Isolation of *Bacillus thuringiensis* and Investigation of Its Crystal Protein Genes

By

Fatoş Tuba ÇETİNKAYA

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ABSTRACT

Bacillus thuringiensis is a ubiquitous, gram-positive and spore-forming bacterium. During sporulation, it produces intracellular crystal proteins (cry proteins), which are toxic to insects. Because of its insecticidal activity, it has been used for nearly fifty years to control certain insect species among the orders Lepidoptera, Coloeptera, and Diptera. However, it is still necessary to search for more toxins to control other insect orders and to provide alternatives for coping with the problem of insect resistance. The genetic diversity of *B. thuringiensis* strains shows differences according to the regions where they were isolated. Thus, each habitat may contain novel *B. thuringiensis* strains, which have some toxic effects on target spectra of insects.

The aim of this study was to isolate *B. thuringiensis* strains from different environments and to identify the crystalline protein gene content of the isolates. Sixty five samples including soil, stored product dust, insect cadavers, and dry leaf residues were collected from Akhisar/Manisa, İzmir, and Ereğli/Konya. Three approaches were applied for the isolation of *B. thuringiensis*: sodium acetate selection, heat treatment, and endospore staining. Polymerase Chain Reaction (PCR) method was used for the characterization of *cry* gene content of *B. thuringiensis* strains. The universal primers specific to *cry* 1, *cry*2, *cry* 3, and *cry* 9 genes were used to detect the type of *cry* gene carried by each environmental isolate of *B. thuringiensis* strains. In addition, 16S rRNA based PCR-restriction fragment length polymorphism (RFLP) was carried out to confirm *B. thuringiensis* strains. Finally, SDS-PAGE analysis was optimized to detect protein profiles of crystal proteins obtained from *B. thuringiensis* isolates.

It was found that, 136 of 359 isolates showed *B. thuringiensis*-like colony morphology and subterminal endospore position. One hundred isolates were screened by PCR and 18 of them were found to contain *cry* genes (5 *cry* 1, 3 *cry*3, and 10 *cry* 9). However, the *cry* 2 gene was not detected from any isolates. 16S rRNA based PCR-RFLP for 18 isolates gave the same restriction pattern as positive controls, indicating that all 18 isolates were *B. thuringiensis*. SDS-PAGE studies for Cry 9 proteins of the isolates exhibited different protein profile from positive control of *B thuringiensis* strain.

ÖZ

Bacillus thuringiensis yaygın olarak yeryüzünün her kısmında bulunabilen, gram-pozitif ve spor oluşturan bir bakteridir. Spor oluşumu sırasında, böceklere karşı toksik, hücre içi kristal proteinleri (cry proteinleri) sentezler. İnsektisidal aktivitesinden dolayı, yaklaşık olarak elli yıldan beri Lepidopteraların, Coloepteraların, ve Dipteraların arasında bulunduğu böcek gruplarını kontrol altında tutmak amacıyla kullanılmaktadır. Bununla birlikte, başka böcek gruplarını kontrol altında tutmak ve mevcut böceklerin bu tür ilaçlara karşı geliştirdiği dirence alternatif olarak, yeni kristal proteinlerinin araştırılması gereklidir. *B. thuringiensis* türlerinin genetiksel çeşitliliği izole edildikleri bölgelere bağlı olarak farklılık göstermektedir. Böylelikle, her bir habitat amaçlanan böcek gruplarına toksik etki gösteren yeni *B. thuringiensis* türlerini barındırabilir.

Bu çalışmanın amacı, farklı ortamlardan *B. thuringiensis* türlerinin izole edilmesi ve bu izolatların içerdikleri kristal protein genlerinin saptanmasıydı. Topraktan, depo tozlarından, böcek ölülerinden ve kuru yapraklardan oluşan altmış beş örnek, Akhisar/Manisa, İzmir, ve Ereğli/Konya'dan toplandı. *B. thuringiensis* izolasyonu için sodyum asetat seleksiyonu, sıcaklık uygulaması ve endospore boyama yöntemlerine başvuruldu. *B. thuringiensis* türlerinin içerdikleri kristal protein genlerinin karakterizasyonu için Polimeraz Zincir Reaksiyonu (PCR) uygulandı. Farklı ortamlardan izole edilen *B. thuringiensis* türlerinin hangi tip kristal geni taşıdığının belirlenmesi için *cry* 1, *cry* 2, *cry* 3, ve *cry* 9 kristal genleri için spesifik üniversal primerler kullanıldı. Buna ek olarak, elde edilen izolatların *B. thuringienis* olduğunu teyit etmek için 16S rRNA based PCR-restriction length polymorphisim (RFLP) metodu kullanıldı. Son olarak, elde edilen izolatların kristal protein profillerinin tespiti için SDS-PAGE metodu optimize edildi.

349 izolattan 136 tanesinin *B. thuringiensis*'e benzer koloni morfolojisi gösterdiği ve subterminal pozisyonda endospore içerdiği bulundu. 100 izolat PCR metodu ile tarandı ve 18 tanesinin *cry* geni (5 *cry* 1, 3 *cry* 3, ve 10 *cry* 9) taşıdığı belirlendi. Bununla birlikte, hiçbir izolatta *cry* 2 geni tespit edilmedi. Pozitif 18 izolatın 16S rRNA based PCR-RFLP ile taranması sonucunda, tip türlerle birlikte aynı restriksiyon profilleri elde edildi ki, bu da 18 izolatın *B. thuringiensis* olduğunu gösterdi. Bu izolatların Cry 9 proteinleri için SDS-PAGE ile yapılan çalışmalarda *B. thuringiensis* tip türünden farklı kristal proteini profili elde edildi.

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Chapter 1

INTRODUCTION

The competition for crops between human and insects is as old as agriculture. The use of chemical substances to control pests was started in the mid-1800s. Early insecticides were some inorganic chemicals and organic arsenic compounds. Organochloride compounds, organophosphates, carbamatespyrethroids and formamides followed them. Many of these chemicals are also being used today. Certain properties made these chemicals useful, such as long residual action and toxicity to a wide spectrum of organisms. However, chemical pesticide applications have caused many environmental problems including insect resistance, toxicity to humans and to beneficial insects (Glazer and Nikaido, 1995).

Like all organisms, insects are susceptible to infection by pathogenic microorganisms. Many of these infectious agents have a narrow host range and, therefore, do not cause uncontrolled destruction of beneficial insects and are not toxic to vertebrates. *Bacillus thuringiensis* is a major microorganism, which shows entamopathogenic activity (Glazer and Nikaido, 1995; Schnepf *et al.*,1998). The organism is a ubiquitous, gram-positive and spore-forming bacteria that forms parasporal crystals during the stationary phase of its growth cycle. Its insecticidal activity depends on parasporal crystals encoded by *cry* genes and this insecticidal activity varies according to insect type. Natural isolates of *B. thuringiensis* have been used as a biological pesticide since the 1950s for the control of certain insect species among the orders Lepidoptera, Coloeptera and Diptera. The genes of *B. thuringiensis* coding parasporal crystals are also a key source for transgenic expression which provide pest resistance in plants (Schnepf *et al.,* 1998). This feature makes *B. thuringiensis* the most important biopesticide on the world market (Bernhard *et al.,* 1997). In 1995, worldwide sales of *B. thuringiensis* based insecticides were estimated at $90 million representing about 2% of the total global insecticide market (Lambert and Pereron, 1992; Schnepf *et al.*,1998).

1.1. Developments of *Bacillus thuringiensis* Research

B. thuringiensis was first isolated by S. Ishiwata in 1901 from a diseased silkworm larvae (*Bombyx mori*) and named the isolate as *Bacillus sotto*. *B. thuringiensis* was not characterized until a decade later. E. Berliner isolated a similar *Bacillus* from a diseased Mediterranean flour moth larvae (*Anagasta kuehniella*), and named his isolate as *B. thuringiensis* (Connon, 1995). *B. thuringiensis* was first to be used as an insecticide in the 1950s in the USA. The first commercial name was Thurincide, which was prepared from *B. thuringiensis* subsp. k*urstaki* (Beegle and Yamamoto, 1992). Dulmage discovered more active *B. thuringiensis* var. *kurstaki* (HD1), which was commercialized in the USA as Dipel (Glazer and Nikaido, 1995). The demand of *B. thuringiensis* based insecticides in agriculture sector declined, in the mid 1970s, because of more effective chemical pesticides. In the 1980s, *B. thuringiensis* research was stimulated by progress in biotechnology. First, Schnepf and Whiteley (1981) cloned a crystal toxin gene from *B. thuringiensis* subsp. k*urstaki* into *E.coli*, since then much research has been performed to improve target spectra and to find out more infectious strains of *B. thuringiensis.*

1.2. Conventional *Bacillus thuringiensis* Preparations

Most *B.thuringiensis* preparations available on the market contain spores with parasporal inclusion bodies composed of δ-endotoxins. In commercial production, the crystals and spores obtained from fermentation are concentrated and formulated for spray-on application according to conventional agriculture practices (Baum *et al*., 1996). Although, there are numerous strains of *B.thuringiensis* having insecticidal activity against insect orders (e.g. Lepidoptera, Diptera, Coloptera, Homoptera, Mollaphoga), nematodes and aphids, only a few of them have been commercially developed.

B. thuringiensis based insecticides are divided into three groups**,** which are summarized on Table 1.1. Group I has been used for the control of Lepidopterans. These groups of insecticides are formulated with *B. thuringiensis* subsp. *kurstaki*. Group II contains the *sandiego* and *tenebrionis* strains of *B. thuringiensis* and has been applied for the control of certain Coloepterans and their larvae. Group III contains the

israelensis strain of *B. thuringiensis*, which has been used to control black flies and mosquitoes

Table 1.1. Examples of products incorporating *Bacillus thuringiensis* δ-endotoxins (Cannon, 1995, Biological Control: Benefits and Risks, Chapter 17, pp 192).

Product	Bt variety	Manufacturer
Products for the control of Lepidopteran larvae		
DIPEL	*kustaki* (HD 1)	Abbott Laboratories
THURICIDE	*kustaki*	Sandoz AG
JAVELIN/DELFIN	*kustaki* (NRD-12)	Sandoz AG
BACTEC BERNAN I, III, V	*kustaki*	Bactec Corporation
CATERPILLER ATTACT	*kustaki*	Ringer Coporation
BIOBIT	*kustaki*	Novo Nordisk
BACTOSPEINE	*kustaki*	Novo Nordisk
TOAROW CT	*kustaki*	Taogosei Chem
TUREX/AGREE[a]	*kustaki/aizawai*	Ciba Geigy
CUTLASS[a]	*kustaki*	Ecogen Inc
MV[b]	*kustaki*	Mycogen Coporation
M PERIL[b]	*kustaki*	Mycogen Coporation
CERTAN PLUS	*aizawai*	Sandoz AG
FLORBAC	*aizawai*	Novo Nordisk
XENTARI	*aizawai*	Abbott Laboratories
BACTEC BERNAN II	*morrisoni*	Bactec Coporation
Products for the control of Dipterous (mosquito and blackfly) larvae		
VECTOBAC	*israelensis*	Abbot Laborates
TECHNAR	*israelensis*	Sandoz AG
ACROBE	*israelensis*	American Cyanamid
MOSQUITO ATTACK	*israelensis*	Ringer Coporation
SKEETAL	*israelensis*	Novo Nordisk
BACTIMOS	*israelensis*	Novo Nordisk
Products for the control of Coloepteran larvae		
NOVADOR	*tenebrionis*	Novo Nordisk
BK-100	*tenebrionis*	Novo Nordisk
FOIL[a]	*kurstaki/tenebrionis*	Ecogen Inc
M-TRAK[b]	*san diego*	Mycogen Coporation
Products for the control of forest defoliating caterpillers		
THURICIDE	*kurstaki*	Sandoz AG
FORAY	*kurstaki*	Novo Nordisk
BIODART	*kurstaki*	ICI (Zenece)
CONDOR	*kurstaki*	Ecogen Inc

[a]- transconjugant
[b]-killed microbial (transgenic)

1.3. General Characteristics of *Bacillus thuringiensis*

B. thuringiensis is a member of the genus *Bacillus* and like the other members of the taxon has the ability to form endospores that are resistant to inactivation by heat, desiccation and organic solvents. The spore formation of the organism varies from terminal to subterminal in sporangia that are not swollen, therefore, *B. thuringiensis* resembles other *Bacillus* species in morphology and shape (Stahly *et al.,* 1991). The organism is a gram-positive and facultative anaerobe. The shape of the cells of the organism is rod. The width of the rod varies 3-5 μm in size when grown in standard liquid media. The most distinguishing feature of *B. thuringiensis* from closely related bacillus species (e.g. *B. cereus, B. anthracis*) is the presence of a parasporal crystal body that is near to the spore, outside the exosporangium during the endospore formation, which is shown in Figure 1.1 (Andrews *et al*., 1985; Andrews *et al*., 1987; Bulla *et al*.,1995).

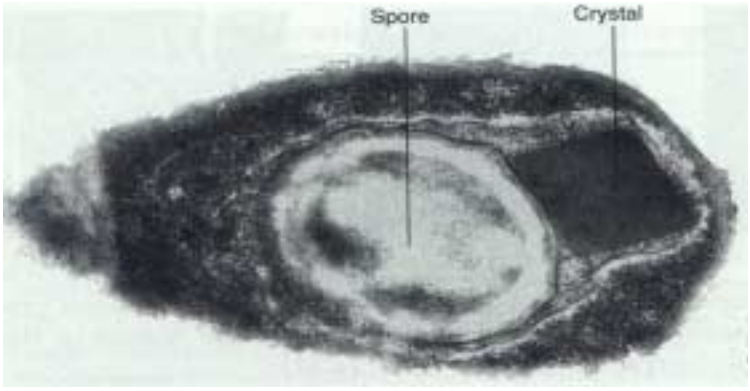

Figure 1.1. Formation of the toxic parasporal crystal in *B. thuringiensis* (Madigan *et al.,* 2000, Brock Biology of Microorganisms, Chapter 12, pp 509).

It is thought that *B. thuringiensis* is an insecticide-producing variant of *B.cereus* (Gordon *et al*., 1973). Several *B. thuringiensis* strains also produce *B .cereus* type enterotoxin (Carson and Kolstø, 1993). Plasmids coding for the insecticidal toxin of *B. thuringiensis* have been transferred into *B. cereus* to make it a crystal producing variant

of *B. thuringiensis* (Gonzales *et al*., 1982). Molecular methods including genomic restriction digestion analysis and 16S rRNA sequence comparison support that *B. thuringiensis, B. anthracis,* and *B. cereus* are closely related species and they should be considered as a single species (Carson, 1994, 1996; Ash *et al*., 1991, Bourque *et al*., 1995; Helgason *et al*., 2000).

1.4. Ecology and Prevalence of *Bacillus thuringiensis*

B. thuringiensis occurs naturally and it can also be added to an ecosystem artificially to achieve insect control. For this reason, the prevalence of *B. thuringiensis* in nature can be defined as "natural" and "artificial". The habitat is considered as natural when *B. thuringiensis* can be isolated when there is no previous record of application of the organism for insect control. The artificial habitats of *B. thuringiensis* are areas sprayed with *B. thuringiensis* based insecticides (usually a mixture of spores and crystals) (Stahly *et al*., 1991).

B.thuringiensis is indigenous to many environments including soil **(**Martin and Travers, 1989; Bernard *et al*., 1997)**,** insect cadavers (Corazzi *et al*., 1991; Kaelin *et al*., 1994; Itaqou-Apoyolo *et al*., 1995; Lopez-Meza and Ibarra, 1996; Cadavos *et al*., 2001), stored product dust (Chambers *et al*., 1991; Meadows *et al*., 1992; Hongyu *et al*., 2000), leaves of plants (Smith and Couche, 1991; Bel *et al*., 1997; Mizuki *et al*., 1999), and aquatic environments (Iriarte *et al*., 2000; Ichimatsu *et al*., 2000). Moreover, *B. thuringiensis* has recently been isolated from marine sediments (Maeda *et al*., 2000), and also from the soils of Antarctica (Forsty and Logan, 2000). Thus, it is obvious that *B. thuringiensis* is widespread in nature. However, the normal habitat of the organism is soil. The organism grows naturally as a saprophyte, feeding on dead-organic matter, therefore, the spores of *B. thuringiensis* persist in soil and vegetative growth occurs when nutrients are available. Because of this, *B. thuringiensis* can also be found in dead insects.

Meadows (1993) suggested three prevailing hypothetical niches of *B. thuringiensis* in the environment: as an entomopathogen, as a phylloplane inhabitant, and as a soil microorganism. However, the true role of the bacteria is not clear. Although, it produces parasporal crystal inclusions that are toxic to many orders of insects, many *B. thuringiensis* strains obtained from diverse environments show no insecticidal activity. For example, Maeda *et al*. (2000) has found that *B. thuringiensis*

strains obtained from marine environments of Japan exhibit no insecticidal activities. The insecticidal activity of *B. thuringiensis* are rare in nature. For instance, Iriarte *et al*. (2000) reported that there is no relationship between mosquito breeding sites and pathogenic action level of *B. thuringiensis* in the surveyed aquatic habitats. However, another study suggests that habitats with a high density of insect mortality were originated by the pathogenic action of this bacterium (Itoqou-Apoyolo *et al*., 1995).

1.5. Insecticidal Crystal Proteins of *Bacillus thuringiensis*

1.5.1. Mode of Action

The crystal proteins of *B. thuringiensis* show host specificity. For this reason, each type of Cry protein can be toxic to one or more specific insect species. Because of this specific toxicity, they do not affect many beneficial insects, plants and animals including humans. The specificity of these insecticidal crystal proteins (ICPs) derives from their mode of action (Adang, 1991; Gill *et al*., 1992).

The parasporal crystals of *B. thuringiensis* contain the ICPs in the form of protoxins. After ingestion of parasporal crystals by the susceptible insect, the crystals are dissolved in alkaline conditions (pH 10-12) in the insect mid-gut, generating 130 to 135 kDa protein chains called protoxin. These proteins are then processed to the actual toxic fragments of 60-65 kDa by the gut proteases (Gill *et al*., 1992; Höfte and Whiteley, 1989; Knowles, 1994). Finally, these activated toxins bind to specific receptors present in the larval mid-gut epithelia. The activated toxin binding to the specific receptors on the cell membrane creates ion channels or pores. The pore formation causes osmotic shock. As a result of this process, the cell membrane lyses, paralysis occurs and consequently, the insect stops feeding and dies from starvation (Knowless, 1994).

1.5.2. Structural Features of Crystal Proteins

B. thuringiensis produce one or more crystalline inclusion (parasporal crystal) bodies during the sporulation of its growth cycle and these can be seen under the phase-contrast microscope. Several terminologies are used for the crystalline inclusions, for example, insecticidal crystal proteins (ICPs), cry toxins or δ-endotoxin. These

parasporal crystals consist of proteins, which exhibit highly toxic insecticidal activity. On the other hand, actively growing cells lack the crystalline inclusions, so that, they are not toxic.

The δ-endotoxins fall into two categories; Cyt and Cry. These two types of δ-endotoxins do not share significant sequence homology, although, both seem to work through pore formation that leads to cell lysis and irreversible damage of the insect mid-gut (Gill *et al*., 1987; Thomas and Ellar, 1983; Chang *et al*., 1993; Guerchcoff *et al*., 2001). The three dimensional structures of the four δ-endotoxins (Cry 1, Cry 2, Cry 3 and Cyt 2A) have been resolved by X-ray crystallography (Li *et al*., 1991; Li *et al*., 1996; Grachulski *et al*., 1995). The Cry 1, Cry 2, and Cry 3 are remarkably similar, each of them consisting of three domains, which is shown in Figure1.2. The N-terminal Domain I consists of seven α-helices. These are six amphipathic helices which around a central core helix. Domain II consists of three β-sheets with three-fold symmetry. This conformation is called 'Greek Key'. The C-terminal, domain III, consists of two antiparallel of β-sheets in a 'jelly-roll' formation. Each domain has a role in the mode of action of the toxin. Domain I is involved in membrane insertion and pore formation. Domains II and III are both involved in receptor recognization and binding. Additionally, a role for domain III in pore function has been found (De Maagd *et al*., 1996, 1996, 2001).

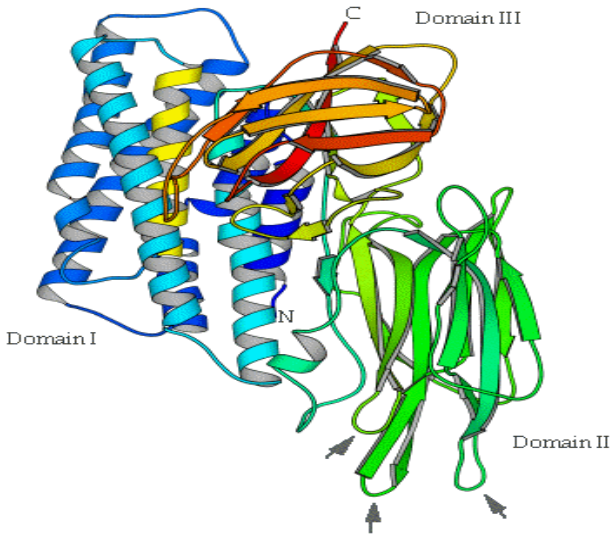

Figure 1.2. The structure of Cry 3A (http:// www.bioc.cam.ac.uk/UTOs/Ellar.html)

However, Cyt 2A structure is radically different from the other three structures (Crickmore et al., 1998). It consists of a single domain, which is shown in Figure 1.3. The structure of the domain is composed of alpha helix outer layers wrapped around a mixed beta-sheet (Schnepf *et al*., 1998).

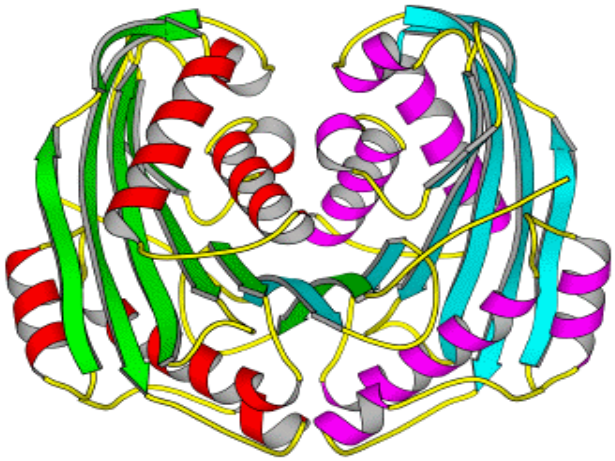

Figure 1.3. The structure of Cyt 2A (http:// www.bioc.cam.ac.uk/UTOs/Ellar.html)

The activated cry toxins have two functions: receptor binding and ion channel activity. The activated toxin binds to the specific receptors on the mid-gut epithelia of susceptible insect (Hofman *et al*., 1988 and 1988). Binding is a two stage process involving reversible and irreversible binding (Van-Rie *et al*., 1989). These steps may include toxin binding to the receptor, insertion of the toxin into apical membrane or both. On the other hand, the Cyt toxins have no specific receptor recognition, although, they cause pore formation.

1.5.3. Other Pathogenic Factors of *Bacillus thuringiensis*

During the active growth cycle, certain strains of *B. thuringiensis* produce extracellular compounds, which might contribute to virulence. These extracellular

compounds include phospholipases, β-exotoxins, proteases, chitinases and vegetative insecticidal proteins (VIPs) (Zhang *et al*., 1993; Levinson, 1990; Estruch, 1996; Lövgren, 1990; Schnepf *et al*., 1998). *B. thuringiensis* also produces antibiotic compounds having antifungal activity (Stabb *et al*., 1994). However, the cry toxins are more effective than these extracelluler compounds and allow the development of the bacteria in dead or weakened insect larvae.

Some strains of *B. thuringiensis* produce a low molecular weight, heat stable toxin called β-exotoxin, which has a nucleotide-like structure. Because of its nucleotide-like structure it inhibits the activity of DNA-dependent RNA polymerase of both bacterial and mammalian cells (Glazer and Nikaido, 1995). *B. thuringiensis* strains also produce a protease, which is called inhibitor A. This protein attacks and selectively destroys cecropins and attacins which are antibacterial proteins in insect. As a result of this, the defense response of the insect collapses. The protease activity is specific, because it attacks an open hydrophobic region near the C-terminus of the cecropin and it does not attacks the globular proteins **(**Dalhambar and Steiner, 1984).

Other important insecticidal proteins, unrelated to Cry proteins, are vegetative insecticidal proteins (VIPs). These proteins are produced by some strains of *B. thuringiensis* during vegetative growth. These VIPs do not form parasporal crystals and are secreted from the cell. For this reason, they are not included in the Cry protein nomenclature. For example, the VIP 1A gene encodes a 100 kDa protein which is processed from its N-terminus. This processing produces an 80 kDa product, which has been shown to be toxic to western corn root warp larvae (Schnepf, 1998).

1.6. Genetics of *Bacillus thuringiensis*

1.6.1. *Bacillus thuringiensis* Genome

B. thuringiensis strains have a genome size of about 2.4 to 5.7 Mb (Carson *et al*., 1994). A physical map has been constructed for *B. thuringiensis* (Carson and Kolstø, 1993). Comparison with the *B. cereus* chromosomal map suggested that all of these chromosomes have a similar organization in the half near the replication origin while displaying greater variability in the terminal half (Carson *et al*., 1996). Most *B. thuringiensis* isolates have several extra-chromosomal elements (plasmids) ranging in

size from 2 to >200 kb. Some of these plasmids are circular and some are linear. The parasporal crystal proteins are generally encoded by large plasmids. Sequence hybridizing studies with *cry* gene probes have been shown that *cry* genes are also found in the bacterial chromosome (Carson *et al*., 1994). *B. thuringiensis* also contains large variety of transposable elements (Mahillon *et al.,* 1994). These transposable elements are thought to be involved in the amplification of the *cry* genes in the bacterial cell. Another possible role of these elements could be mediating the transfer of plasmid by a conduction process involving the formations of cointegrate structures between self-conjugative plasmids and chromosomal DNA or nonconjugative plasmids. The last function of these elements may be the horizontal dissemination of genetic material, including *cry* genes, within *B. cereus* and *B. thuringiensis* species (Schnepf *et al*., 1998).

1.6.2. The *cry* Genes

The genes coding for the insecticidal crystal proteins are normally associated with plasmid of large molecular mass (Gonzales and Carlton, 1980). Many Cry protein genes have been cloned, sequenced, and named *cry* and *cyt* genes. To date, over 100 *cry* gene sequences have been organized into 32 groups and different subgroups on the basis of their nucleotide similarities and range of specificity (Crickmore *et al*., 1998; Bravo *et al.,* 1998). For example, the proteins toxic for lepidopteran insects belong to the Cry 1, Cry 9, and Cry 2 groups. The toxins against coleopteran insects are the Cry 3, Cry 7, and Cry 8 proteins and Cry1Ia1, which is a subgroup of Cry 1 proteins. The Cry 5, Cry 12, Cry 13 and Cry 14 proteins are nematocidal, and the Cry 2Aa1, which is a subgroup of Cry 2 proteins, Cry 4, Cry 10, Cry 11, Cry 16, Cry 17, Cry 19, and Cyt proteins are toxic to dipteran insects (Zeigler, 1999).

Each of the *B. thuringiensis* strains can carry one or more crystal toxin genes, and therefore, strains of the organism may synthesize one or more crystal protein. Transfer of plasmids among *B. thuringiensis* strains is the main mechanism for generating diversity in toxin genes (Thomas *et al*., 2001).

1.6.3. *Cry* Gene Expression

The insecticidal crystal proteins are synthesized during the stationary phase of the bacterial life cycle growth. These proteins generally accumulate in the mother cell. The dry weight of the proteins account for up to 25% in sporulated cells of *B. thuringiensis*. The high level of crystal protein synthesis in *B. thuringiensis* is controlled by a variety of mechanisms. These mechanisms may occur at the transcriptional, post-transcriptional and post-translational levels (Agassie and Lereclus, 1995).

The sporulation-specific genes control *cry* gene expression. However, some of *cry* gene expression occurs during the vegetative growth. Thus, the expression of *cry* gene mechanisms have been grouped in two groups, sporulation-dependent and sporulation independent. The *cry* 1Aa gene, encoding toxins active against lepidoptera, is a typical example of a sporulation-dependent *cry* gene. This gene is only expressed during the sporulation phase. On the other hand, *cry* 3Aa gene, isolated from the coloepteran-active *B. thuringiensis* var. *tenebrionis*, is expressed during the vegetative growth and also during the stationary phase. In the stationary phase, the expression of this gene has been found to be less than the vegetative phase (Sekar *et al*., 1988; De Souza *et al*., 1993).

The stability of *cry* mRNA is an important contributor to high levels toxin production at the post-transcriptional level. The half-life of *cry* mRNA is about 10 minutes which is at least fivefold greater than the half-life of an average bacterial mRNA (Glathorn and Rapoport, 1973). The putative transcriptional terminator of the *cry*1Aa (a stem loop structure) acts as a positive retroregulator (Wong and Chang, 1986). The fusion of a DNA fragment carrying this terminator to the 3′ end of the heterogonous genes increases the half-life of their transcripts by two to threefold. The stability of *cry* mRNA is also increased by the 3′-stem loop structures. Three-fold structure reduces the movement of 3′-5′ exoribonucleases. For example, the *cry* 1Aa transcriptional terminator sequence increases *cry* mRNA stability by protecting it from exonucleotytic degradation at the 3’ end (Schnepf *et al*., 1998).

The crystal proteins are generally found in the form of crystalline inclusion in the mother cell compartment. The crystal shape depends on the protoxin composition. This ability of the protoxins to crystallize may decrease their susceptibility to premature proteolytic degradation. The factors, including the secondary structure of the protoxin,

the energy of the disulphide bonds and the presence of additional *B. thuringiensis* specific components affect the structure and the solubility characteristics of *cry* proteins (Schnepf *et al*., 1998)

1.7. Strain Collections of *Bacillus thuringiensis*

Intensive screening programs have identified *B. thuringiensis* strains from soil, plant surfaces, dead insects, and stored grain samples. The screening for novel isolates has led to the discovery of strains with toxic activity against a broad range of insect orders, including Lepidoptera, Coloeptera, Diptera, Hymenoptera, Homoptera, Molophoga, and Acari (Feitelson *et al*., 1992). Furthermore, *B. thuringiensis* strains able to control other insect orders such as Nemalthelmintes, Platyhelmintes, and Sarcomastigophora have been found (Feitelson, 1993). Some strains of *B. thuringiensis* have also been found to be toxic to nematodes, mites and protozoa (Feitelson *et al*., 1992; Feitelson, 1993; Edwards *et al*., 1988) It is still necessary to search for more toxins, since a significant number of pests remain to be uncontrolled with the available Cry proteins. It is also very important to provide alternatives to overcome the problem of insect resistance, especially, with regard to the expression of *B. thuringiensis* genes encoding insecticidal proteins in transgenic plants (Van-Rie, 1991).

The genetic diversity of *B. thuringiensis* strains shows differences according to the regions where they were isolated. In fact, each habitat may contain novel *B. thuringiensis* which may have some toxic effects on a target spectra of insects. The characterization of *B. thuringiensis* strain collections may help in the understanding of the role of *B. thuringiensis* in the environment and the distribution of *cry* genes. Several *B. thuringiensis* strain collections have been described in the literature (Chak *et al*., 1994; Ben-Dov *et al*., 1997; Juárez-Pérez *et al*., 1997; Bravo *et al*., 1998; Thenius *et al*., 1998; Iriarte *et al*., 2000; Kaelin *et al*., 1994; Smith and Couche *et al*., 1991; Bernard *et al*., 1997).

1.7.1. Isolation Methods to Establish *Bacillus thuringiensis* Strain Collections

Isolation of *B. thuringiensis* from soil and other natural environments is greatly facilitated by the use of selective techniques. These techniques improve the isolation rate of the organism from environmental samples. There are many selective

enrichment methods described in the literature (Travers *et al*., 1987; Johnson and Bishop, 1996). The sodium acetate selection method has been used routinely for the isolation of *B. thuringiensis* from environmental samples (Martin and Travers, 1989; Carozzi *et al*., 1991; Ben-Dov *et al*., 1997; Bravo *et al*., 1998; Hongyu *et al*., 2000). The germination of spores in crystal forming bacilli, including both *B. thuringiensis* and *B. sphaericus*, is inhibited by sodium acetate concentrations of approximately 0.25M. Soil containing up to 10^9 bacteria/g is inoculated into a nutrient medium containing the sodium acetate. After a period of growth, the vegetative cells are eliminated by heat treatment and remaining spores are isolated on a nutrient medium without acetate. The survivors from this treatment range from 20-96% *B. thuringiensis* and *B. sphaericus*. These two species are easily differentiated by observation of colonial and cellular morphology (Travers *et al*., 1987).

B. thuringiensis is usually isolated together with *B. cereus* from the environment. Selective mediums for *B. cereus* can also be used for the isolation of *B. thuringiensis* from environmental samples (Mizuki *et al*., 1999; Ichimatsu *et al*., 2000). *B. thuringiensis* can be differentiated from *B. cereus* by the observation of parasporal crystals under phase contrast microscope. Recently, *B. thuringiensis* has also been isolated from extreme environments (Maeda *et al*., 2000; Forsty and Logan, 2000), therefore, isolation methods have been adapted according to the requirements of *B. thuringiensis* strains that isolated from such extreme conditions. For example, Maeda *et al*. (2000) isolated *B. thuringiensis* strains from marine sediments in Japan by the use of *B. cereus* selective medium containing 3% NaCl.

1.7.2. Morphological Properties of *Bacillus thuringiensis*

Colony morphology can help to distinguish *B. thuringiensis* colonies from other *Bacillus* species. The organism forms white, rough clonies, which spread out and can expand over the plate very quickly. *B. thuringiensis* strains have unswollen and ellipsoidal spores that lie in the subterminal position. The presence of parasporal crystals that are adjacent to the spore in the mother cell is the best criteria to distinguish *B. thuringiensis* from other closely related *Bacillus* species. The morphology, size, and number of parasporal inclusions may vary among *B. thuringiensis* strains. However, four distinct crystal morphologies are apparent: the typical bipyramidal crystal, related to Cry 1 proteins (Aronson *et al*., 1976); cuboidal inclusions related to Cry 2 proteins

and usually associated with bipyramidal crystals (Ohba and Aizawi, 1986); amorphous and composite crystals releated to Cry 4 and Cyt proteins (Federici *et al*., 1990); and flat, square crystals, related to Cry 3 proteins (Hernstadt *et al*., 1986 ; Lopez-Meza and Ibarra, 1996). Spherical and irregular pointed crystal morphologies can also be observed in *B. thuringiensis* strains.

There is a relationship between toxic activity and crystal shape, so that the observation of crystal morphology by phase contrast microscopy can provide important clues. For instance, Maeda *et al*. (2000), collected 22 isolates of *B. thuringiensis* from marine sediments in Japan. Two isolates of *B. thuringiensis* subsp. *kurstaki*, which are toxic to lepidopteran larvae formed typically bipyramidal inclusions, whereas isolate *higo*, which is toxic to mosquitoes, formed spherical crystals.

The observation of crystal morphology is the first step for establishing *B. thuringiensis* strain collections. Ohba and Aizawa (1986) isolated 189 isolates of *B. thuringiensis* from 136 soil samples from nonagricultural areas of Japan. The classification was based in part on the possession of parasporal bodies. Bernard *et al*. (1997) isolated 5303 *B. thuringiensis* from 80 different countries and 2793 of them were classified according to their crystal shape. They reported that the proportion with bipyramidal shaped crystals was 45.9%, while 14 % were spherical and 4 % rectangular.

1.7.3. Characterization Methods to Establish *Bacillus thuringiensis* Strain Collections

An important aspects for establishing *B. thuringiensis* strain collections is to have a method which allows for rapid and exact characterization. Many methods have been described for characterization of *B. thuringiensis* strains, such as bioassay, serotyping (De Barjac and Franchon, 1990), southern blot analysis for search of known homologous genes (Kongstad and Whiteley, 1986), analysis for reactivity to different monoclonal antibodies (Höfte *et al.*, 1988), and electrophoretic analysis of PCR products using specific primers (Corazzi *et al.*, 1991). Analysis of δ-endotoxin genes by bioassays is an exhaustive and time consuming process, since it is necessary to screen all target insect isolates. Serotyping is also an impractical method, it does not reflect the specific *cry* gene classes of strains . The main disadvantage of the analysis of reactivity

to different monoclonal antibodies is cross-reaction. Because of this, Polymerase Chain Reaction (PCR) is the best alternative of such methods.

PCR is a highly sensitive and rapid method to detect and identify the target DNA sequences (*cry* genes). It requires very little DNA and allows quick, simultaneous screening of *B. thuringiensis* strains isolated from the environment. PCR has also been exploited to predict insecticidal activities of *B. thuringiensis* strains, to determine the distribution of *cry* genes, and to detect new genes. Corazzi *et al.* (1991) reported the sequence of twelve PCR primers, which distinguish three major classes of *cry* genes (*cry* I, *cry* III, and *cry* IV). These primers were exploited to predict insecticidal activities of *B. thuringiensis* strains collected from soil samples and insect cadavers.

Each *cry* gene group is divided into different subgroups, which can show toxic effect to different species of an insect order. Multiplex-PCR method has been used to detect subgroups of a cry gene family. This method is based on two sets of primers. The first set of primers, the universal primer set is applied to detect the related *cry* gene family, and the other set of primer called the specific primer set is used for the detection of the subgroup of the *cry* gene family. The universal primers are chosen from highly conserved regions of a *cry* gene family. The specific primers, on the other hand, are designed from variable regions of these gene family sequences. For instance, Ceron *et al.* (1994, 1995) exploited this method to detect subgroups of *cry* I and *cry* III genes from soil collected *B. thuringiensis* strains. Detection of novel *cry* genes has also been achieved by this method. For example, Juarez-Perez *et al*. (1997) applied PCR technology to detect new *cry* genes. For this purpose, degenerate primers, which exist between two conserved regions of *cry* 1 gene family, and specific primers were used together to detect *cry* 1 type of novel *cry* genes. By this method, a novel *cry* 1B was reported.

The genetic diversity and distribution of *cry* genes varies according to the regions where they were isolated, so that each habitat can contain a novel *cry* gene group that has a different insecticidal activity. Because of this, PCR-based methods have been used extensively to establish *B. thuringiensis* strain collections. For instance, it was reported that the distribution of *cry* 1-type genes in the Taiwan strain collection is dependent on geographic location and some of these isolates may contain novel cry IC-type genes (Chak *et al.,* 1994). In Russian strain collection, six pairs of universal primers (*cry* 1, *cry* 2, *cry* 3, *cry* 4, *cry* 7/8, and *cry* 9) were used to determine the distribution of *cry* genes, and also specific primers (20 *cry* 1, 3 *cry* 2, 4 *cry* 3, 2 *cry* 4, 2

cry 7, 3 *cry* 8, and 4 *cry* 9) were applied to detect new *cry* genes from 124 soil isolates of *B. thuringiensis*. Strains containing *cry*1 type genes were found to be most abundant, while strains with *cry* 3 were absent and it was also reported that 43 of 124 field collected strains of *B. thuringiensis* might contain new *cry* gene or genes (Ben-Dov *et al.,* 1997, 1999). A similar study was done by Bravo *et al*. (1998). They isolated 496 *B. thuringiensis* strains from Mexico. The analysis of strains was based on Multiplex-PCR with general and specific primers that could detect cry 1, cry 2, cry 3, cry 5, cry 7, cry 8, cry 9, cry 11, cry 12, cry 13, cry 14, cry 21, and cyt genes. They reported that cry 5, cry 12, cry 13, cry 14, and cry 21 carrying strains were absent in their collection.

The main drawbacks of the PCR approaches are the detection of already-known genes and the failure to detect and identify novel cry genes. Kuo and Chank (1996) have proposed a method to increase the efficiency of PCR- based methods. This method is PCR-based Restriction Length Polymorphism (PCR- based RFLP). PCR-RFLP is essentially based on the amplification of *cry*-type gene family with known universal primers and PCR products are then cut with restriction enzymes to detect the differences in a cry gene group.

1.8. Objectives

Because the genetic diversity and distribution of cry genes in *B. thuringiensis* strains vary based on geographical location. Each habitat may contain novel *B. thuringiensis* isolate that have more toxic effects on target spectra of insects. Intensive screening programs have been identified *B. thuringiensis* strains from soil, plant surfaces and stored product dust samples. The screening for novel isolates of *B. thuringiensis* is a worldwide project. Therefore, many strain collections have been described in the litrature, such as Assian (Chak *et al.,* 1994; Ben-Dov *et al.,* 1997, 1999) and Mexican (Bravo *et al*., 1998) strain collections. Therefore, the main goals of this study were:

1) To isolate *B. thuringiensis* strains from different environments in Turkey.
2) To identify crystalline protein gene content of the isolates by PCR analysis with universal primers specific to *cry* 1, *cry* 2, *cry* 3, and *cry* 9 genes.
3) To confirm the B. thuringiensis isolates based on 16S rRNA based PCR-RFLP analysis.
4) To determine crystal protein profiles of isolated *B. thuringiensis* strains by SDS-PAGE analysis.

Chapter 2

MATERIALS AND METHODS

2.1. Materials

Nutrient broth was purchased from Oxoid. Bacteriological agar, mineral oil, safranin, malachite green, methylene blue, commassie brillant blue, ethidium bromide were from Merck Chemical Company. Bacteriological plates were obtained from Greiner. Sodium acetate (monobasic), crystal violet, ammonium oxalate, ammonium per sulfate, iodine, potassium iodide, EDTA, Tris-base, agarose, and Direct Load Range DNA Molecular Weight Marker, acrylamide solution (40%), bromophenol blue, TEMED were purchased from Sigma Chemical Company. Glycerol was from Applichem Chemical Company. Taq DNA Polymerase kit and dNTPs were obtained from Promega. Restriction enzymes, Taq I and Hae III, Protein Molecular Weight Marker were from Fermentas. Primers were synthesized by Integrated DNA Technologies, INC. Reference strains of *Bacillus thuringiensis*; ***B. thuringiensis*** subsp. ***kurstaki***, ***B. thuringiensis*** subsp. ***aizawai***, ***B. thuringiensis*** biovar. ***tenebrionis***, ***B. thuringiensis*** biovar. ***israelensis***, and ***B. thuringiensis*** subsp. ***kumamotoensis***, were kindly supplied by Bacillus Genetic Stock Center, Ohio State University, USA.

2.2. Methods

2.2.1. Sample Collection

Sixty five samples including soil, marshy areas, dead insects, dry leaf residues, animal feces, and stored product dust samples were collected from Akhisar/ Manisa, İzmir, and Ereğli/ Konya, where there is no previous record of application of *B. thuringiensis* based insecticides. The collected samples are summarized on Table 2.1. The soil samples were taken 2 to 5 cm below the surface, after scraping of the surface

material with sterile spatula. Finally, collected samples were stored in sterile plastic bags at 4 °C

Table 2.1. Types, locations, and numbers of collected samples.

Sample Type	Location	Sample Number
Soil	İzmir	30
	Ereğli/ Konya	14
Stored product dust	Akhisar/ Manisa	3
	İzmir	3
	Ereğli/ Konya	2
Dead insect	Akhisar/ Manisa	1
	İzmir	2
Animal feces	İzmir	3
Dry leaf residues	İzmir	3
Marshy Areas	İzmir	4

2.2.2. Isolation of *Bacillus thuringiensis*

The sodium acetate/ heat treatment method was applied to isolate *B. thuringiensis* from environmental samples. Approximately, 0.25 g of each sample were suspended in 18×180 mm test tubes containing 10 ml nutrient broth with concentrations of sodium acetate 0.12M and 0.25M [pH: 6.8]. The samples were also suspended in nutrient broth without sodium acetate as negative control. Next, suspensions were vortexed vigorously and incubated overnight at 37 °C in a shaking water bath. Afterwards, the samples were pasteurized for 5 minute at 80 °C in order to kill vegetative bacterial cells and to eliminate non-sporeforming bacterial cells. Following heat treatment, the samples were plated on nutrient agar plates, which were incubated overnight at 35 °C. Finally, bacterial colonies were separated by their colony morphology. The colonies, which showed *B. thuringiensis*-like colony morphology were rough, white and spread out over the plate. These colonies were subcultured on nutrient agar plates and incubated for 48h at 35 °C to check the position of the spore in

the bacterial cell by light microscopy. For this purpose, endospore and simple staining methods were carried out.

Simple staining means that one dye and a one step procedure was used to stain microbial cells. Spores of *Bacillus* species do not stain, and they may be seen as unstained bodies within bacterial cells stained with methylene blue. Smears of *Bacillus* isolates were prepared and they were fixed by heat. The bacterial smears were then floded with methylene blue. Staining lasted for 5 min. Finally, destaining was performed by washing under the tap water and stained bacterial colonies were observed under an oil–immersion objective. In addition, endospore staining with malatchite green was performed for a better observation of Bacillus spores. This staining procedure involved primary staining with malatchite green for 5 min and steam heat to drive the stain into spores. This stain was retained by endospores but washed out of the rest cells with water. Cells were then counterstained with the red dye safranin. The spores appeared green and cells appeared red after staining by this procedure.

Isolates having ellipsoidal and subterminal spores in unswollen bacterial cells were identified as *B. thuringiensis* and stored in nutrient broth containing 50 % glycerol at –80 °C.

2.2.3. *Bacillus thuringiensis* Strains

Reference strains of *B. thuringiensis*, which are shown in Table 2.2, were received absorbed onto paper disks. The disks were aseptically plated on nutrient agar. Two drops of nutrient broth were used to hydrate disks. Then, the plates were incubated overnight at 35 °C. A single colony was then subcultured on the nutrient agar plate. Finally, subcultured strains were stored in nutrient broth containing 50 % glycerol, at –80 °C.

Table 2.2. Reference strains of *B. thuringiensis*

Strains	BGCS Code	Original Code	Genotype	Genes
B. thuringiensis subsp.*kurstaki*	4D1	HD1	serotype 3a3b	*cry* 1,2
B. thuringiensis subsp.*aizawai*	4J3	HD133	serotype 7	*cry 1,2,9 cry 7/8*
B. thuringiensis biovar.*tenebrionis*	4AA1	tenebrionis	serovar tenebrionis	*cry 3*
B. thuringiensis biovar.*israelensis* ONR60A	4Q2	HD500	serotype 14	*cry* 4,11
B.thuringiensis subsp.*kumamotoensis*	4W1	HD867(3-11)	wildtype	*cry 7/8*

2.2.4 DNA Isolation

DNA extraction was performed as described by Bravo *et al*. (1998). Reference *B. thuringiensis* strains, which were used as positive controls, and the environmental isolates were grown overnight on nutrient agar plates at 35 °C. A loopfull of cells were transferred into 0.1 ml of sterile distilled water. The mixture was then frozen for 20 minutes at –80 °C. Thereafter, the mixture was immediately transferred into a boiling waterbath for 10 minutes to lyse the cells. Finally, the resulting cell lysate was centrifuged (Henttich, Micro 12-24 Ependorf Model) for 10 seconds at 10 000 rpm. 15 µl of the supernatant were used as the DNA template.

2.2.5. Oligonucleotide Primers for PCR

The primers used in this study have been described by Ben-Dov *et al*. (1998, 1999). One pair of universal primers (e.g., Un1 direct and reverse) for each four homology groups was applied to amplify a specific fragment. Their sequences and the expected sizes of their PCR products are shown in Table 2.3.

Table 2.3. Universal Primers

Universal Primers	Expected PCR Product Size
For cry 1 genes Un1, D_1 5'- CATGATTCATGCGGCAGATAAAC-3' R_1 5'- TTGTGACACTTCTGCTTCCCATT-3'	274-277 bp
For cry 2 genes Un2, D_2 5'- GTTATTCTTAATGCAGATGAATGGG-3' R_2 5'- CGGATAAAATAATCTGGGAAATAGT-3'	689-701 bp
For cry 3 genes Un3, D_3 5'- CGTTATCGCAGAGAGATGACATTAAC-3' R_3 5'- CATCTGTTGTTTCTGGAGGCAAT-3'	589-604 bp
For cry 9 genes Un9, D_6 5'- CGGTGTTACTATTAGCGAGGGCGG-3' R_6 5'- GTTTGAGCCGCTTCACAGCAATCC-3'	351-354 bp

2.2.6. PCR Analysis of *cry* Genes

All PCR reactions were carried out in 50 µl reaction volumes. 15 µl of template DNA was mixed with reaction buffer containing 200 µM deoxynucleoside triphosphate mix, 0.5 to 1 µM (reverse and direct) primers, 3mM magnesium chloride, and 2U of Taq DNA polymerase. Amplifications were carried out in a DNA thermal cycler (Techno-Progen). The conditions for PCR were as follows: a single denaturation step for 3 min at 95 °C, a step cycle program set for 35 cycles with a cycle of denaturation step for 1 min at 95 °C, annealing for 1 min at 52 °C, and extension for 1 min at 72 °C. Finally, an extra extension step for 10 min at 72 °C was used. Each experiment was performed with positive (a standard template) controls.

Following the amplification, 10 µl of each PCR sample was electrophoresed on 1.2 % agarose-ethidium bromide gel in Tris-Actate/ EDTA (TAE) electrophoresis buffer (0.04M Tris-Acetate, 0.001M EDTA [pH 8]) at 100V for 40 min.

2.2.7. 16S rRNA based PCR-RFLPs

PCR amplifications were routinely carried out in a 50 µl reaction volume that contained 15µl of DNA template; primers EGE 1: (5′-agagtttgatcctggctcag-3′) and EGE 2: (5′-ctacggctaccttgttacga-3′) at 10 pmol each; 1.5 mM magnesium chloride; dNTP mix at 200 µM; and 1.5 U Taq DNA polymerase. Two drops of mineral oil was used to cover PCR mixture. The conditions for PCR were as following: a step program set for 40 cycles with a cycle of denauration for 1 min at 94 °C, annealing for 1 min at 56 °C, and extension for 1 min at 72 °C. An extra extension step for 10 min at 72 °C was also used. After amplification, 10 µl of each PCR product was electrophoresed on 1% agarose-ethidium bromide gel in TAE buffer (0.04M Tris-Acetate, 0.001M EDTA [pH 8]) at 100V for 40 min. Finally, the PCR products were extracted for RFLP analysis.

Chloroform extraction method was carried out to PCR products. 60 µl of TE buffer were added to adjust the reaction volumes to 100 µl. Mineral oil was then removed from the mixture by centrifugation for 5 min at 10 000 rpm and PCR samples were transferred into sterile 1.5 ml eppendorf tubes. Two volumes of chloroform (200 µl) were added and mixed thoroughly. The samples were then centrifuged for 6 min at 10 000 rpm. The upper phase of samples was taken and 200 µl of chloroform was added. Thereafter, the mixtures were vortexed thoroughly and centrifuged for 2 min at 10 000 rpm. Upper phase of the 100 µl samples were transferred into eppendorf tubes containing 10 µl of 3M sodium acetate (pH 5.2) and vortexed thoroughly. Two volumes of 95% ethanol was added. The samples were then incubated for 30 min at –20 °C. After that, the samples were centrifuged for 15 min at 10 000 rpm and supernatants were discarded without disturbing the pellets. The DNA pellets were washed in 300 µl of 70% ethanol. The samples were then centrifuged for 5 min at 10 000 rpm and supernatants were removed. Washing step was repeated once more. Finally, dried pellets were dissolved in 10 µl of 1* TE and the resuspended samples were centrifuged at 6000 rpm for 3 second. 5 µl of each sample were transferred into 0.5 ml of PCR tubes in order to be used for restriction enzyme digestion.

Restriction enzyme digestions were carried out in a 20 µl reaction volume that contained 2U of restriction enzyme. The samples were digested with Taq I (at 65 °C) and Hae III (at 37°C) restriction enzymes. The samples were then incubated overnight. Additionally, mineral oil was used in order to avoid evaporation for Taq I. Finally, the

samples were electrophoresed on 1 % agarose-ethidium bromide gel in TAE buffer at 100 V for 40 min.

2.2.8. Protein Profiling

Purification of crystal proteins was done according to the method of Bel *et al.* (1997). *B. thuringiensis* isolates were cultured and allowed to sporulate on nutrient agar plates. When sporulation was assumed to be completed, two loops of the colony material were removed from the plate and transferred to sterile eppendorf tubes containing 1 ml of ice-cold sodium hydroxide. Resuspended samples were centrifuged for 5 min at 13 000 rpm and supernatants were discarded. After that, pellets were resuspended in 140 µl of 1 % SDS-0.01 % β-mercaptoethanol, and boiled for 10 min to dissolve the crystals. Samples were then centrifuged for 10 min at 13 000 rpm and supernatants were removed to be applied to TCA precipitation. For protein precipitation, 140 µl of 20 % TCA (thricholoroaceticacid) was added onto protein pellet and incubated for 10 min on ice. Thereafter, the resuspended pellets were centrifuged for 15 min at 10 000 rpm and supernatants carefully removed. An equal volume of cold acetone was then added and centrifuged for 5 min at 12 000 rpm. Finally, supernatants were removed to be used for electrophoresis by resuspending the pellets in SDS-PAGE loading buffer (0.15M Tris/Cl pH 8.8, 3.75mM EDTA, 0.75M sucrose, 0.075 % bromophenol blue, 2.5 % SDS, and 7.4 mM dithiothreitol) in equal volumes. The samples were then boiled for 10 min. Electrophoresis was carried out in 10 % polyacrylamide gel, at 150V.

After electrophoresis, the gel was fixed with a solution containing 10 ml of acetic acid, 20 ml of methanol, and 70 ml of sterile distilled water. The coomassie brilliant blue R250 was used for the staining. The stain was prepared by dissolving 1.25 g of dye in 450 ml of methanol: H_20 (1:1 v/v) and 50 ml of glacial acetic acid. The staining was carried out overnight in a glass tray containing 500 ml of staining solution at room temperature on an orbital shaker. Finally, the gel was destained by soaking it in the methanol/ acetic acid solution containing 90 ml of methanol: H_2O (1:1 v/v) and 10 ml of acetic acid on an orbital shaker for 4h. After destaining, the gel was dried in gelatin.

Chapter 3

RESULTS AND DISCUSSION

3.1. Sample Collection and Isolation

Sixty five samples comprising soil, soil from marshy places, dead insects, dry leaf residues, animal feces, and stored product dust samples, were collected from Akhisar/ Manisa, İzmir and Ereğli / Konya. Locations of samples are shown in Table 2.1 of the material and method section.

Travers *et al.,* (1987) tested 37 strains of spore-forming bacteria in four sodium acetate concentrations (0.06M, 0.12M, 0.25M, and 0.5M) in order to determine their ability to germinate in acetate buffered medium. They reported that the germination of *B. thuringiensis* strains was usually inhibited by 0.25M sodium acetate concentration, while other spore-formers germinated. Several *B. thuringiensis* isolates lacked the ability to germinate in the presence of 0.12 M acetate buffer and, while other *B. thuringiensis* (non-acetate selected isolates) strains could germinate in high sodium acetate concentrations. Similar to their isolation study, in this study, two different sodium acetate concentrations (0.12M and 0.25M) and also nutrient broth without sodium acetate (negative control) were used to increase the efficiency of the isolation rate of *B. thuringiensis* strains from environmental samples and to compare sodium acetate selection results with negative control. Pasteurization of samples at 80°C for 5 min was performed to kill vegetative cells of other spore-formers and to eliminate non-spore formers. Both sodium acetate/heat treatment and negative control with heat treatment gave similar isolation rate of *B. thuringiensis*, however, more of the other spore-formers were isolated with negative control, and overall 359 *Bacillus* species were isolated from 65 environmental samples.

Based on literature, the colony morphology of *B. thuringiensis* is rough, white and spread out over the plate, and the cellular morphology of the organism is a rod-like shaped. The bacteria have ellipsoidal spores located at subterminal or paracentral position in the unswollen mothercell (Thiery and E. Frachon, 1997). In this study, the discrimination of *Bacillus* isolates were done according to their colony morphology and ten different morphologies were observed for 359 *Bacillus* species, which are displayed

in Table 3.1. After that, the cellular morphology of the isolates, which was randomly chosen, was observed to identify *B. thuringiensis* strains by a light microscopy. Two methods were used for the microscopic examinations; simple staining and endospore staining. According to the colonial and cellular observations, 136 of 359 (38%) isolates were provisionally identified as *B. thuringiensis* strains. The isolates exhibiting A, C, I, and D type colonies were considered to resemblance *B. thuringiensis.*

The isolates were named according to the sample number, where they were isolated; the sodium acetate concentration; and colony morphology such as 2-K-C and 2-0.12-A. The "2" shows sample number, "K" represents negative control, "0.12" displays sodium acetate concentration, "C, and A" shows colony morphology.

Table 3.1. The colony morphologies of *Bacillus* isolates

Colony Code	Morphology	Number of Isolates	% of Total Isolate Number
A	Spread, white, and wavy	97	27%
B	Small, yellow, smooth and bright	91	25%
C	Round, white and rough	31	8%
D	Spread, white and round with raised margin	25	6%
E	Yellow, irregular and spreading	21	5%
F	Yellow, and round with radiating margin	9	2%
G	Resembles to colony C, but brighter than C	22	6%
H	Resembles to colony D, but the color is flue	3	0.8%
I	Spread, white, wavy and round with scalloped margin	32	8%
J	Drop like and runny	28	7%

Martin and Travers (1989) reported that the normal habitat of *B. thuringiensis* is soil, since the spores of the organism persist in soil. Therefore, 44 soil samples were collected from agricultural areas in this study and the most of the *B. thuringiensis* strains were isolated from these soil samples. Hongyu *et al*., (2000) and Meadows *et al.*, (1992) reported that stored product dust samples are rich with *B. thuringiensis* strains. However, in our study, among the 8 stored product samples, only one *B. thuringiensis* strain was isolated. Meadows *et al*., (1992) also isolated *B. thuringiensis* from samples of bird and mammalian feces and suggested that *B. thuringiensis* multiplied in the cadavers of insects, and these cadavers were ingested by birds and mammals. For this reason, three animal feces samples were used in our study and four *B. thuringiensis* strains were isolated from them.

3.2. Identification of *cry*-type Genes From Environmental *Bacillus thuringiensis* Isolates

The *cry* gene contents of 100 *B. thuringiensis* isolates were determined by PCR analysis of *cry* 1, *cry* 2, *cry* 3, and *cry* 9 genes. Universal primers and their expected PCR products are shown in Table 2.2. of material and method section. Genomic DNA from each *B. thuringiensis* isolate served as template in PCR reactions. Reactions without any DNA template served as negative control in each PCR experiment. Reactions with Un 1 (direct and reverse), Un 2, Un 3, Un 9 primers were carried out to detect *cry* 1, *cry* 2, *cry* 3, and *cry* 9 genes, respectively. A similar PCR analysis for *cry*-type genes was previously reported by Ben-Dov *et al.*,(1997, 1999). Each PCR analysis was checked with the appropriate positive control strains of *B. thuringiensis*. These strains were *B. thuringiensis* subsp. *kurstaki* for *cry* 1 and *cry* 2 gene groups, *B. thuringiensis* biovar. *tenebrionis* for *cry* 3 gene groups, and *B. thuringiensis* subsp. *aizawai* for *cry* 9 gene group. PCR conditions were optimized using the control strains of *B. thuringiensis* before screening the isolates. All positive controls gave the expected PCR products (Figure 3.1.A, and B).

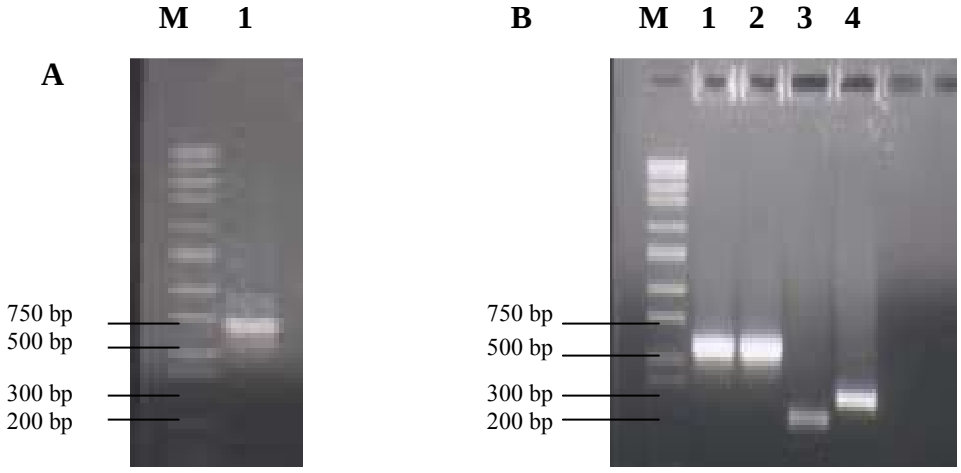

Figure 3.1. Agarose gel (1%) electrophoresis of PCR products from positive controls. **(A):** Lane M: 0.5-10kb DNA MW marker, Lane 1: *B. thuringiensis* subsp.*kurstaki* as *cry* 2 positive control; **(B):** Lane 1: *B. thuringiensis* biovar.*tenebrionis* as *cry* 3 positive control, Lane 2: *B. thuringiensis* biovar.*tenebrionis cry* 3 positive control, Lane 3: *B. thuringiensis* subsp.*kurstaki* as *cry* 1 positive control, Lane 4: *B. thuringiensis* subsp.*aizawai* as *cry* 9 positive control.

3.2.1. *cry* 1 Gene Analysis of *Bacillus thuringiensis*

When the template DNA from environmental isolates of 100 *B. thuringiensis* samples was amplified with PCR in the presence of primers for *cry* 1 gene, five isolate were shown to contain *cry* 1 gene (Figure 3.2.A, lane 2; B, lanes 2,3,4,5) as in the positive control (Figure 3.2., A, lane 1, B, lane 1). Universal primers produced PCR products of expected size at around 270bp for the isolates 7-K-A, 7-0.12-C, 7-0.12-D, and 10-0.12-D (Figure 3.2., A, lane 2, B, lanes 2, 3, 5). However, isolate 8-0.25-C produced a strong band at 350bp, which was larger than the expected size (Figure 3.2., B, lane 4). Because we used universal primers to detect each *cry* gene group, it was not possible to determine the *cry* gene sub-type in this present study. Therefore, the band at 350bp could correspond to a different sub-type of *cry* 1 gene. Corazzi *et al.* (1991) suggested that novel isolates containing novel *cry* genes may give PCR products different in size relative to the standard or may completely lack PCR products. Therefore, homology to known *cry* genes for these strains can be analyzed by using additional primers. In this present study, some *B. thuringiensis* isolates (8-0.25-C) containing *cry* 1 gene produced PCR product profile that was different from that of reference strain. This *B. thuringiensis* isolate most probably contains subgroups of *cry* 1

gene. Future experiments with specific primers for *cry* 1 gene subtypes is expected to reveal the type of *cry* gene subgroup and also new cry gene carried by this isolate.

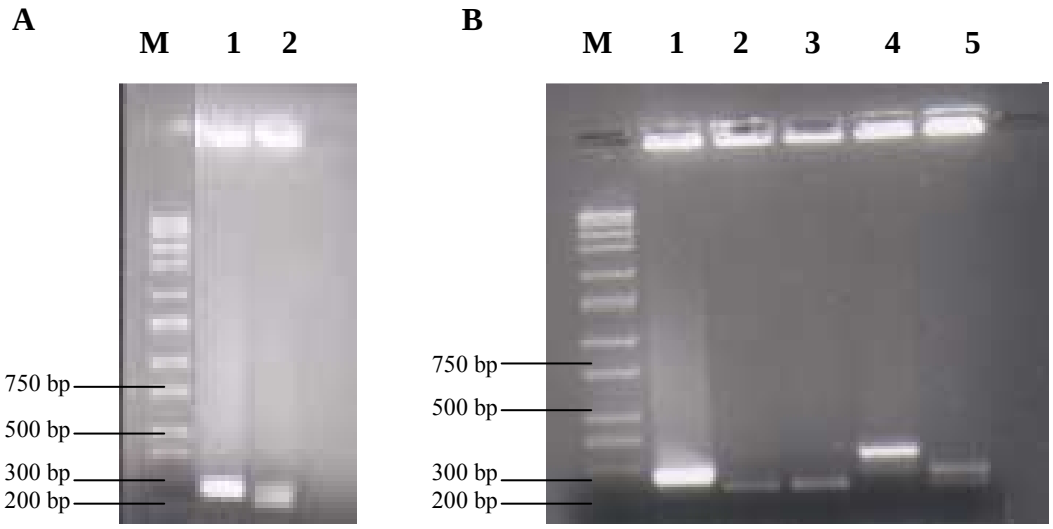

Figure 3.2. Agarose gel (1.2%) electrophoresis of PCR products for *cry* 1 genes. **(A):** Lane M: 0.5-10kb DNA MW marker, Lane 1: *B. thuringiensis* subsp. *kurstaki* as *cry* 1 positive control, Lane 2: 7-0.12-D; **(B):** Lane M: 0.5-10kb DNA MW marker, Lane 1: *B. thuringiensis* subsp. *kurstaki* as *cry* 1 positive control, Lane 2: 7-K-A, Lane 3: 7-0.25-C, Lane 4: 8-0. 25-D, Lane 5: 10-0.12-D

3.2.2. *cry* 3 Gene Analysis of *Bacillus thuringiensis*

One hundred environmental isolates of *B. thuringiensis* were screened with PCR for the presence of *cry* 3 gene. Three of them produced PCR product at about 600bp in length, the size as the band obtained in positive strains of *B. thuringiensis* (Figure 3.3., A and B, lane 1). These isolates, which were *cry* 3 positive, were 21-K-B, 46-K-A, and 46-0.25-A (Figure 3.3., A, lane 2, B, lanes 4 and 7 respectively). The environmental isolates, 46-K-A and 46-0.25-A produced 500 bp PCR product. These strains may contain a few *cry*-gene subgroups, which might have homology with the *cry* 3 gene universal primer-binding region. In every PCR reaction, a negative control without template DNA was included in order to see if there was any cross-contamination in PCR reagents. As it can be seen in Figure 3.3., B lane 8, there was no cross

contamination in the reagents, because no PCR band was observed in the absence of DNA template.

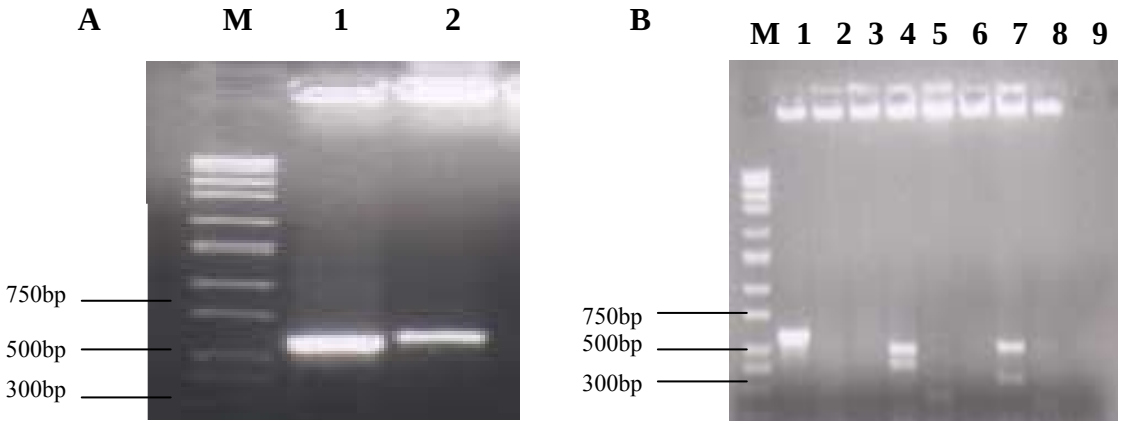

Figure 3.3. Agarose gel (1.2%) electrophoresis of PCR products for *cry*3 genes. **(A):** Lane M: 0.5-10kb DNA MW marker, Lane 1: *B. thuringiensis* biovar.*tenebrionis* as *cry* 3 positive control, Lane 2: 21-K-B; **(B):** Lane M: 0.5-10kb DNA MW marker, Lane 1: *B. thuringiensis* biovar.*tenebrionis* as *cry* 3 positive control, Lane 2: 38-K-A, Lane 3: 38-0.25-A, Lane 4: 46-K-A, Lane 5: 46-0.12-A Lane 6: 46-0.12-I, Lane 7: 46-0.25-A, Lane 8: 47-K-A, Lane 9: negative control (without DNA template)

3.2.3. *cry* 9 Gene Analysis of *Bacillus thuringiensis*

Ten of the 100 environmental isolates of *B.thuringiensis* produced positive results in PCR with Un9 universal primers for *cry* 9 gene (figure 3.4.). Although, a single band at 300bp was obtained with *B. thuringiensis* subsp. *aizawai*, which served as positive control, the field-collected strains of *B. thuringiensis,* except isolates 16-K-A, 18-K-B, and 15-K-B (Figure 3.4., B, lanes 2, 3, 7) produced an extra band at 500bp. This could due to the genetic diversity of *B. thuringiensis* strains isolated from environmental samples. These strains may contain a few *cry*-gene subgroups, which might have homology with the *cry* 9 gene universal primer-binding region. The isolate 9-0.12-A (Figure 3.4., B, lane 6) also produced an interesting PCR profile containing three extra bands. The expected band for *cry* 9 gene of this isolate was very intense at 300bp. In addition, two additional weak bands at around 550bp and 800bp were observed. Similar to previous isolates, it is probable that the isolate 9-0.12-A may

contain other *cry* 9 subgroups which have certain degree of homology to the primers chosen for *cry* 9 gene amplification. Similar to this present study, Kuo and Chak (1996) reported such extra PCR products with *cry* 1 universal primers. They also reported that this might be due to the non-specific priming of the oligonucleotide primers or the difference between the sizes of an unknown *cry* gene and predicted size of the known *cry* gene. Brown and Whiteley (1992) reported that two or three *cry* genes might be positioned next to each other, forming an operon. In this case, priming of an oligonucleotide primer to the neighbor *cry* gene may produce an extra PCR product larger than expected size. In the studies of others (Ben-Dov *et al.,* 1997; 1999), a set of bands for a specific *cry* gene group was shown in multiplex PCR analysis. In order to specifically identify such *cry* gene groups, these isolates will be examined with primers specific for subgroups of the each *cry* genes.

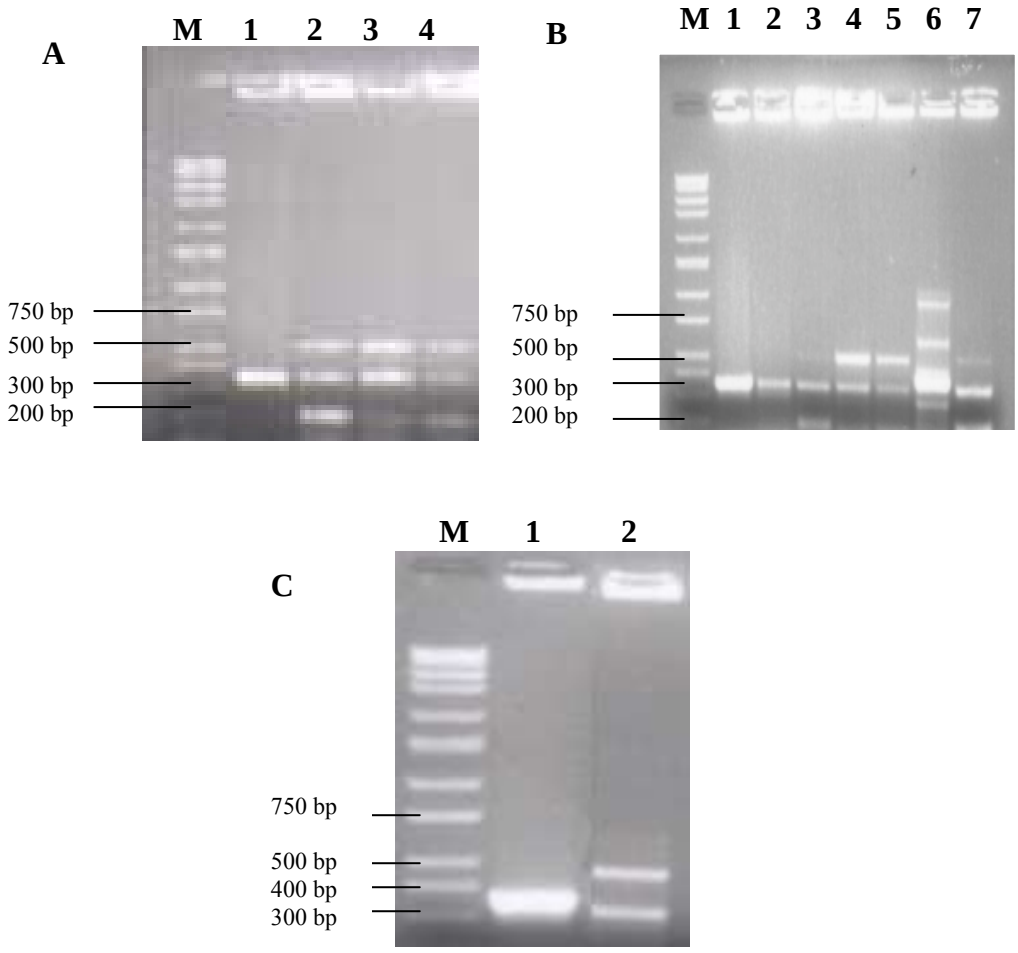

Figure 3.4. agarose gel (1.2%) electrophoresis for PCR products of *cry* 9. **(A):** Lane M: 0.5-10kb DNA MW marker, Lane 1: *B. thuringiensis* subsp. *aizawai* as *cry* 9 positive control, 33-

0.12-A, Lane 3: 37-K-A, Lane 4: 37-K-D; **(B):** Lane M: 0.5-10kb DNA MW marker,Lane 1: *B. thuringiensis* subsp. *aizawai* as *cry* 9 positive control, Lane 2: 16-K-A, Lane 3: 18-K-B, Lane 4: 20-0.12-B, Lane 5: 23-K-A, Lane 6: 9-0.12-A, Lane 7: 15-K-B., **(C):** Lane M: 0.5-10kb DNA MW marker, Lane 1: *B. thuringiensis* subsp. *aizawai* as *cry* 9 positive control, Lane 2: 13-K

Overall, 359 *Bacillus* members were isolated from 65 environmental samples. One hundred and thirty of them (38%) were identified as *B. thuringiensis* strains based on microscopic observation of spore position in the cell. One hundred of these 136 isolates were examined by PCR in order to identify the type of cry gene content of these isolates. Universal primers only for *cry* 1, *cry* 2, *cry* 3, and *cry* 9 were used for PCR. Among the 100 isolates, only 18 of them were found to be positive for the *cry* genes tested. The reason only 18% of isolates were positive with PCR is most probably due to the use of limited *cry* gene primers. In fact, 32 different *cry* gene groups were reported by Crickmore *et al*., (1998). Similar to the results of this study, Ben-Dov *et al.,* (1997) found that 89 of 215 field-collected strains of them did not produce PCR products with these universal primers. Therefore, we expect to see more *cry* gene positive isolates by using different sets of *cry* gene primers in the future.

3.3. The Distribution of cry Genes

Diversity, distribution and abundance of cry gene type are dependent on the geographical area where *B. thuringiensis* strains were collected. In this study, *cry* 9 was found in 10 % of *B. thuringiensis* isolates; therefore, this gene group was the most abundant one. According to the literature, Bravo *et al*., (1998) screened 496 field-isolated *B. thuringiensis* by PCR and detected *cry* 9 genes in 2.6 % of their Mexican strain collection. In addition, Ben-Dov *et al*., (1999) detected *cry* 9 genes in 10.2% of their 215 soil isolated *B. thuringiensis* strains from Israel, Kazakhstan, and Uzbekistan.

The *cry* 3 and *cry* 1 containing strains were detected in 3% and 5 % of all *B. thuringiensis* isolates, respectively, in this study. Ben-Dov *et al*., (1997) reported that strains containing *cry* 1 genes were most abundant, while strains with *cry* 3 were absent in soil isolated *B. thuringiensis* strains from Israel, Kazakhstan, and Uzbekistan. Another study (Chak *et al*., 1994) reported that soil isolated 225 *B. thuringiensis* strains

from Taiwan did not harbor *cry* 3 genes; however, *cry* 1A gene containing strains were the most abundant of them. Although, these two studies suggested that *cry* 3 genes were absent in Asia, three isolates containing *cry* 3 were isolated in this study. In addition, among the one hundred isolates screened by PCR, none of them was positive with *cry* 2 primers. The distribution of *B. thuringiensis* isolates in this study is summarized in Table 3.2.

Table 3.2. Type and distribution of *B. thuringiensis* isolates determined by morphological and PCR analysis.

IsolateCode	*cry* Gene	Morphology	Spore Position	Crystal Shape	Sample Type	Place	% of Isolates Tested by PCR
7-K	*cry* 1	A	Subterminal	ND	Dried leaf	Kalkıç /İzmir	**5 %**
7-0.12	*cry* 1	D	Terminal	IP	Dried leaf	Kalkıç /İzmir	
7-0.25	*cry* 1	C	Subterminal	B, R, IP	Dried leaf	Kalkıç /İzmir	
8-0.25	*cry* 1	D	Subterminal	ND	Soil	Kalkıç /İzmir	
10-0.12	*cry* 1	D	Terminal	A, IP	Soil	Kalkıç /İzmir	
21-K-B*	*cry* 3	A	Subterminal	S	Insect cadaver	Urla /İzmir	**3 %**
46-K	*cry* 3	A	Subterminal	ND	Soil	Ereğli /Konya	
46-0.25	*cry* 3	A	Subterminal	ND	Soil	Ereğli /Konya	
9-0.12	*cry* 9	A	Subterminal	IP	Soil	Kuşcenneti /İzmir	**10%**
13-K	*cry* 9	A	Subterminal	A	Stored Product Dust	Bayraklı/İzmir	
15-K-B*	*cry* 9	A	Subterminal	IP	Soil	İzmir	
16-K	*cry* 9	A	Subterminal	IP	Soil	Kuşcenneti /İzmir	
18-K-B*	*cry* 9	A	Subterminal	S, IP	Soil	Kuşcenneti /İzmir	
20-0.12-B*	*cry* 9	A	Subterminal	IP	Soil	Çiğli /İzmir	
23-K	*cry* 9	A	Subterminal	IP	Soil	Çiğli /İzmir	
33-0.12	*cry* 9	A	Subterminal	IP	Soil	Dikili /İzmir	
37-K	*cry* 9	A	Subterminal	IP	Soil	Dikili /İzmir	
37-K	*cry* 9	D	Terminal	ND	Soil	Dikili /İzmir	

ND: Not determined; **B:** Bipyramidal, **R:** Rectangular, **S:** Spherical, **A:** Amorphous, **IR:** Irregularly Pointed

3.4. 16S rRNA based PCR-RFLP

Because 16S rRNA ribotyping is one of the most reliable methods for the identification of bacteria at the species level, ribotyping of 16S rRNA from 18 *B. thuringiensis* isolates was carried out. DNA isolated from each *B. thuringiensis* isolates was amplified in the presence of primers for variable regions of 16S rRNA. As it can be seen from Figure 3.5, each environmental isolates gave only one band at expected size, which is same as the band produced from positive control of *B. thuringiensis* strains (Lanes 7, 8, 9, 10, 11, 12).

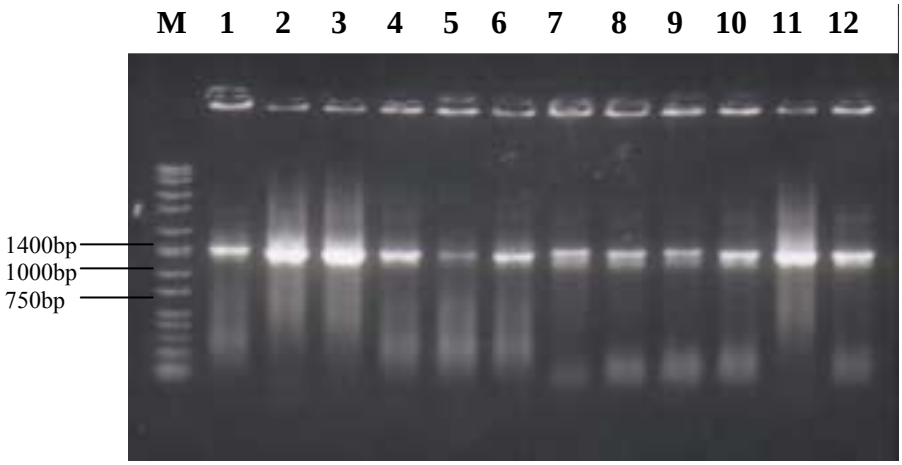

Figure 3.5. Agarose gel (1%) electrophoresis for 16s rRNA –PCR. Lane M: DNA MW Marker, Lane 1: *B. thuringiensis* subsp. *kurstaki*, Lane 2: *B. thrungiensis* biovar. *tenebrionis*, Lane 3: *B. thuringiensis* subsp. *kumamotoensis*, Lane 4: *B. thuringiensis* subsp. *aizawai*, Lane 5: *B. thuringiensis* biovar. *israelensis*, Lane 6: B. *thuringiensis* subsp. *kurstaki*, Lane 7: 7-K-A, Lane 8: 7-0.12-D, Lane 9: 7-0.25-C, Lane 10: 10-0.12-D, Lane 11: *B. thuringiensis* subsp. *tenebrionis*, Lane 12: 21-K-B

Two restriction enzymes, Taq I and Hae III, were used to cut PCR products in order to see if they will give the same restriction pattern in all isolates (Figure 3.6). After cutting the PCR products with restriction enzyme Hae III, three bands were obtained in all *B. thuringiensis* positive samples (lanes 1, 2, 3), and in all environmental

isolates (Lanes from 5 to 15). Some weak bands were obtained in lanes 4. 6. and 11. This was because of insufficient chloroform extraction of the restriction products. Restriction of 16S rRNA PCR products with Taq I enzyme resulted in the same restriction pattern with two bands in both positive control (Figure 3.7., lanes 1, 2, 3) and environmental isolates (Lanes from 4 to15). Therefore, 16S rRNA based PCR-RFLP results indicated that 18 environmental *B. thuringiensis* isolates characterized by morphological and PCR analysis were *B. thuringiensis*. In other words, 18 isolates were strongly confirmed by ribotyping experiments.

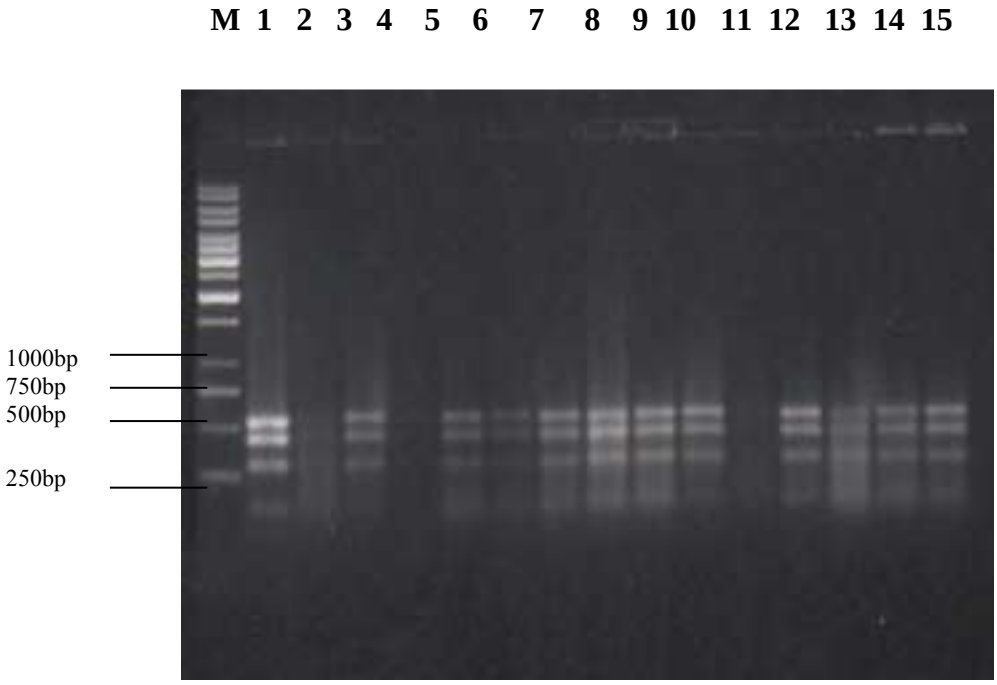

Figure 3.6. Agarose gel (1%) electrophoresis for Hae III. Lane M: DNA MWM Marker, Lane 1: *B. thuringiensis* subsp. *kumamotoensis*,Lane2: *B. thuringiensis* biovar. i*sraelensis*,Lane 3: *B. thuringiensis* subsp. *kurstaki*,Lane 4: 7-K-A, Lane 5: 7-0.12-D, Lane 6: 7-0.25-C, Lane 7:10-0.12-D, Lane 8: *B. thrungiensis* biovar. *tenebrionis*, Lane 9: 21-K-B, Lane 10: *B. thuringiensis* subsp. *aizawai*, Lane 11: 15-K-B, Lane 12: 37-K-A, Lane 13: 13-K, Lane 14: 23-K-A, Lane 15: 18-K-B

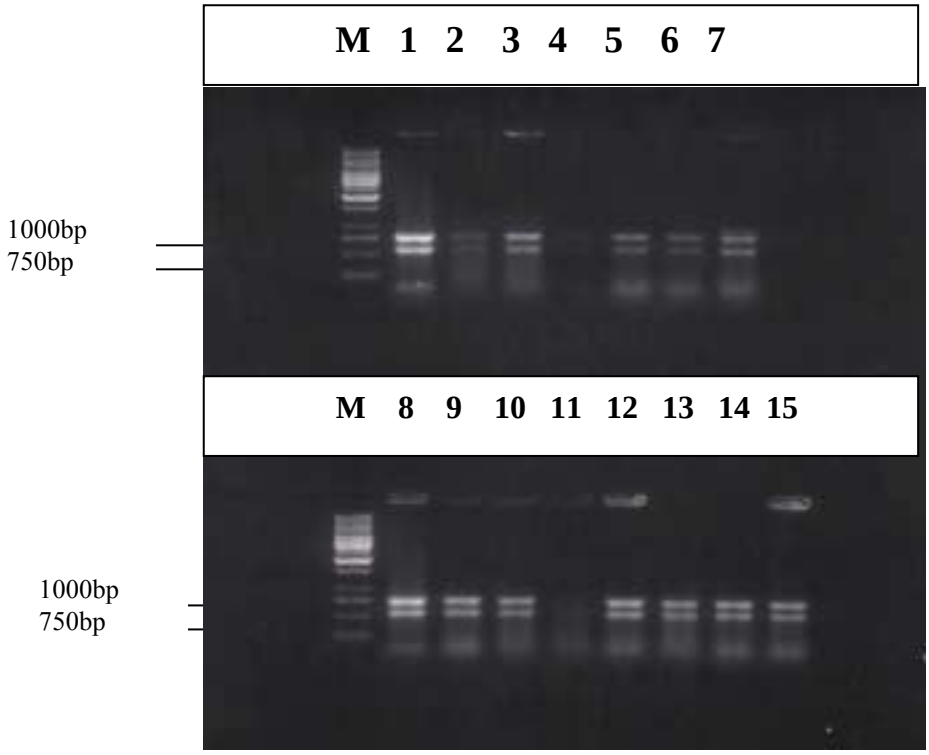

Figure 3.7. Agarose gel (%1) electrophoresis for Taq I. Lane M: DNA MW Marker, Lane 1: *B. thuringiensis* subsp. *kumamotoensis* , Lane 2: *B. thuringiensis* biovar. *israelensis,* Lane 3: *B. thuringiensis* subsp. *kurstaki,* Lane 4: 7-K-A, Lane 5: 7-0.12-D, Lane 6: 7-0.25-C, Lane 7:10-0.12-D, Lane 8: *B. thrungiensis* biovar. *tenebrionis*, Lane 9: 21-K-B, Lane 10: *B. thuringiensis* subsp. *aizawai*, Lane 11: 15-K-B, Lane 12: 37-K-A, Lane 13: 13-K, Lane 14: 23-K-A, Lane 15: 18-K-B

3.5. Crystal Morphology of *Bacillus thuringiensis* Isolates

The morphology, size, and the number of parasporal inclusions may vary among the *B. thuringiensis* strains. However, some distinct morphologies are apparent: the typical bipyramidal crystal, related to Cry 1 protein, (Aronson *et al*., 1976); cuboidal inclusions related to Cry 2 proteins (Ohba and Aizawi, 1986); and flat, square, and spherical crystals related to Cry 3 proteins (Hernstadt *et al*., 1986). Therefore, the crystal morphologies of thirty of the 18 positive isolate were observed by phase contrast microscopy in this study. Five distinct crystal morphologies were observed: bipyramidal, rectangular, amorphous, spherical and irregularly pointed (Table 3.2). All of the isolates collected from soil produced irregularly pointed parasporal inclusion.

Based on the litrature, Cry 1 type of crystal proteins encoded by *cry* 1 gene group usually associated with the bipyramidal crystals, so that, cry 1 gene carrying isolate 7-0.25-C produced the biypiramidal crystal protein and also contained rectangular and irregular pointed secondary crystals in this study(Figure 3.8.). Similar to our study, Maeda *et al*. (2000) reported that crystal inclusions of two non-insecticidal *B. thuringiensis* isolates were the mixture of two morphotypes: bipyramidal and spherical. Baum and Malvar (1995) reported that the different *cry* genes are with in operons which include open reading frames (*orf's*) encoding proteins with undefined functions. Therefore, the presence of these secondary crystals may be due to the different cry genes expressed during the sporulation phase. In addition, other crystal types were detected in the isolates. For example, the spherical crystal morphology was observed only in isolate 21-K-B, which was isolated from insect cadaver (Figure3.9). Most of the *cry* 9 containing isolates produced one type crystal protein which was irregularly pointed crystal protein (data not shown).

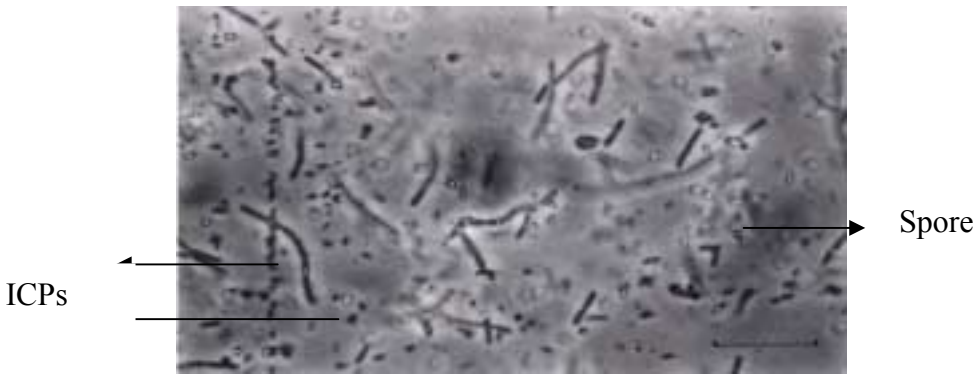

Figure 3.8. Photomicrograph of sporangia, crystals and vegetative cells of *cry* 1 positive 7-0.25-C isolate. Bar represents 10µm.

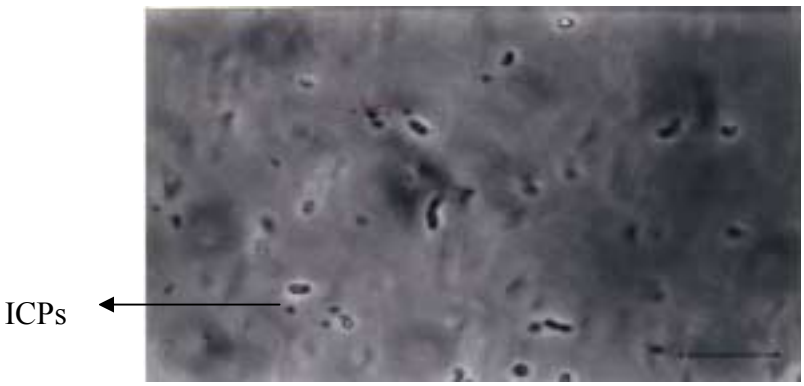

Figure 3.9. Photomicrograph of sporangia, crystals and vegetative cells of *cry* 3 positive 21-K-B isolate. Bar represents 10µm.

3.6. Crystal Protein Profiles of *Bacillus thuringiensis* Isolates

Sodium dedocyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis is generally used to compare protein profiles of *B. thuringiensis* isolates. For example, Bel *et al*., (1997) used SDS-PAGE analysis in order to determine the differences among the *B. thuringiensis* isolates collected from different olive tree environments. In the present study, before examining crystal protein profiles of 18 *B. thuringiensis* isolates, SDS-PAGE was optimized with positive controls (Figure 3.10). *B. thuringiensis* subsp. *kurstaki* containing *cry* 1 and *cry* 2 genes produced 75 kDa and 25 kDa protein (lanes 1,3); *B. thuringiensis* subsp. *aiwazai* containing *cry* 1, 2, 9, 7/8 genes produced 120 kDa protein (lane 4); *B. thuringiensis* biovar. t*enebrionis* containing *cry* 3 gene gave rise to 77 kDa, 33 kDa and 25 kDa protein, respectively (lanes 2, 5).

The size of Cry 1 and Cry 2 proteins of *B. thuringiensis* subsp. *kurstaki* were different from the study of Höfte and Whiteley (1989), who reported that Cry 1 type of protein was between 130-150 kDa; Cry 2 type protein was at 70 kDa; Cry 3 type protein was at 75 kDa and Cry 9 type protein was at 130-140 kDa. The reason for this is most probably due to different cry gene groups and also different subtypes of cry genes carried by each different strain. In fact, *B. thuringiensis* can produce one or more crystalline inclusion; therefore, the protein profile of the organism shows differences among the strains. Another possible reason for the differences between protein profiles of the same *cry* gene in two different studies could be that some environmental factors can turn on or turn off expression of some *cry* genes (Agassie and Lereclus, 1995). There may also be post transcriptional and post-translational regulation of *cry* gene proteins; therefore, *B. thuringiensis* strains can exhibit different protein profile regardless of the type of *cry* genes, which they carry. In other words, the same protein profile cannot be expected from two different strains, which carry *cry* 1 gene.

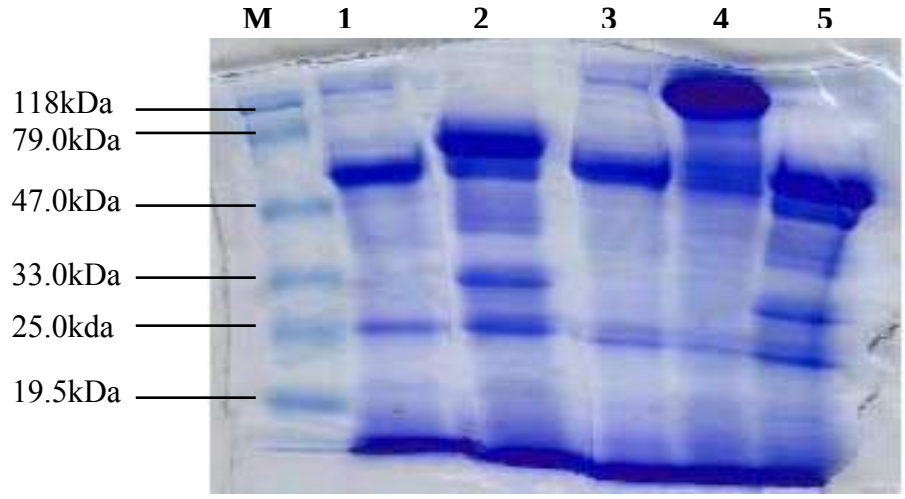

Figure 3.10. SDS-PAGE (%10) with reference strains of *B. thuringiensis*. Lane M: Protein MW Marker, Lane 1: *B. thuringiensis* subsp. *kurstaki* containing *cry* 1,2 genes, Lane 2: *B. thuringiensis* biovar.*tenebrionis* containing *cry* 3 gene, Lane 3: *B. thuringiensis* subsp. *kurstaki* containing *cry* 1,2 genes, Lane 4: *B. thuringiensis* subsp *aizawai* containing *cry* 1, 2, 9, 7/8 genes, Lane 5: *B. thuringiensis* biovar.*tenebrionis* containing *cry* 3

Crystal proteins were purified from environmental *B. thuringiensis* isolates carrying *cry* 9 gene. SDS-PAGE analysis indicated that protein profile of the isolate 13-K exhibited a strong band at 80 kDa and two weak additional bands at around 42 and 50 kDa (Figure 3.11., lane 2). However, reference strain *B. thuringiensis* subsp. *aizawai* gave rise to a strong protein band at around 120 kDa (Figure 3.11., lane 1). In other words, the protein profiles of the reference strain and 13-K isolate were different from each other, even though they both carry the *cry* 9 gene. Again this difference is most probably because of different subtypes of *cry* 9 genes carried by the reference strain and our isolate. In addition, more importantly, these reference strains carry not only *cry* 9 gene but also *cry* 1, 2, 7/8 genes. Therefore, at this point we could not tell if *cry* 9 gene or other *cry* genes were expressed at the protein level. Western blot analysis with a specific antibody for *cry* 9 gene would reveal this point. In addition, the isolates 18-K-B and 37-K-A showed a strong band at 80 kDa, similar to isolate 13-K (data not shown). Moreover, crystal protein profiles of other isolates were analyzed on SDS-PAGE, but the intensity of the protein bands was very weak. Insufficient protein concentration

could be the reason for that; and therefore, protein bands were not detectable with coomassie brilliant blue staining. More sensitive staining techniques such as silver staining kit could be alternative to visualize the bands. For example, Iriarte *et al.* (2000) found that a strain of *B. thuringiensis* have 66 kDa protein, which was not visible on SDS-PAGE gel staining with commassie brilliant blue 250, but it was detectable after silver staining. The main reason for less protein amount in environmental samples than that of reference strains could probably be due to the differences in sporulation time of the strains. Because the time for completion of sporulation varies from strain to strain and depends on growth medium conditions, parasporal crystal formation was always checked with phase contrast microscope before isolating crystal proteins from *B. thuringiensis* isolates in the literature. Therefore, crystal protein isolation will be carried out after verification of crystal formation by phase contrast microscope in future studies.

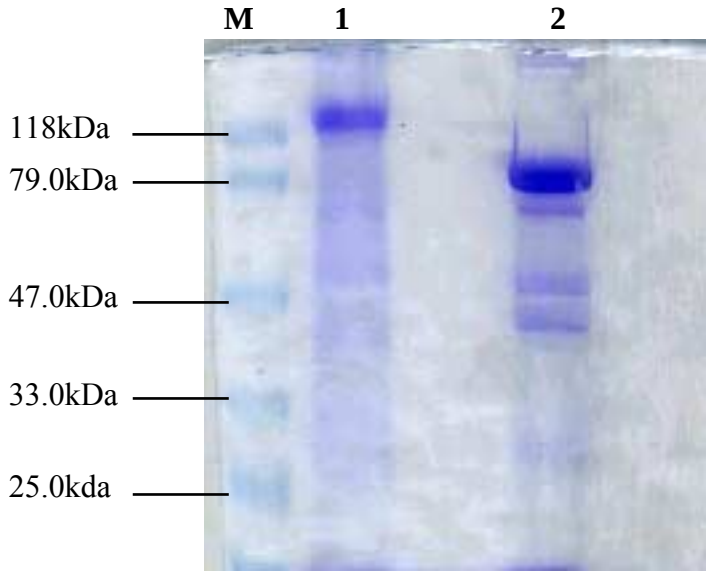

Figure 3.11. SDS-PAGE (%10) of Cry 9 proteins: Lane M: Protein MW Marker, Lane 1: *B. thuringiensis* subsp. *aizawai* containing *cry* 9 gene, Lane 2: 13-K

Chapter 4

CONCLUSION AND FUTURE EXPERIMENTS

Bacillus thuringiensis is a ubiquitous, gram-positive and spore-forming bacterium. The organism produces intracellular crystal proteins, which are toxic to insects, during the stationary phase of its growth cycle. Because of its insecticidal activity, *B. thuringiensis* has been used for a long time as a biopesticide. However, it is still necessary to search for more toxins to control insect orders, which have the ability to develop resistance against such pesticides, and to also provide alternatives for chemical insecticides. The genetic diversity of *B. thuringiensis* is directly related to geographical areas. Thus, toxic activity of the bacteria varies according to regions where the organism was isolated. Therefore, the purpose of this study was to isolate *B. thuringiensis* strains that are collected from Turkey, and to identify the *cry* gene content of these isolates, which may carry new crystal genes coding effective crystal proteins.

As a result of the study, 359 Bacillus species were isolated from 65 environmental samples collected from agricultural areas, warehouses and marshy places. One hundred and thirty six of them (38%) were identified as *B. thuringiensis* strains based on colony morphology and microscopic observation of spore position in the cell. One hundred of these 136 isolates were examined by PCR using for *cry* 1, *cry* 2, *cry* 3, and *cry* 9 universal primers in order to identify the type of *cry* gene content of these isolates. Among the 100 isolates, only 18 of them were found to be positive for the *cry* genes tested. In addition, these 18 isolates were confirmed to be *B. thuringienis* strains by carrying out 16S rRNA-based PCR-PFLP experiments. The reason that only 18% of isolates were positive with PCR could most probably be due to the use of limited *cry* gene primers. Therefore, use of different sets of *cry* gene universal primers will enable us the identification of more *B. thuringiensis* strains containing other type of *cry* gene groups. Furthermore, these 18 PCR positive isolates may contain novel *cry* genes coding for more effective insecticidal crystal proteins. In the future, specific

primers will be used to screen these isolates again in order to detect subgroups of these genes.

Five distinct crystal morphologies were observed for 13 isolates of 18 PCR positive strains by phase contrast microscopy. These were spherical, amorphous, rectangular, bipyramidal, and irregularly pointed. All of the observed isolates produced irregularly pointed crystals; on the other hand, one of the isolates contained three parasporal crystals, which were bipyramidal, rectangular and irregular pointed. Among the 13 isolates, only one isolate produced spherical crystal. Finally, SDS-PAGE analysis was carried out to determine crystal protein profiles of *B. thuringiensis* isolates and to discriminate these bacterial strains from each other. First of all, SDS-PAGE technique was optimized for crystal proteins of *B. thuringiensis.* SDS-PAGE analysis of reference strains resulted in protein bands which were distinct from each other. Similarly, protein profiles of our isolates carrying *cry* 9 gene produced a major protein band at around 80kDa, indicating that *B. thuringiensis* isolates of this study had different subgroups of *cry* 9 gene from reference *cry* 9strains, or reference strain is expressing another *cry* gene. Moreover, protein profiles of other *B. thuringiensis* isolates in this study were examined but the intensity of protein bands was not strong enough. In a future study, after conformation of crystal protein formation by phase contrast microscopy, crystal protein isolation will be carried out in order to allow sufficient protein isolation for SDS-PAGE analysis.

In a future study, the bioactivity of crystal proteins purified from the 18 *B. thuringiensis* isolates will be examined on different insect groups. After that, preliminary studies on culture conditions of *B. thuringiensis* strains with higher insecticidal activity will be carried out for large scale production of crystal protein. Because, crystalline genes are mostly carried on the plasmids, plasmid profiles will also be prepared and the *cry* genes, they contain, will be cloned.

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