NGS) have been used for grain storage for more than 500 years and the percent of samples yielding Bt was 62%. In fact, Bt indexes of NGS and Nikfer are very similar with the values of 0.27 and 0.26, respectively. This shows a similar degree of occurrence of Bt in two places with similar background.

The Bt index serves as a measurement of success in isolating Bt. After acetate selection for Bt isolation, no growth was observed in six of the grain samples. In all regions, the percentage of grain samples yielding Bt was relatively low in grain samples (23%) when compared with those of soil (81%), stored product dust (70%) and straw (67%) samples (Table 1). This indicates that grain is not as good a source as the others for Bt. An average Bt index was found to be 0.33 for all samples but the index changes according to sample types and origins (Table 1). The abundance of Bt was the highest in all soil samples, with a Bt index of 0.40. It decreases to 0.26 in all stored dust product samples and to 0.20 in all grain and animal faeces. Unlike our study, Bravo *et al.* (1998) collected soil samples from cultivated fields in Mexico and obtained a Bt index of about 0.24, nearly twofold lower than our index. However, Martin & Travers (1989) found the highest Bt index as 0.85 in the soil samples collected from Asia, nearly twofold greater than ours. This may be related to climate and geographic conditions. In addition, Hongyu *et al.* (2000) and Bernhard *et al.* (1997) reported that Bt is more abundant in stored product environments than in soil. Taken together, these studies show that the level of Bt index changes from region to region and between types of samples.

Because there is a relationship between toxic activity and crystal shape of Bt strains (Maeda *et al.* 2000), observation of crystal morphology by PC microscope can provide valuable information about toxic activity of Bt isolates. In fact, observation of crystal morphology is the first step for establishing Bt strain collection (Ohba & Aizawa 1986; Bernhard *et al.* 1997). Therefore, when the crystal shape of the isolates was examined using a PC microscope, it was found that 42% of the isolates had more than one crystal shape; however, 58% of them had only one crystal shape (Figure 2). More definitive results about toxic activity of the isolates will be obtained from bioactivity assay of each isolate in the future study because a discrepancy exists between predicted *cry* gene type and its insecticidal activity (Shisa *et al.* 2002). For example, even though the *cry1* gene product is toxic against Lepidoptera, Shisa *et al.* have reported that the *cry1* gene product of native Bt strains was toxic to only Diptera.

In addition, it was observed that a Bt isolate was sometimes positive for two or more *cry* genes even though it had only one type of crystal morphology. This may be due to lack of expression of all different *cry* genes at protein level. Moreover, 99 isolates exhibited spore and colony morphology similar to that of Bt whereas no crystal formation was observed by PC microscope. On the other hand, when PCR analysis was performed for nine of them, seven isolates were positive for *cry* genes examined. This is also related to the absence of gene expression at the protein level. In fact, crystal protein synthesis in Bt is controlled by a variety of mechanisms at the transcriptional, post-transcriptional or post-translational levels (Agassie & Lereclus, 1995).

PCR screening of 163 crystal-forming isolates indicated that 103 of them were positive with primers for the five different *cry* genes examined. The number of isolates containing the *cry9* gene was the greatest (21) compared to that of isolates containing the *cry1* gene (17), *cry*2 gene (7) and *cry3* gene (10) and *cry4* gene (8). However, Bravo *et al.* (1998) have found *cry1* genes the most frequent (49.5%), then *cry3* gene as highly abundant (21.7%) and *cry9* gene less abundant (2.6%). These results show how different geographic regions affect diversity of *cry* gene content of Bt strains. In addition, it is probable that the remaining 60 isolates negative for the observed *cry* genes may contain different *cry* genes from the ones examined in this study because 32 different *cry* gene groups and many subgroups have been defined in the literature (Schnepf *et al.* 1998; Crickmore *et al.* 1998).

PFGE patterns of restriction enzyme-digested genomic DNA is known to be a useful technique to identify closely related bacterial isolates (Bygraves & Maiden 1992; Tenover *et al.* 1995). A recent study by Rivera & Priest (2003) has indicated that PFGE is a better technique than H-serotyping for discriminative typing of Bt strains. In the present study, we carried out PFGE analysis in order to see if isolates carrying the same *cry* gene are identical. Although there were some similarities among PFGE patterns of the isolates (Figure 6), none of them were the same. Based on Rivera & Priest (2003), if PFGE patterns differed by changes up to 3 bands and more than three bands, strains are described as closely related and unrelated, respectively. Therefore, our isolates carrying the *cry9* gene could be unrelated strains. In addition, results show that *cry9*-positive isolates may be heterogenous because Rivera & Priest (2003) have reported that serovars *canadensis* and *entomocidus* exhibited unique patterns and were described as heterogenous. Similar to their results, our findings also showed that there is no exact correlation between *cry* gene content and PFGE patterns. This is possible because *cry* genes are often carried on plasmids and plasmid exchange between strains as well as recombination between *cry* genes from different backgrounds occur in Bt strains (De Maagd *et al.* 2001). As a result, extensive genetic characterization and PFGE patterns will give more definite results about diversity of Bt strains with different *cry* genes.

In conclusion, this study is the first for isolation and characterization of Bt native strains in Turkey. Different PFGE patterns of isolates carrying the same *cry* gene indicates wide range of biodiversity among Bt strains in Anatolia. Planned further studies related to Bt isolation from different parts of Anatolia and detailed genetic characterization as well as toxic activity will give more comprehensive results about biodiversity of Bt strains.

Acknowledgements

This work was partially supported by grants from IYTE (2002 IYTE43) and from DPT (2002K-1207390).

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