ISOLATION AND CHARACTERIZATION OF *Bacillus thuringiensis* FROM OLIVE TREE-RELATED HABITATS

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by
Çelenk ÇINAR

May 2005
İZMİR

We approve the thesis of **Çelenk ÇINAR**

Date of Signature

..... **04 May 2005**

Assoc. Prof. Dr. Hatice GÜNEŞ
Supervisor
Department of Biology
İzmir Institute of Technology

..... **04 May 2005**

Prof. Dr. Şebnem HARSA
Co-Supervisor
Department of Food Engineering
İzmir Institute of Technology

..... **04 May 2005**

Assoc. Prof. Dr. Ali Fazıl YENİDÜNYA
Co-Supervisor
Department of Molecular Biology and Genetics
Cumhuriyet University

..... **04 May 2005**

Assist. Prof. Dr. Figen KOREL
Department of Food Engineering
İzmir Institute of Technology

..... **04 May 2005**

Assist. Prof. Dr. Ahmet KOÇ
Department of Biology
İzmir Institute of Technology

..... **04 May 2005**

Prof. Dr. Şebnem HARSA
Head of Biotechnology and Bioengineering
Program
İzmir Institute of Technology

.....

Assoc. Prof. Dr. Semahat ÖZDEMİR
Head of the Graduate School

ACKNOWLEDGEMENTS

I would like to give thanks to my advisor Assoc. Prof. Dr. Hatice GÜNEŞ for her academic support, valuable help and comments.

I also would like to thank my co-advisor Prof. Dr. Şebnem HARSA for her support.

I also feel deeply indebted to Assoc. Prof. Dr. Ali Fazıl YENİDÜNYA for his help, guidance, and constructive criticism.

I wish to express my sincere gratitude to my good friend and colleague Zehra Seda KARHAN for her invaluable technical input, emotional support, endless help through difficult part of my life and Ali Sinan KARHAN for his technical help, intelligence, illustrations, and professional support.

I am grateful to Mert SUDAĞIDAN for his constant support, encouragement, friendship, professional approach, and creativity.

I also thank to Dr. Ali AYDIN at Department of Food Hygiene and Technology of İstanbul University for his technical help.

Thanks are also extended to all other colleagues at Biology Department of İzmir Institute of Technology for their practical and academic support especially to Elif YAVUZ, Burcu OKUKLU, Özgür APAYDIN, Zeynep YEĞİN, and Çisem BULUT.

Special thanks go to my entire family for enduring all the difficulties during my life, especially İmge and Ekim for their love.

ABSTRACT

Bacillus thuringiensis (commonly referred to as *Bt*) is a Gram-positive, spore-forming soil bacterium that produces insecticidal crystal proteins during sporulation. These crystals are referred as *Bt* toxins or δ-endotoxins. The most important characteristics of the toxins are their insecticidal activity against many insects. Since their insecticidal potential has been discovered, it has been produced commercially and used as microbial pesticides all over the world. Therefore, the aim of this study was to isolate *Bt* from olive tree-related habitats, and to determine the phenotypic and genotypic characteristics of the isolates. To accomplish this purpose, 240 samples were collected in the Aegean Region. The phase-contrast microscopy results showed the presence of crystals in 54 environmental samples, corresponding to 100 *Bt* isolates. The crystal morphologies were spherical, bipyramidal, cuboidal, irregular pointed, and irregular shaped. The greatest proportion of samples yielding this organism was from the soil. The remaining were from olive leaf residue, green olive leaves, animal faeces, dust samples, and olive pomace. The isolates were characterized on the basis of biochemical characters, *cry* and *cyt* gene content, plasmid profiling, 16S-ITS rDNA RFLP.

Biochemical tests included protease (caseinase and gelatinase), lecithinase, amylase, nuclease, urease, esculinase, arginine dihydrolyse activity; fermentation of sucrose, salicin, mannose, cellobiose, and maltose, production of acetyl-methyl-carbinol, methyl red reaction. Polymerase chain reaction (PCR) has been applied for the identification of *cry1*, *cry2*, *cry4*, *cry9*, *cry11*, *cry13*, *cyt1*, and *cyt2* genes. 68% of the isolates amplified *cry1* gene; 57% amplified *cry4*; 20% amplified *cry11*; 26% amplified *cry9*; 20% amplified *cry2* genes, and none of the isolates harbored *cry13* gene. *Cyt1* gene was found in 40% of the isolates while *cyt2* gene was present in 80% of the isolates. The most abundant genotype of *cry* genes was *cry1* and *cry4*. Most of the isolates (58%) possessed more than one *cry* gene. In addition, different combinations of *cry* and *cyt* genes were obtained. Plasmid profiling showed the presence of plasmids in all isolates and the number of plasmids was usually more than one. Also, the discrimation effect of 16S-ITS rDNA RFLP was tested to differentiate certain isolates and reference strains, which showed similar biochemical characteristics.

ÖZET

Bacillus thuringiensis (*Bt*) Gram-pozitif, sporlu bir toprak bakterisi olup, sporlanma döneminde insektisidal özellik gösteren kristal protein üretir. Bu kristal proteinlere *Bt*-toksin veya δ-endotoksin adı verilir. Toksinlerin en önemliği özelliği birçok böcek türüne karşı insektisidal etki göstermesidir. İnsektisidal potansiyeli keşfedildiğinden beri, dünyanın pekçok yerinde *Bt* ticari olarak üretilmekte ve mikrobiyal pestisit olarak kullanılmaktadır. Bu çalışmanın amacı, zeytinle ilgili alanlardan *Bt* izole etmek, bu izolatların fenotipik ve genotipik özelliklerini belirlemektir. Bu amaçla, Ege Bölgesinden 240 adet örnek toplanmıştır. Faz-kontrast mikroskobu ile 54 örnekten izole edilen 100 adet *Bt* izolatının kristal protein ürettiği belirlenmiştir. Kristal proteinlerin morfolojisi, küresel, bipiramidal, kübik, düzensiz noktasal, ve düzensiz şekilli olarak farklılık göstermektedir. En fazla izolat, toprak örneklerinden elde edilmiştir. Diğer izolatlar, yaprak kalıntısı, taze zeytin yaprağı, fekal örnekler, toz, ve zeytin pirinasından izole edilmiştir. İzolatlar, biyokimyasal özelliklerine, *cry* ve *cyt* gen içeriğine, plazmid ve 16S-ITS rDNA RFLP profillerine göre karakterize edilmiştir.

Biyokimyasal testler ile proteaz (kazeinaz ve jelatinaz), lesitinaz, amilaz, nükleaz, üreaz, eskülinaz, arginin hidrolizi aktivitelerine, sukroz, salisin, mannoz, sellobioz ve maltozu fermente etme yeteneklerine ayrıca asetil-metil-karbinol üretme yeteneğine bakılmış ve metil kırmızısı testi uygulanmıştır. Polimeraz zincir reaksiyonu (PCR) *cry1*, *cry2*, *cry4*, *cry9*, *cry11*, *cry13 cyt1*, ve *cyt2* genlerini belirlemek için uygulanmıştır. İzolatların %68'i *cry1*; %57'si *cry4*; 20%'si *cry11*; 26%'sı *cry9*; 20%'si *cry2* genlerini amplifiye etmiştir; izolatlar *cry13* genini içermemektedir. *Cyt1* geni izolatların %40'ında; *cyt2* geni izolatların 80%'inde bulunmaktadır. İzolatlarda en fazla *cry1* ve *cry4* genotipine rastlanmıştır. İzolatların büyük bir çoğunluğu (58%) birden fazla *cry* geni içermektedir. Plazmid profilleri izolatların tamamının plazmid içerdiğini, çoğu izolatın ise birden fazla plazmid taşıdığını göstermiştir. Benzer biyokimyasal özellik gösteren bazı izolat ve referansları sınıflandırmak için 16S-ITS RFLP yönteminin ayrım gücü test edilmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

LIST OF ABBREVIATIONS

AMC : Acetyl-methyl-carbinol

bp : Base pair

Bt : *Bacillus thuringiensis*

cry : Crystal

cyt : Cytolytic

DNA : Deoxyribonucleic Acid

dNTP : Deoxynucleotide triphosphate

EDTA : Ethylenediamine tetra acetic acid

FAO : Food and Agricultural Organization

h : Hour

IOOC : International Olive Oil Council

ITS : Internally Transcribed Spacer

kb : Kilo base

Mb : Mega base

min : Minutes

µl : Microliter

µM : Micromolar

mM : Milimolar

MR : Methyl Red

PCR : Polymerase Chain Reaction

subsp. : Subspecies

sp. : Species

s : Seconds

TAE : Tris Acetate EDTA

TBE : Tris Borate EDTA

TE : Tris EDTA

U : Unit

UV : UltraViolet

VP : Voges-Proskauer

CHAPTER 1

INTRODUCTION

A pesticide is defined as the compound used for a wide variety of purposes to control a range of insects (Renwick 2002). Pesticides that include insecticides, herbicides, and fungicides are employed in modern agriculture to control pests and to increase crop yield. In both developed and developing countries, the use of chemical pesticides has increased dramatically during the last few decades. Since 1950s, organochlorine compounds, organophosphorus compounds, arsenic and mercury compounds, phenoxy acid herbicides, atrazine, pyrethroids, and dithiocarbamates have been the most popular pesticides (Dich *et al*. 1996).

Control of pests with chemicals results in several problems. The residues of these synthetic insecticides cause toxic effects on wild life (e.g., birds, beneficial insects like honeybees). The overdose usage contributes the development of resistant pest variants that can be no longer killed by chemicals. These insecticides also induce harmful effects on nontarget insects such as predators, parasites, etc. (Barkay *et al*. 1989). They can also be harmful to humans and domestic animals. Other environmental concern is the contamination of ground water (Lacey *et al*. 2001).

The geographic and climatic conditions make Turkey suitable for cultivating a wide range of agricultural crops. Among the cultivated crops, olive production has an economic value because Turkey is the fifth country in olive production in the world and exports 54% of it to many European countries (IOOC 2004). To protect the olives against pests, mostly organophosphates are used. In Turkey alone, 13,169 metric ton of insecticides were consumed against olive pests and 31% of these were organophosphates in 2001 (WEB_1, 2004).

Microbial control agents can be effective and used as alternatives to chemical insecticides. *Bt* is the most widely used microbial control agent. The broad spectrum of susceptible hosts, production on artificial media and ease of application by conventional equipment have caused the widespread use of this bacterium against several pests in agriculture, forest, and aquatic habitats (Lacey and Siegel 2000). The utility of this bacterium has been enhanced by the discovery of new varieties which are active against Lepidoptera, Coleoptera, and Diptera (Lacey *et al*. 2001). *Bt*-based biological pesticides

inreasingly account for 80-90% of all biological pest control agents worldwide, and 2% of all insecticides in use (Schnepf *et al*. 1998, Glare and O'Callaghan 2000).

1.1. Microbial Insecticides

Microbial insecticides offer an alternative to chemical insecticides with increased specificity and safety therefore they are used in integrated pest management programmes (Anagnou-Veroniki *et al*. 1996, Peňa and Schaffer 1997). These insecticides contain microbials or biochemicals produced by microorganisms.

The advantages of using microbial control agents are their efficiency, safety for humans and other nontarget organisms, reduction of pesticide residues in food, preservation of other natural enemies, and increased biodiversity in managed ecosystem (Lacey *et al*. 2001). Also, microbial agents are highly specific against target pests so they facilitate the survival of beneficial insects in treated crops (Meadows 1992).

Microbial insecticides are being developed as biological control agents during the last three decades. The widely known and used bacteria as insect pathogens belong to *Bacillus* genus and these are *Bt*, *B. lentimorbus*, and *B. sphaericus* (Sutherland and Khoo 1987). The most common biopesticides applied in many agroecosystems are the commercial formulations of *Bt* (Entwistle *et al*. 1993).

1.2. The Genus *Bacillus*

The genus *Bacillus* is composed of many saprophytic bacteria capable of producing an endospore (Slepecky and Leadbetter 1994). The bacteria belong to this genus are rod-shaped, usually Gram-positive, catalase-positive, and aerobic or facultatively anaerobic (Thiery and Frachon 1997). Most Gram-positive endospore-forming bacteria are soil microorganisms (Slepecky and Leadbetter 1994).

Bacillus has been divided into three morphological groups based on spore shape and swelling of the sporangium (Gordon *et al*. 1973). Group I is characterized by the presence of ellipsoidal spores that do not swell the mother cell (Priest 1993). This group comprises a large number of species living in soil such as *Bt*, *B. sphaericus*, *B. subtilis*, *B. anthracis*, and *B. cereus*. Some of these species are very closely related and form

different groups within the group I. One of these subgroups includes the *B. cereus* group.

1.2.1. The *Bacillus cereus* Group

This group includes *B. cereus*, *B. mycoides*, *Bt*, *B. anthracis*, *B. pseudomycoides*, and *B. weihenstephanensis* (Chen and Tsen 2002, Helgason *et al*. 2000). Systematists consider the former three species as subspecies of *B. cereus* because they are closely related (Leonard *et al*. 1997).

The genetic and phenotypic characteristics of *Bt* are very similar to *B. cereus* (Toumanoff and Vago 1951, Priest 2000). The only difference between these two species is the formation of large proteinaceous parasporal inclusions observed in *Bt.* These inclusion bodies, crystals have unique toxic activities against certain insects and some other invertebrates (Charles *et al*. 2000), against human cancer cells (Mizuki *et al*. 1999, 2000), and human pathogenic protozoa (Kondo *et al*. 2002).

1.3. *Bt*

Bt is an aerobic, Gram-positive, rod-shaped, spore-forming bacterium. This bacterium has filamentous appendages (or pili) on the spores (Des Rosier and Lara 1981, Smirnova *et al*. 1991, Zelansky *et al*. 1994). Colonies have a dull or frosted glass appearance and often andulate margin from which extensive outgrowths do not develop (Sneath 1986).

Under aerobic conditions, *Bt* grows in a simple culture medium such as nutrient broth. After nutrients are depleted, it produces a spore along with one or several parasporal crystals (Figure 1). There are seven stages during the sporulation. Parasporal protein synthesis starts at about stage II or III of sporulation, and the crystal reaches its maximum size (approximately spore size) by stage V. The crystals are made of proteins varying in size. These crystal proteins are called as δ-endotoxins or insecticidal crystal proteins. When the spore matures, cells lyse. Then, free spores and crystals are released into the environment (Aranson *et al*. 1986, Asano *et al*. 2003).

Figure 1.1. Phase-contrast photograph of the isolate 16Q showing crystal protein and spore

1.3.1. *Bt* δ-endotoxins

Two types of δ-endotoxin are produced by *Bt* strains. They are named Cry and Cyt proteins. Each insecticidal crystal protein is the product of a single gene. The genes synthesize these endotoxins are often located on large, transmissible plasmids. Cry and Cyt proteins differ structurally. The most important feature of these proteins is their pathogenicity to insects and each crystal protein has its distinct host range.

The number and type of δ-endotoxins produced determine the bioactivity of a *Bt* strain (Crickmore *et al*. 1995, Kumar *et al*. 1996, Schnepf *et al*. 1998, Höfte and Whiteley 1989). Based on the amino acid homology, over 300 *cry* genes have been classified into 47 groups and 22 *cyt* genes have been divided into two classes (WEB_2, 2005).

1.3.1.1. The Cry Proteins

Cry proteins are the predominant type. The crystal proteins are encoded by *cry* genes. The accumulation of Cry protein in a mother cell can make up 20-30% of the dry weight of the sporulated cells (Agaisse and Lereclus 1995, Baum and Malvar 1995). Each crystal protein has its own insecticidal spectrum. Therefore, Cry proteins have been classified on the basis of their host specificity and their amino acid compositions. (Höfte and Whiteley 1989, Schnepf *et al*. 1998, Jensen *et al*. 2003). The crystal proteins have different forms such as bipyramidal (Cry1), cuboidal (Cry2), flat rectangular

(Cry3A), irregular (Cry3B), spherical (Cry4A and Cry4B), and rhomboidal (Cry11A) (Schnepf *et al*. 1998).

Cry1, Cry2, and Cry9 proteins show strongest toxicity to Lepidopterans (Crickmore 2000). Proteins belonging to the class Cry4 and Cry11 are specifically toxic to Dipterans. Cry3, Cry7, Cry8, Cry14, Cry18, Cry34, and Cry35 (Ellis *et al*. 2002, de Maagd *et al*. 2001) proteins show insecticidal activity against Coleopterans. Some Cry proteins on the other hand display toxicity to more than one insect order. For example, Cry1I is both active against Lepidopterans and Coleopterans (Tailor *et al*. 1992), whereas Cry1B shows toxicity against Lepidoptera, Coleoptera, and Diptera (Zhang *et al*. 2000).

1.3.1.2. The Cyt Proteins

Beside Cry proteins, some *Bt* strains also synthesize cytolytic proteins encoded by *cyt* genes. *Cyt* means a parasporal inclusion (crystal) protein from *Bt* that exhibits hemolytic activity, or any protein that has obvious sequence similarity to a known Cyt protein (Crickmore *et al*. 1998). This class of δ-endotoxins differs in amino acid composition and action mechanism from Cry toxins (Thomas and Ellar 1983, Höfte and Whiteley 1989, Butka *et al*. 1997). These toxins act synergistically with mosquitocidal Cry toxins (Poncet *et al*. 1994). Cyt toxins differ from the Cry toxins; the protoxin mass of Cyt toxins (30 kDa) is smaller than the Cry toxins (Thomas and Ellar 1983, Du *et al*. 1999). The Cyt toxins are only found in Dipteran specific strains, while the Cry toxins are present in many *Bt* strains with wide host range. One Cyt toxin is found in a given *Bt* strain, but two or more subclasses of Cry toxins can exist in a strain. Although both the activated forms of these toxins can lead to pores in lipid bilayers, only the Cyt toxins cause the cytolysis of various eukaryotic cells including erythrocytes (Gill *et al*. 1992, Knowles *et al*. 1989, Slatin *et al*. 1990).

Cyt toxins may be used to overcome insecticide resistance and to increase the activity of microbial insecticides (Guerchicoff *et al*. 2002). Cyt1 and Cyt2 are two cytolytic classes of Cyt toxins that have been identified on the basis of the amino acid identity and are divided into 22 subclasses (WEB_2, 2005). Among these subclasses, Cyt1Aa and Cyt2Aa display the highest mosquitocidal activity (Koni and Ellar 1994). Cyt1A may be used as a practical tool to manage resistance against *B. sphaericus*,

which is also a mosquitocidal bacterium. Also other Cyt proteins may increase the insecticidal activity of non-Cyt proteins to other insects (Wirth *et al*. 2000).

1.3.2. Classification of Insecticidal Crystal Proteins

In previous nomenclature, crystal proteins are classified as CryI (Lepidoptera-specific), CryII (Lepidoptera- and Diptera-specific), CryIII (Coleoptera-specific), CryIV (Diptera-specific), and CytA for cytolytic proteins (Diptera-active) based on their structure and host range (Höfte and Whiteley 1989). This nomenclature replaces with the one proposed by Crickmore (1998).

In this nomenclature, Cry proteins are named on the basis of the amino acid similarity to established holotype and Cry proteins showing similar amino acid sequences are grouped together. For example, Cry proteins with the same Arabic number share at least 45% amino acid sequence identity for example Cry4; the same Arabic number and upper case letter at least 75% sequence identity (Cry4B), the same arabic number and upper and lower case letter 95% sequence identity (Cry4Ba).

1.3.3. Toxin Structure

X-ray crystallography has been used to solve the three-dimensional structure of the activated forms of Cry3A, Cry1Aa and Cyt2A toxins (Schnepf *et al*. 1998). Cry toxins share a similar conformation and a common three-domain structure. The N-terminal domain I, is a bundle of seven α-helices. Six of these helices are surrounded by a central core helix, α-5. Domains II and III contain β-sheets in different conformations. Domain II is formed by three antiparallel β-sheets and has the most variable structure between the Cry toxins. The C-terminal domain III is a β-sandwich of two antiparallel β-sheets. The β-sandwich region provides the structural integrity of the toxin. Domain I is responsible for membrane insertion, structural stability, and pore formation. Domains II and III have functions in receptor recognition and specific binding. Furthermore, domain III is also responsible for modulating ion channel activity (Schnepf *et al*. 1998, Shimizu and Morikawa 1996, Li *et al*. 1991, de Maagd *et al*. 2001). The C-terminal region of the protoxins contains lysine and cysteine residues that are essential in the assembly and solubilization of the crystals (Choma *et al*. 1990). N-terminal region has

carbohydrate functionality that provides the binding of the Cry toxins to glycoproteins like receptors such as N-aminopeptidases or E-Cadherins in the insect midgut (Burton *et al*. 1999, Angst *et al*. 2001).

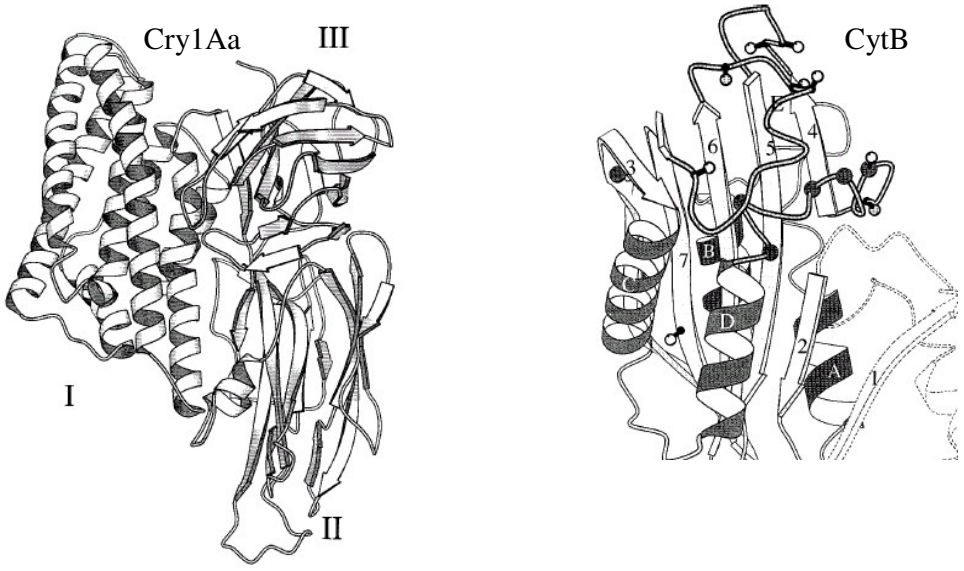

Figure 1.2. Structure of Cry1Aa and CytB δ-endotoxin (Source: Li *et al*. 2001)

Cyt2A contains a single domain in which two outer layers of α-helix wrap around a mixed β-sheet. Cyt1A is also thought to have a similar structure (Schnepf *et al*. 1998). Figure 2 represents the structure of Cry1A and CytB δ-endotoxins.

1.3.4. Action Mechanism of δ-endotoxins

Crystals are formed as protoxins by *Bt*. To become active; a suspectible insect must ingest them. After being ingested, the crystals are solubilized in the alkaline environment in the insect midgut (pH>10). After solubilization, midgut proteases convert the protoxins into active toxins. The active toxin binds to specific receptors on the membranes of epithelial midgut cells; this interaction provides the insertion of the toxin into the lipid bilayer and formation of pores (0.5 to 1 nm). As a result, pore formation leads to gut paralysis. Finally, insect larvae stop feeding and die from lethal septicemia (Aranson *et al*. 1986, Knowles and Ellar 1987, Höfte and Whiteley 1989,

Lereclus *et al*. 1989, Adang 1991, Gill *et al*. 1992). Figure 3 illustrates the action mechanism of Cry proteins.

The mode of action of Cyt toxins has not been fully determined. It has been suggested that these toxins could also be involved in colloid-osmotic lysis like Cry toxins but the formation of lesions in the cell membrane may be different (Butko *et al*. 1996, Butko *et al*. 1997, Crickmore *et al*. 1995, Höfte and Whiteley, 1989). All Cyt toxins react directly with phospholipids without the need for a membrane protein receptor (Thomas and Ellar 1983).

Serine proteases such as chymotrypsin, thermolysin, elestase are important in both solubilization and activation of protoxins (Yang and Davies 1971, Spiro-Kern 1974, Borovsky 1986, Dai and Gill 1993). Besides these digestive proteases, a novel DNase from an insect has been found to act synergistically with the crystal protein and to convert it to the active DNA-free toxin in the larval gut (Clairmont *et al*. 1998, Milne and Kaplan 1993).

Spores are known to synergize the insecticidal activity of crystals when tested against insects. This may be related to the invasion of haemocele through the ulcerated midgut, and the subsequent development of septicemia (Li *et al*. 1987).

The efficiency and potency of Cry toxins to control insects could be increased by the addition of enzyme chitinase in *Bt* preparations. The chitinase acts on the peritrophic membrane which is composed of a network of chitin and proteins (Smirnoff 1973). This enzyme hydrolyses the β-1,4 linkages in chitin so it may distrupt the peritrophic membrane by creating holes and facilitates the contact between δ-endotoxins and membrane receptors in the midgut epithelium (Regev *et al*. 1996). Some factors such as pH, enzymes, peritrophic membrane, enzyme detoxification, and antimicrobial characteristics of gastric juice of insect gut make insects resistant to the toxin (Davidson 1992).

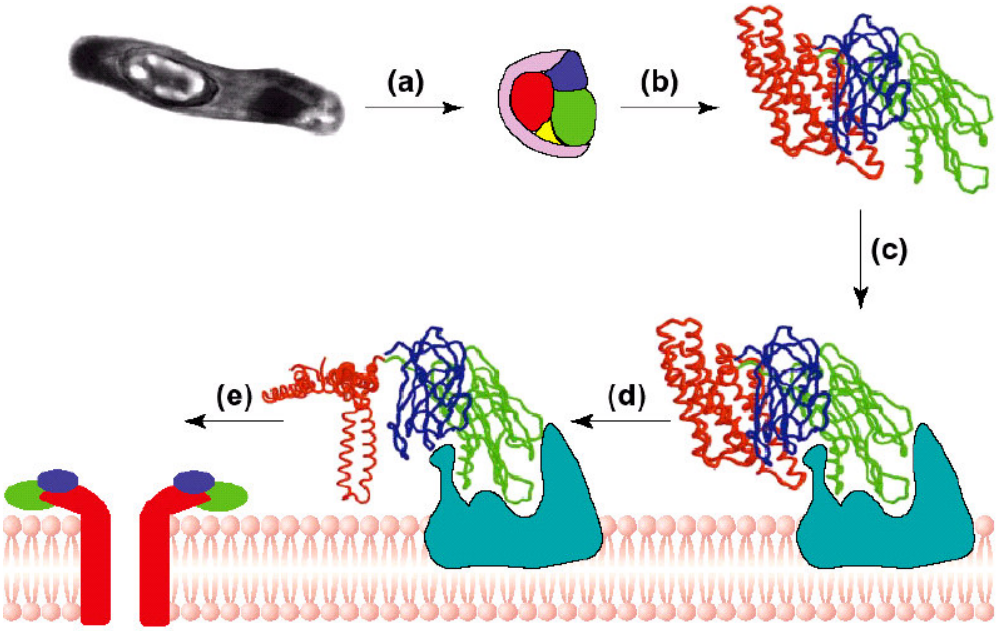

Figure 1.3. Action Mechanism of Cry δ-endotoxin (Source: de Maagd *et al.* 2001)

1.3.5. Natural Habitats of *Bt*

Natural habitat of *Bt* is not known exactly but it has often been detected in soil (Martin and Travers 1989, Ohba and Aizawa 1986), settled dust (De Lucca *et al*. 1982), insect cadavers (Heimpel 1967), phylloplanes of many plants (Mizuki *et al*. 1999, Ohba 1996, Smith and Couche 1991) and in fresh water (Ichimatsu *et al*. 1998). This shows that this organism is found among the predominant sporeformers in natural environments (Maeda *et al*. 2000). The Coleopteran and Lepidopteran-active *Bt* subspecies are mainly associated with soil and phylloplane, while the Dipteran active ones are mostly found in aquatic environments.

1.3.6. Insecticidal Spectrum of *Bt* δ-endotoxins

More than 3000 insect species included in 16 orders have been found to be susceptible to different crystal proteins (Lin and Xiong 2004). Insecticidal crystal proteins are toxic to insects within the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera, Mallophaga as well as non-insect organisms such as nematodes, mites, protozoa, and plathelmintes (Feitelson 1993).

The toxicity is high against the insects belonging to the first three orders. Lepidopteran and Coleopteran insects are leaf-feeders with chewing mouthparts, whereas Dipterans feed by filtering water. These two different feeding behaviours provide the possible intake of *Bt* spores /crystals (Borror *et al*. 1989).

1.3.7. Safety of *Bt* Biopesticides

Bt-based insecticides have been used commercially to control selected insect pests for approximately 40 years (Baum *et al.* 1999). During this period, these products have caused no adverse effects on human health or environment (McClintock *et al*. 1995, EPA 1998). In the 1990s, the development of natural and recombinant *Bt* products have broadened the insect host range in pest management programs. New formulations based on conventional or genetic engineering methods (encapsulation of the toxins and/or feeding stimulants to increase ingestion), screening of the interactions of *Bt* with insect herbivores and plant allelochemicals or natural enemies of the pests to improve the formulation of biological control strategies, and information and management of insect resistance increased the uses of *Bt* (Navon 2000). Short persistence and complete biodegradability are other benefits of *Bt* toxins (Bohorova *et al*. 1997, Copping 1998).

Over synthetic pesticides, the advantages of this organism include lack of polluting residues, high specificity to target insects, safety to non-target organisms such as mammalians, birds, amphibians and reptiles as well as its relatively low costs of development and registration (Flexner and Belnavis 1999).

In spite of these economical benefits, the use of *Bt*-based biopesticides is much less than synthetic chemicals (Flexner and Belnavis 1999).

1.3.8. Advantages of *Bt*-protected Plants

Recently, the gene(s) encoding the insecticidal proteins have been cloned (Schnepf and Whiteley 1981) and expressed in genetically modified plants to make them naturally resistant against harmful insects (Fischhoff *et al*. 1987, Vaeck *et al*. 1987, Perlak *et al*. 1990). Cotton, potato, sugarcane, tomato, peanut, and rice expressing

cry genes have been produced (Adang *et al*. 1993, Nayak *et al*. 1997, Singsit *et al*. 1997, Van der Salm *et al*. 1994).

These plants provide some benefits compared to chemicals; proteins are generally not toxic to humans and animals and they do not bioaccumulate in fatty tissue or stay in the environment like some halogenated chemical pesticides (Sjoblad *et al*. 1992). Also, Cry proteins show a high degree of specificity for the target and closely related insect species and must be ingested to be effective. The potential exposure of Cry proteins to human and nontargets is extremely low. These proteins are found within the plant tissue in microgram quantities and reproduced at low levels in the pollen (McClintock *et al*. 1995, EPA 1988, 1998).

1.3.9. Non-insecticidal δ-endotoxins

Another class of δ-endotoxins is mammalian cell-recognizing crystal proteins. In natural environments non-insecticidal *Bt* strains are more widely distributed than insecticidal ones (Ohba and Aizawa 1986, Ohba 1996, Mizuki *et al*. 1999a). This is related to the absence of trypsin recognition sites on the crystal proteins (Gill *et al.* 1992). Human cancer cell-killing activities are associated with parasporal inclusions which do not exhibit insecticidal and haemolytic activity (Mizuki *et al*. 1999b).

1.3.10. Other Metabolites

Some subspecies of *Bt* produce α-, β-, γ-endotoxins, a loose factor, and a bacillogenic factor into the growth medium besides δ-endotoxins (Steinhaus 1960). Entomopathogenic bacilli also produce various exoenzymes such as lecithinase, protease, chitinase, and collagenase that can also contribute to pathogenicity to insects (Smirnoff and Berlinquet 1966, Kreig and Lysenko 1979, Sela *et al*. 1998).

Some serotypes of *Bt* produce a thermostable exotoxin, called thuringiensin or β-exotoxin. Its expression occurs during the vegetative growth like other extracellular insecticidal toxins. This compound is an ATP analog, and this similarity makes it an inhibitor of DNA-dependent RNA polymerases. β-exotoxin has a broader spectrum of effectivity against many insects when compared to α-endotoxin (Farkas *et al*. 1976, Sebesta and Horská 1970, Sebesta and Sternbach 1970, Mohd-Salleh and Lewis 1982,

Cantwell *et al*. 1983, Krieg and Langenbruch 1981). On the other hand, the use of β-exotoxin is not registered in many parts of the world because it interferes with RNA biosynthesis resulting in a broad-spectrum toxicity in vertebrates and invertebrates and considered as an environmental hazard by the Environmental Protection Agency. Commercial formulations of *Bt* are produced from isolates that do not contain this exotoxin (McClintock *et al*. 1995).

α-toxin is a phospholipase C and primarily affects the phospholipids on the cell membrane (Heimpel 1954, Bonnefoi and Beguin 1959). γ-toxin is a heat-labile toxin and toxic to sawflies. The action mechanism of this toxin has not been determined (Heimpel 1967).

Some strains also produce insecticidal proteins, other than the Cry proteins, in the vegetative growth phase. They are termed as vegetative insecticidal proteins (VIP). Although Cry proteins are accumulated in the cell, VIP is secreted from the cell into the culture medium, which limits its field application. These proteins are active against many agronomically important pests especially to Lepidopterans that are less sensitive to δ-endotoxins (Estruch *et al*. 1996, Yu *et al*. 1997).

This organism was found to produce *B. cereus*-like extracellular enterotoxins suggesting the potential risk of diarrhea in humans. It is therefore important to develop insecticides from strains with low enterotoxin activity (Damgaard 1995).

1.3.11. Genetic Diversity of *Bt*

The genetic diversity of *Bt* arises from the presence of many different plasmids in each strain, conjugation transfer mechanism, and the transposon-like inverted repeats flanking the endotoxin genes, facilitating a high frequency of DNA rearrangements. Horizontal transfer of protoxin encoding-plasmids may lead to strains producing two different parasporal inclusions. In most species, the major protoxin gene is carried on a low copy number large plasmid (one plasmid per cell).

The number and sizes of plasmids vary. There is a very broad range in size, from 4 to 100 MDa. Protoxin genes are often found in plasmids which are >30 MDa. The *Bt* species have transposable elements, including insertion sequences and transposons. Insertion sequences (IS) are especially found in large plasmids and many of these sequences carry protoxin genes. Plasmids that do not include protoxin genes

also play a role in the regulation of protoxin synthesis. Plasmids also enhance and provide supplementary growth factors when nutrients are limitted. If protoxin gene is found on a transposable element, it can move into and out of the chromosome. Because of this movement, protoxin sequences may sometimes be present in the chromosome of some subpecies (Aronson *et al*. 1986).

The numbers of both large and small plasmids are between 2 and 11 in one cell (Gonzalez *et al*. 1981, Lereclus *et al*. 1993).

If plasmids are lost, it will be impossible to distinguish *Bt* from *B. cereus* (Crickmore *et al*. 1995, Höfte and Whiteley 1989, Thorne 1993).

1.3.12. Isolation of *Bt* from Environmental Samples

Screening samples from different environments may be useful to obtain *Bt* strains with broader host ranges and new toxic properties (Höfte and Whiteley 1989). The abundance of the bacterium depends mainly on the type of environmental sample. Soil has been shown as the main source of *Bt* novel isolates (De Lucca *et al*. 1981) as it has been recovered from 70% of soil samples from all over the world (Martin and Travers 1989).

There are some suggestions about the high recovery of *Bt* from soil. First, while collecting sample, the surface is always rejected and the material is taken from at least 5 cm under the surface where UV light damage is not possible and temperature is more stable (Trindade *et al*. 1996). Second, the soil can act as a reservoir of spores (Akiba 1986, Martin and Travers 1989, Meadows 1993, Ohba and Aratake 1994, Lereclus 1996).

The efficiency of isolation also depends on the method used. Enrichment techniques are not useful because it has a lower detection limit which is about 10^3 bacteria per gram of soil. Immunofluorescence-based methods also have a lower detection limit of 10^5 bacteria per gram of soil in spite of their direct enumeration. The most efficient isolation method so far has been the sodium acetate selection, combined with heat tratment.

Bt spores do not germinate in the medium buffered with high concentration (0.25 M) of sodium acetate (Travers 1982). This method eliminates most sporeforming and all nonspore-forming bacteria. Germination of *Bt* spores is selectively inhibited by sodium

acetate, which allows the germination of other spore-formers. At that time, *Bt* remains in the quiescent state. Then, heat treatment is applied to kill all undesired bacteria, which have entered the vegetative stage (Martin and Travers 1987).

Bt can live as a spore and/or vegetative cell on leaves (Damgaard PhD thesis). Appendages on the spore may facilitate the attachment of spores to the surface of plant leaves, leading to the settlement of spores and the colony formation on phylloplane (Mizuki *et al*. 1998). Isolation of *Bt* from leaf samples is simpler than soil because the number of acrystalliferous sporeforming bacteria are significantly lower in leaves. A moderate heat treatment at 65 ^{o}C also eliminates contamination of fungi and non-sporeforming bacteria and promotes the germination of *Bt* spores and other sporeformers (Smith and Couche 1990).

Serial dilution method on the other hand can be more effective than this method for the isolation of *Bt* from phylloplanes because most of the viable cells on leaves are found in the vegetative stage and the use of heat treatment can kill these cells (Maduell *et al*. 2002).

1.3.13. Common Techniques for the Characterization

1.3.13.1. Phenotypic Characterization

Phenotypic characterization methods include description of crystal morphology, biochemical reactions, H-serotyping, bioassay, SDS-PAGE, and cellular fatty acid analysis. The characterization of *Bt* is mainly based on the presence of Cry proteins that are detected by phase-contrast microscopy. This method is very useful to screen novel environmental isolates (Bravo *et al*. 1998) and it may be the best way to observe parasporal bodies (Ammons and Rampersad 2002). The toxicity of strains is also more related to the morphology of crystal proteins than to the serotypes (Mikkola *et al*. 1982, Higuchi *et al*. 1998). Generally, bipyramidal inclusions are active against Lepidoptera, ovoidal inclusions to Diptera, and rhomboidal inclusions to Coleoptera, cuboidal inclusions to both Lepidoptera and Diptera (Höfte and Whiteley 1989).

Biochemical characterization of the strains is important because each serotype has specific physiological characters and one serotype can be subdivided into biotypes

based on the different enzymatic reactions. These biotypes also show different toxicity (de Barjac 1981).

H-serotyping requires very motile bacterial cultures for the preparation of flagellar suspensions. H-antigen serum is produced by the injection of the flagellar suspension of reference strains chosen from each serotype into rabbits. The flagellar suspension of bacteria is titrated against the serum of defined serotype (Thiery and Frachon 1997). This method is independent from the classification of strains based on the parasporal protein production or their biological activities (Shisa *et al*. 2001). Thus, the prediction of insecticidal activity of the strains by serological identification is not possible because there is not any direct correlation between the insecticidal activity and serovar. Therefore, serological characterization must be combined with the toxicity bioassays to predict the host range of *Bt* strains (Porcar *et al*. 2000). The catalogue of the Pasteur Institute (2001) listed 71 serotypes comprising 84 subspecies based on flagellar H-serovars.

Bioactivity tests must be conducted against insects for each strain to determine toxicity because the expression of a *cry* gene can not be detected by PCR. Also these tests can be used to improve the commercial formulations and understand the mechanism action of toxins (Aronson *et al*. 1986). In large-scale production, the final product is bioassayed against an accepted international standard using a specific test insect (Dulmage *et al*. 1981).

Protein composition of the strains is determined by SDS-PAGE. Cellular fatty acid analysis by gas chromatography is used to distinguish two isolates belonging to the same serotype (Thiery and Frachon 1997).

1.3.13.2. Genotypic Characterization

DNA-based methods used for characterization are specific primed PCR, random amplified polymorphic DNA (RAPD), DNA: DNA colony hybridization (Hansen *et al*. 1998), rRNA-based probe (Akhurst *et al*. 1997). Strains with similar plasmid profiles can belong to the same subspecies. Therefore, strains can be classified by comparing the plasmid and crystal patterns (Carlton and González 1985, González *et al*. 1982).

PCR is a molecular method widely used to characterize the insecticidal bacterium *Bt.* It provides the determination of the presence of a target gene by the

amplification of specific DNA fragments. The identification of toxin genes by PCR can be used to predict the insecticidal activity of a given strain. This method has largely substituted bioassays used in preliminary classification of *Bt* collections because of its rapidity and reliability (Porcar and Juárez-Pérez 2002). This method also allows the identification of *cyt* gene content of a strain (Bravo *et al*. 1998). In the premilinary screening, primer pairs designed from highly conserved regions are used to recognize entire *cry* gene subfamilies. Recently, specific primers selected from a variable region are used for amplification (Ben-Dov *et al*. 1997). The prediction results of PCR must be combined with bioassays in order to decide the potential of isolates as biopesticides (Masson *et al*. 1998). Strains yielding unusual PCR profiles can be selected for further analysis to search whether they contain novel *cry* genes (Carozzi *et al*. 1991).

Hybridization is an alternative way to PCR-based methods (Porcar and Juárez-Pérez 2002). Because of the homology between *cry* gene sequences, hybridization techniques are developed to detect and characterize putative new *cry* genes by using specific probes (Crickmore *et al*. 1998).

DNA sequence homology is a procedure, which is used for the confirmation of a novel *cry* gene. Novel *cry* gene must have a significant sequence similarity to one or more toxins within the established nomenclature or Cry protein must exhibit pesticide activity or experimentally verifiable toxic effect to a target organism with a new name (Crickmore *et al*. 1998).

1.4. 16S-ITS rDNA RFLP

The ribosomal operon is useful to trace genetic relationships and to identify strains rapidly (Amann *et al*. 1997). Te Giffel *et al*. (1997) reported differences in 16S rDNA sequences of a limited number of *Bt* and *B. cereus*. The internal transcribed spacer (ITS) located between 16S and 23S ribosomal DNA is frequently used as a molecular marker to identify microbial species and analyze the phylogenetic relationship between strains. ITS can be present in multiple copies in most bacterial genomes (Daffonchio *et al*. 1998, Gürtler and Stanisich 1996, Jensen *et al*. 1993, Gürtler 1999).

Yavuz *et al*. (2004) presented the identification of extracellular enzyme producing thermophilic bacillus by 16S-ITS rDNA RFLP and showed that 16S-ITS

rDNA RFLP was a rapid method for the molecular characterization of environmental isolates.

1.5. Olive Production in Turkey

Olive (*Olea europaea* L.) is an evergreen tree and has been traditionally grown for olive oil and table fruit consumption (Lopez and Villatta 1998). There are about 805 million olive trees in the world, occupying approximately 24 million acres. Almost 98% of these trees are cultivated in the Mediterrenean region that accounts for 97% of the total olive production and 91% of world consumption (Alcaide *et al.* 1996)

Western and southern Turkey has a Mediterranean climate and a considerable olive oil production. Olive oil trees cover 4 % of the all-agricultural areas in Turkey. The olive oil production in the regions of Turkey is 75-80% in the Aegean, 10% in the Mediterranean area and around 10% in the areas of Marmara and South East Anatolia (Olgun *et al.* 2001). According to International Olive Oil Council (2004), Turkey produced 102.000 tonnes table olives and 75.000 tonnes olive oil within the 2003/2004 period.

1.6. Olive Pests and Their Harmful Effects

Mild winters and long dry summers provide suitable environmental conditions for many insect populations in the Mediterranean climate. The olive fruit fly *Bactrocera* (*Dacus*) *oleae* (Gmelin) (Diptera: Tephritidae) is the major insect pest of olives in the region (Cabras *et al.* 1997). The larvae, which feed on olive fruits, cause severe and economically important damages. Persistent infestation by this fly also affects the quality of the produced oil and the crop's export market potential (Arambourg 1986, Fimiani 1989). The female olive fly lays a single egg under the skin of non-mature olives. Larvae emerge after two or three days and feed by creating tunnels (approximately 15 mm long) and destroying a fourth or a fifth of the olive's interior. Fruits fall prematurely and their oil has low quality and higher acidity (Dominguez 1993). The effected olive fruit is also totally unsuitable for eating. If no control measures are taken, it is estimated that > 60% of the harvest is affected (Navrodizis *et al.* 2000).

1.6.1. Chemical Control of Olive Fruit Fly

The control of the olive fruit fly using chemical pesticides, although effective, has proven to be unsafe and ecologically disruptive. Generally, less than 1% of the pesticides used reach the target pest. The rest is blown in the environment (Cirio *et al.* 1997).

This pest has been controlled by spraying with chemical pesticides that also kills the natural enemies of the fly and can thus lead to the rapid development of insecticide resistance. Insecticidal treatments, applied from an aircraft over vast areas, have both ecological and toxicologic consequences such as environmental contamination, destruction of non-target organisms, severe outbreaks of other secondary pests, and the presence of insecticide residues in the olive oil (Delrio 1992).

Insecticides used to control this pest mainly belong to the organophosphorus pesticides class (Cabras *et al.* 1997). 41.38% of the insecticides registered in Turkey were organophosphates (Zeren *et al.* 2002). These compounds show higher acute toxicities than chlorinated pesticides and have the advantage of rapid degradation in the environment (Rastrelli *et al.* 2002). More extensively used ones are dimethoate, parathion-methyl, fenthion, parathion-ethyl, and methidathion (Dugo *et al*. 2005).

Pesticides cause toxic effects to humans in many ways. Harmful health effects arise from formulating and applying the pesticides, accidental or incidental exposure, and residues in food products. Pesticide residues on agricultural products can be transferred directly to humans, with serious health effects (Thapinta and Hudak 2000). These insecticides for example inactivate cholinesterase enzyme, which plays an important role in the nervous system, and may cause acute and chronic poisoning (Bardin *et al.* 1994, Lotti 1995). Rastrelli *et al.* (2002) observed that fenthion was found in 27% of olive oil samples, and dimethoate was present in 44% of the samples analysed. Their finding indicated that the most important residue in olive oil was dimethoate (Heim 1984).

1.6.2. Biological Control of Olive Fruit Fly

Alternative ways such as release of *Opius concolor* which is the most important parasitoid of the olive fruit fly, sterile flies and mass trapping were developed to avoid or to substitute for chemical control however these methods showed only partial success (Kapatos 1991).

Although *Bt*-based biopesticides have been used for the control of many other insect species, there is no systematic study on the effectiveness of this bacterium for the control of the olive fruit fly (Schnepf *et al*. 1990, Lambert and Peferoen 1992, Payne *et al*. 1995, Robacker *et al*. 1996, Nault and Kennedy 1999). The use of *Bt* against this insect may solve many of the problems.

Navrodizis *et al*. (2000) demonstrated that *Bt* spores and crystals affected several stages of the life cycle of this pest in both field and laboratory experiments.

1.7. Thesis Objectives

The purpose of this study was to isolate *Bt* strains from olive tree-related habitats and to determine their distribution in these habitats. The Cry proteins have site-specific properties that only affect the insects found in the locations where isolation has been achieved. Because of this reason, commercially produced *Bt*-biopesticides may not show the expected lethal effect on insects.

Spain, Italy, Greece that are known to be the leading olive-producers countries have conducted some studies to isolate and characterize *Bt* from olive tree-related habitats to find the active strains which can be used against olive fruit fly. Although Turkey is found in the top order based on the olive production, any studies have not been done about the isolation of *Bt* from these habitats.

In the lights of this information, the main aim was to characterize these isolates both phenotypically and genotypically. The characterization methods included determination of crystal protein composition by phase contrast microscopy, biochemical characteristics, plasmid contents, insecticidal gene content (*cry* and *cyt* genes) by PCR.

Finally, in order to differentiate the isolates and to find correlation with the biotypes, 16S-ITS rDNA RFLP analysis was carried out.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used in this study were shown in Appendix A.

2.1.2. Media

Media were listed in Appendix B.

2.1.3. Reagents and Solutions

Reagents and solutions were presented in Appendix C.

2.2. Methods

2.2.1. Sample Collection

Two hundred-forty samples were collected from olive groves, olive-oil factories, cherry groves, high land areas in Aegean region including; 124 samples of soil, 19 samples of green olive leaves, 31 samples of olive leaf residue, 5 samples of animal faeces, 38 samples of olive pomace, 22 dust samples, and one sample of insect cadaver. The sources of these samples had not been sprayed with any of the *Bt*-biopesticide before. Soil, dust, olive leaf residue, green olive leaves, insect cadaver, faeces were collected from olive groves, whereas olive pomace, olive leaf residue and dust were collected from olive-oil factories (Table 2.1). Also, soil samples were taken from cherry groves and three altitudinal levels located between 600 and 700 m above sea level.

Samples were collected between the end of summer and in autumn when the olive pests were the most harmful and active on olive trees. From each tree a minimum of 10 leaves were collected that were not exposed to direct sunlight. During leaf sampling, soil contamination was avoided. Soil samples were taken from at least 10 cm below the ground level by scraping off the surface with a sterile spatula.

The samples were placed in sterile plastic bags aseptically, sealed and stored at +4 ^{o}C until they were analyzed.

Table 2.1. Sampling Sites, Sources and Numbers of Collected Samples

Sampling Site	Origin of Sample	No. of Samples Collected
Eski Foça Ilıpınar village	Soil	17
	Green olive leaves	10
	Olive leaf residue	8
	Faeces	1
Dikili Bademli village	Soil	16
	Green olive leaves	7
	Faeces	1
	Olive leaf residue	9
Aliağa	Soil	9
	Olive leaf residue	1
Çeşme	Soil	4
Kemalpaşa Kımız Farm	Soil	19
	Faeces	3
	Olive leaf residue	1
Kuşadası	Soil	16
Güzelbahçe Küçükkaya village	Soil	4
Konak Tırazlı village	Soil	2
Balçova Teleferik	Green olive leaves	1
Urla	Soil	3
	Olive leaf residue	1
Turgutlu Irlamaz village Dalbahçe village	Soil	24
	Green olive leaves	1
Kemalpaşa	Soil from cherry grove	5
Bergama Sulu and Tunçyağ Olive oil factory	Dust	17
	Olive pomace	31
Turgutlu Yıldırım and Canöz Olive oil factory	Dust	5
	Olive pomace	7
	Olive leaf residue	11
		Total: 240

2.2.2. *Bt* Reference Strains

Reference strains used in this study were obtained from Bacillus Genetic Stock Center (Ohio, USA).

B. thuringiensis subsp. *kurstaki* BGSC 4D1

B. thuringiensis subsp. *aizawai* BGSC 4J3

B. thuringiensis biovar. *tenebrionis* BGSC 4AA1

B. thuringiensis subsp. *israelensis* BGSC 4Q2

B. thuringiensis subsp. *galleriae* BGSC 4G1-4G6

B. thuringiensis subsp. *alesti* BGSC 4C1-4C3

B. thuringiensis subsp. *finitimus* BGSC 4B1-4B2

B. thuringiensis subsp. *thompsoni* BGSC 4Q1

B. thuringiensis subsp. *morrisoni* BGSC 4K1-4K3

B. thuringiensis subsp. *kumamtoensis* BGSC 4W1

2.2.3. *Bt* Isolation

Bt isolation was done by using two different methods based on the sample type. The shaken-flask technique (Smith and Couche 1990) was used for the leaf samples to increase the surface area. In this technique, 5 to 6 mm sections were cut from three different leaves per sample aseptically. The leaf sections were placed in an Erlenmeyer flask (100 ml) containing 10 ml of Luria Bertani (LB) broth and shaken at 250 rpm for 4 h at 30 ^{o}C. After the incubation, 200 µl aliquots of the resulting suspensions were placed in glass test tubes (10 by 70 mm) and incubated in an 80 ^{o}C water bath for 10 min. The heat-treated suspension was cooled on ice, and 100 µl aliquots were spread on nutrient agar plates. After a 72 h incubation at 30 ^{o}C, pale white colonies with a matt texture (*B. cereus* morphology) were plated again for single colony purification on LB agar plates until pure culture was obtained.

The technique described by Travers *et al* (1987) based on acetate selection was used to screen the remaining of the samples for the presence of *Bt* with the following modifications:

Approximately 0.25 gr of sample was suspended in 10 ml of 2M LB broth-sodium acetate medium (50% LB broth 2x: 2% tryptone, 1% yeast extract, 2% sodium

chloride; 12,5% sodium acetate 2 M, pH 6.8), vortexed vigorously and incubated at 180 rpm and 37 ^{o}C in a rotary shaker overnight. The sample suspension was pasteurized for 10 min at 80 ^{o}C in a water bath in order to kill vegetative cells and non-spore forming bacteria.

After cooling at room temperature, the suspension was streaked on nutrient agar and incubated for 48 hr at 37 ^{o}C. Colonies showing *B. cereus* type morphology were selected and streaked again on nutrient agar to obtain pure cultures.

2.2.4. Phenotypic Characterization

2.2.4.1 Examination of Crystal and Spore Morphology

The isolates were characterized on the basis of the morphology of vegetative cells, spores and parasporal crystals by phase contrast microscopy.

Isolates were grown on *Bt* sporulation medium for 48 h at 37 ^{o}C. The cells were suspended in 3-5 µl of sterile distilled water on the microscope slides and covered with lamel. The slide was examined under phase-contrast microscope for endospore position, crystal production and morphology. The isolates having visible parasporal crystals next to the spore in the sporangium cells were identified as *Bt*.

These colonies showing a different morphology and crystal shape were scraped off from the plates and transferred into 1.5 ml sterile vials containing 1 ml of nutrient broth in 20 % glycerol. After vortex mixing, they were stored at -80 ^{o}C as stock cultures for further analysis.

2.2.4.2. Catalase Test

Catalase is an enzyme that catalyzes the breakdown of hydrogen peroxide to water and oxygen. The presence of catalase is important in the prevention of toxic by products of oxygen metabolism that can kill the cell.

$$2H_2O_2 \rightarrow 2H_2O_+O_2$$

Isolates were grown on nutrient agar at 37 ^{o}C overnight. Catalase activity was detected by adding a drop of 3% hydrogen peroxide solution onto an isolated colony.

Immediate and vigorous bubbling indicated a strong catalase reaction whereas scant or no bubble formation indicated a negative test.

2.2.4.3. Proteolytic Activity

Proteolysis means the excretion of proteases by the bacterial cells during growth. Proteolytic activity is mostly determined by hydrolysis of gelatin (Thiery and Frachon 1997). Also, proteolytic activity can be determined against casein, the primary protein in cheese.

2.2.4.3.1. Hydrolysis of Casein

Caseinase activity was detected by spotting a very small amount of bacteria onto the center of a dried plate of milk agar. After incubation for 24 h at 37 ^{o}C, clear zones around the colonies were taken as the evidence for caseinolytic activity.

2.2.4.3.2. Hydrolysis of Gelatin

The method of Collins *et al*. (1995) was used with some minor modifications to carry out gelatin hydrolysis. A tube of nutrient gelatin was inoculated by stabbing with a heavy inoculum from 24 h culture and incubated for up to 48 h at 37 ^{o}C. The culture and an uninoculated tube were stored in a refrigerator for 24 h before checking them for hydrolysis.

The test was ready to read as soon as the control tube was solidified. Liquefaction of the medium indicated the gelatin hydrolysis although the control tube remained solid. Negative tests needed to be reincubated and tested every 24 h for up to 2 weeks (Harrigan 1998).

2.2.4.4. Lecithinase Activity

Lecithinase activity was detected by spotting or streaking a very small amount of bacteria onto the center of the nutrient agar plate supplemented with egg yolk emulsion (100 ml/L). After incubation for up to 48 h at 37 ^{o}C, lecithinase-producing isolates were

determined by the formation of white precipitate around the colonies. *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *galleriae* were used as lecithinase-positive and lecithinase-negative controls, respectively.

2.2.4.5. Amylase Activity

A plate of starch-nutrient agar plate was streaked once with the organism. After incubation for 24 h at 37 ^{o}C, plates were flooded with 5-10 ml of Gram's iodine solution. Any clear area around the growth the culture indicated the breakdown of starch by the organism due to its production of amylase. Unhydrolyzed starch formed a blue colour with the iodine.

2.2.4.6. DNase Activity

Microorganisms produce DNase that breaks down DNA into smaller fragments. Extracellular nuclease activity was determined on DNase test agar (Oxoid) as described by the manufacturer. Isolates were spotted onto the DNase agar. Following 18 h incubation at 37 ^{o}C, plates were flooded carefully with 1 N HCl that precipitated DNA and allowed to stand for a few min.

DNase positive cultures showed a distinct clear zone around the growth. *S. aureus* was used as positive control for the determination of DNase production.

2.2.4.7. Urease Activity

Urease (urea amidohydrolase) hydrolyses urea to produce ammonia and carbamate. The latter compound spontaneously decomposes to yield another molecule of ammonia and carbanic acid. The net effect of these reactions is an increase in pH (Mobley *et al*. 1995).

Urea hydrolysis was detected on Urea Agar Base (Oxoid) as described by the manufacturer. The surface of a Urea Agar slant containing 2 % final concentration of urea was streaked with a heavy inoculum. Tubes were incubated for 24-48 h at 37 ^{o}C. Urease-producing isolates changed the color of the medium from the yellow-orange to the purple-red.

2.2.4.8. Esculin Hydrolysis

Esculin is hydrolysed to esculetin by the bacterial enzyme esculinase. Afterwards, esculetin reacts with ferric ions in the medium to produce a black iron-complex giving a brown-black halo.

Esculin → Esculetin+ß-D-Glucose → Esculetin+Ferric salts→ Phenolic iron complex

Isolates were streaked onto Listeria Selective Agar Base (Oxford Formulation) and incubated for 48 h at 37 ^{o}C. The hydrolysis of esculin turned the medium black from greenish-yellow and esculinase-positive isolates produced brown-black colored colonies with a black halo.

2.2.4.9. Fermentation of Carbohydrates

Acid production by the fermentation of carbohydrates may occur because of the liberation of ammonia from proteinaceous material such as peptone in the medium (Thiery and Frachon 1997). Therefore, bromcresol fermentation broth and agar without peptone was used to identify acid generation from carbohydrates. The result of the process was determined by the color change of the bromcresol purple. This pH indicator turned yellow under acid conditions and purple under alkaline conditions.

2.2.4.9.1. Sucrose Hydrolysis

A small amount of growth from 24 h culture was inoculated either a straight line or a zigzag onto the bromcresol purple sucrose nutrient agar containing 10% sucrose. After incubation for 24 h at 37 ^{o}C, if the isolates were able to utilize the sucrose, an acid byproduct was created, which turned the media into yellow.

2.2.4.9.2. Salicin, Mannose, Cellobiose and Maltose Hydrolysis

Sugar solutions (10% w/v) were prepared and filter sterilized. Bromcresol purple broth base was supplemented individually with sugar solution (1% final concentration) after sterilization and cooling. One ml of bromcresol purple nutrient broth containing

each sugar was inoculated with a heavy inoculum from 24 h culture in an eppendorf tube. Tubes were incubated at 37 ^{o}C and carbohydrate utilization was assessed at the 24 and 48 h. The fermentation test was considered to be positive when the pH indicator in the medium became yellow after incubation.

2.2.4.10. Arginine Hydrolysis

This test determines the presence of arginine dihydrolyse (ADH). Arginine dihydrolyse pathway is a major source of energy for many microorganisms. In the first step, this pathway deaminates arginine to citrulline and splits citrulline into ornithine and carbamyll phosphate. Glucose fermentation decreases the pH of the medium so the color of the indicator changes to yellow. The second step occurs if ADH is present. ADH hydrolyzes arginine into ornithine, ammonia and CO_2. As a result, the end product is an alkaline that causes the pH to increase, and changing the color of the indicator back to purple (Thiery and Frachon 1997).

One ml of bromcresol purple LB broth (pH 6.8) supplemented with 1% (w/v) of aminoacid L-arginine was inoculated with a loopful of organism from 24 h culture in an eppendorf tube. Broths were overlaid with 400 µl sterile mineral oil to protect them from air. Tubes were incubated for 24 h to 4 days at 37 ^{o}C and examined daily for a purple color change. Before interpretation, tubes were shaken gently. Any trace of purple color was considered as positive and bright clear yellow was considered as negative.

2.2.4.11. Voges-Proskauer and Methyl Red Tests

Voges-Proskauer (VP) is used to identify organisms that are able to produce neutral end-products such as acetyl-methyl-carbinol and 2,3-butanediol from the degradation of glucose during 2,3-butanediol fermentation. Acetyl-methyl-carbinol is converted into diacetyl through the action of potassium hydroxide and atmospheric oxygen.

Isolates were grown in peptone water with vigorous shaking at 180 rpm for 24 h at 37 ^{o}C. After incubation, 4 ml of glucose phosphate broth was inoculated with 200 µl culture grown in peptone water. Tubes were incubated at 180 rpm for 48 h at 37 ^{o}C or until the turbidity obtained. After the incubation, 1 ml culture was transferred into a

sterile tube. 200 µl α-napthol was added and shaken gently, and then 200 µl KOH solution was added and shaken. Open tubes were placed on a slant to increase the contact with the air. Development of a cherry red coloration at the surface within 30-45 min was taken as a positive indication for Voges-Proskauer test.

Methyl Red (MR) test is used to identify bacteria that produce stable acid end-products at a pH of 4.4 in the media due to mixed acid fermentation of glucose. At a pH of 4.4 or less, methyl red is bright cherry red and yellow at a pH of above 4.4.

For the MR test, 200 µl of methyl red reagent was added into the remaining glucose phosphate buffer. Within just a few s, red coloration indicated the positive result.

E. coli was used as positive control for MR and negative for VP during the experiments.

2.2.4.12. Antibiotic Resistance

Isolates were tested for resistance against penicillin (10 µg/ml), ampicillin (100 µg/ml) and chloramphenicol (25 µg/ml) on nutrient agar plates for 48 h at 37 ^{o}C (Kaur *et al.* 2001).

2.2.5. Genotypic Characterization

2.2.5.1. Plasmid DNA Isolation

The method of O'Sullivan and Haenhammer (1993) with some minor modifications was used for the isolation of plasmid DNA. Bacterial cultures were grown on nutrient agar plates at 37 ^{o}C overnight. The cells were collected and resuspended in 1 ml of sterile water. The sample was spinned at 7,000 rpm for 10 min to pellet the bacteria. The supernatant was discarded and the pellet was suspended in 200 µl lysozyme solution (25% sucrose containing 30 mg/ml lysozyme) and incubated for 15 min at 37 ^{o}C. After lysis was completed, 400 µl of alkaline SDS solution (3% SDS, 0.2 N NaOH) was added and incubated at room temperature for 7 min. After this step, 300 µl of ice-cold 3 M sodium acetate (pH 4.8) was added. The solution was mixed immediately and centrifuged for 20 min at 8,000 rpm at 4 ^{o}C. The supernatant was then

transferred into a new eppendorf tube and 650 µl of isopropanol was added and mixed well. After centrifugation for 20 min at 8,000 rpm at 4 ^{o}C, the supernatant was discarded and the pellet was resuspended in 320 µl sterile distilled water. Two hundred µl of ammonium acetate (7.5 M) containing ethidium bromide (0.5 mg/ml) was added into the solution and mixed. Then, 350 µl of phenol/chloroform solution was added, mixed well and centrifuged for 10 min at 10,000 rpm at room temperature. The aqueous phase was transferred into a new eppendorf tube. The DNA was precipitated by adding 1 ml of ethanol (99%, -20 ^{o}C) and spinning for 20 min at 8,000 rpm at 4 ^{o}C. Then, the pellet was washed with 500 µl of 70% ethanol (at room temperature) and centrifuged for 5 min at 10,000 rpm. The pellet was air-dried and resuspended in 40 µl of TER solution (TE, pH 7.8 and RNase, 0.1 mg/ml). After incubation for 60 min at 37 ^{o}C, plasmid DNA was electrophoresed at 90 V for 2 h and visualized in a gel documentation system (Vilber Lourmat, France).

2.2.5.2. Oligonucleotide Primers for PCR

Universal primers, reported by Bendov *et al*. (1997) and Bravo *et al*. (1998), *gral-cry1, gral-cry2, gral-cry4*, *gral-cry9*, and *gral-cry11* were used to detect the presence of *cry1, cry2, cry4, cry9, cry11* genes, respectively. To detect the presence of *cry13Aa,* a specific primer was used. Also, *gral-cyt1* and *gral-cyt2* universal primers reported by Ibarra *et al*. (2003) were used for the amplification of *cyt1* and *cyt2* genes, respectively. The sequences of universal and specific primers as well as their predicted product sizes were shown in Table 2.2. These primers were synthesized by Integrated DNA Technologies, INC., MWG. and Thermo Hybaid.

Table 2.2. Characteristics of Universal and Specific Primers

Primer Pair	Sequence[a] (5'→3') of primer	PCR Product Size (bp)
gral-cry1	CATGATTCATGCGGCAGATAAAC (d) TTGTGACACTTCTGCTTCCCATT (r)	274-277
gral-cry2	GTTATTCTTAATGCAGATGAATGGG (d) CGGATAAAATAATCTGGGAAATAGT (r)	689-701
gral-cry4	GCATATGATGTAGCGAAACAAGCC (d) GCGTGACATACCCATTTCCAGGTCC (r)	439
gral-cry9	CGGTGTTACTATTAGCGAGGGCGG (d) GTTTGAGCCGCTTCACAGCAATCC (r)	351-354
gral-cry11	TTAGAAGATACGCCAGATCAAGC (d) CATTTGTACTTGAAGTTGTAATCCC (r)	305
spe-cry13	CTTTGATTATTTAGGTTTAGTTCAA (d) TTGTAGTACAGGCTTGTGATTC (r)	313
gral-cyt1	CCCCAATCAACAGCAAGGGTTATT (d) TGCAAACAGGACATTGTATGTGTAATT (r)	477-480
gral-cyt2	ATTACAAATTGACAAATGGTATTCC (d) TTTCAACATCCACAGTAATTTCAAATGC (r)	355-356

[a]d and r, direct and reverse primers, respectively.

2.2.5.3. *cry* and *cyt Gene* Identification by PCR

Selected sequences of *cry* and *cyt* genes were amplified by PCR using a reaction mixture containing 5µl Taq 10X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each of the DNA primers, 2U of *Taq* DNA polymerase (Fermentas) and 500 ng of plasmid DNA. The volume was completed to 50 µl with sterile dH_2O. DNA sample was transferred into 0.5 ml PCR tube. A master mix, excluding DNA and *Taq* polymerase, was prepared. It was mixed gently by pipetting and *Taq* polymerase was added into the master mix thoroughly and centrifuged for a few s. The PCR mix was aliquoted into each PCR tube. 50-75µl of mineral oil were layered on top of the reaction mixture. All the steps were performed on ice. Reactions were performed in a Techne Progen thermal cycler (England) with the following amplification conditions: a denaturation step for 1 min at 94 ^oC, 35 amplification cycles: denaturation 1 min at 94 ^oC, annealing 1 min at 60 ^oC (for *cry1, cry2, cry4, cry9, cry13*), 62 ^oC (for *cry11*) and 1 min extension at 72 ^oC; finally an extra extension step of 10 min was carried out at 72 ^oC.

For the amplification of *cyt* genes, these steps were used for PCR: a denaturation step for 2 min at 95 ^oC, 35 cycles of amplification with 1 min denaturation at 95 ^oC, 1 min of annealing at 52 ^oC (for *cyt1*), 50 ^oC (for *cyt2*) and 1 min of extension at 72 ^oC. An extra extension step of 5 min was carried out at 72 ^oC after 35 cycles.

After the completion of PCR reaction, 1.5 % agarose-ethidium bromide gel was prepared by adding 1.5 gr agarose to 100 ml 1xTAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0) and boiling. The solution was cooled to 40-50 ^oC before adding 15 µl of ethidium bromide (10 mg/ml). The solution was poured into a gel tray and allowed to solidify. 10 µl of each PCR product were mixed with 1/5 volume of gel-loading dye before loading and electrophoresed at 70 V for 40 min in 1xTAE buffer. The bands were visualised and recorded in a gel documentation system (Vilber Lourmat, France).

2.2.5.4. Isolation of Bacterial Genomic DNA

Isolation of genomic DNA was carried out by the combination of two methods (Ausubel *et al.* 1994, Cardinal *et al.* 1997). *Bt* strains were grown in 10 ml of nutrient broth at 37 ^oC with shaking overnight. Cells were harvested by centrifuging at 4,850 rpm for 10 min and the cell pellet was resuspended in 200 µl sucrose solution (25%

sucrose and 30 mg lysozyme/ml in 1xTE), followed by the incubation for 1 h at 37 ^{o}C. After the cell lysis, 370 µl 1xTE containing 1 mg proteinase K/ml and 30 µl 10% SDS solution were added and mixed gently. The preparation was incubated for 1 h at 37 ^{o}C. 100 µl 5 M NaCl and 80 µl CTAB/NaCl solution (10% cetyltrimethylammonium bromide, 0.7 M NaCl) were then added and mixed by inverting the tubes. The cell suspension was incubated for 10 min at 65 ^{o}C in a water bath. For chloroform extraction, one volume of chloroform/isoamyl alcohol (24:1) was added and mixed effectively and centrifuged at 8,000 rpm for 5 min. The upper phase was gently transferred into a new eppendorf tube. Chloroform extraction was performed once more. DNA wool was obtained by the addition of one volume of isopropanol to the aqueous phase. The DNA wool was tranferred into an eppendorf tube with a pipette tip containing 500 µl 70% ethanol. After centrifugation at 8,000 rpm for 5 min, the supernatant was discarded and the washed pellet was dried at 37 ^{o}C for 10 min and dissolved in 100 µl 1xTE containing 100 µg/ml RNase. After incubation for 1 h at 37 ^{o}C, the sample volume was adjusted to 400 µl with 1xTE. The solubilization of DNA was achieved by alternating heat shocks, for 10 min at 80 ^{o}C, and 20 min at -20 ^{o}C. The lysate was extracted with phenol/chloroform. For this step, one volume of phenol was added, mixed well. After centrifugation for 5 min at 8,000 rpm, the upper phase was gently transferred into an eppendorf tube. One volume of chloroform/isoamyl alcohol (24:1) was added, mixed well and centrifuged at 8,000 rpm for 5 min. The aqueous phase was transferred into another tube and 0.5 M NaCl final concentration and two volumes of 99% ethanol were added to precipitate the DNA. After spinning at 10,000 rpm for 10 min, pellets were washed in 500 µl 70% ethanol by centrifugation for 5 min at 8,000 rpm. Finally, the washed DNA pellets were dried and resuspended in 50-200 µl 1xTE, depending on the amount of the DNA pellet. Samples were stored at -20 ^{o}C until further use.

2.2.5.5. Identification of Isolates by 16S ITS-rDNA RFLP

2.2.5.5.1. Amplification of the 16S ITS-rDNA Region

PCR amplifications were performed in 50 µl-final reaction volumes containing 500 ng of genomic DNA as the template, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 pmol each of the primers, 1xPCR buffer (Fermentas) and 1.25U *Taq* DNA polymerase (Fermentas).

It was mixed gently by pipetting and *Taq* polymerase was added into the master mix thoroughly and centrifuged for a few s. The PCR mix was distributed into each of the PCR tubes. 50-75µl of mineral oil were layered on top of the reaction mixture. All the steps were performed on ice. Amplification was performed in a Techne Progen thermal cycler (England) with this cycling program:

An initial denaturation at 94 ^{o}C for 5 min; followed by 40 cycles of 94 ^{o}C for 1 min, 42 ^{o}C for 1 min and 72 ^{o}C for 1 min; and a final extension at 72 ^{o}C for 10 min.

Two primers used for the amplification;

Forward, L1: 5$^{'}$–AGAGTTTGATCCTGGCTCAG-3$^{'}$ (Mora *et al.*1998)

Reverse, EGE1: 5$^{'}$ –CAAGGCATCCACCGT-3$^{'}$ (Jensen *et al.* 1993).

2.2.5.5.2. Electrophoresis of Amplified 16S ITS-rDNA Fragments

To estimate the concentration of DNA, the PCR products were electrophoresed in 0.8 % agarose-ethidium bromide gel. The gel was made by adding 0.8 gr agarose to 100 ml 1xTAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0) by boiling. The solution was cooled to 40-50 ^{o}C before adding 15 µl of ethidium bromide (10 mg/ml). The solution was poured into a gel tray, combs were placed. After the gel was solidified, the combs were removed. The casting tray was placed into the tank containing 1xTAE buffer. 5 µl of each PCR product mixed with 1µl of 6xgel-loading dye and 4 µl of 1 kb DNA molecular weight marker were loaded into the wells of agarose gel. The electrophoresis was performed for approximately 2.5 h at 60 mA. The bands were visualised on an UV illuminator and recorded in a gel documentation system (Vilber Lourmat, France).

2.2.5.5.3. Chloroform Extraction of PCR Products

The volume of the PCR products was adjusted to 100 µl with 1xTE (10 mM Tris and 1 mM EDTA, pH 8.0). Two volumes of chloroform/isoamyl alcohol solution (24:1) were added onto the samples and vortexed for 15 s. They were then centrifuged for 2 min at 10,000 rpm. The lower phase was discarded and then two volumes of chloroform/isoamyl alcohol solution were added. They were vortexed for 15 s. The two phases were separated by centrifugation for 2 min at 10,000 rpm and the top layer was transferred into a clean eppendorf tube and 10 µl 3 M sodium acetate (pH 5.2) were added. The solution was mixed well by pipetting. Two and a half volumes of 99% ethanol were then added and vortexed for 15-20 s. The DNA was precipitated by spinning for 15 min at 14,000 rpm. The supernatant was removed and the pellet was carefully washed with 300 µl 70% ethanol. After centrifugation for 5 min at 14,000 rpm, ethanol was removed. The pellets were dried for 15-20 min at 37 ^{o}C and resuspended in 20 µl 1xTE. Purified PCR products were stored at -20 ^{o}C until use.

2.2.5.5.4. RFLP

The restriction enzymes used to digest the PCR products were *Taq*I (T'CGA) and *Hae*III (GG'CC). The amplification products were digested with *Taq*I and *Hae*III, separately, then together. Digestion mixture included 6 µl of purified PCR product, 5U of *Taq*I (Fermentas) and 5µl of 10x restriction enzyme buffer in a total volume of 50 µl. The samples were overlaid with a few drops of mineral oil and were incubated overnight at 65 ^{o}C in a water bath. *Hae*III digestions were performed overnight at 37 ^{o}C. After the digestion, DNA extraction was carried out as described in section 2.2.5.5.3. and dissolved in 10 µl 1xTE.

2.2.5.5.5. Electrophoresis of Restriction Fragments

The digestion products were electrophoresed in 1.6 % agarose ethidium bromide gel. The gel was prepared by adding 1.6 gr agarose to 100 ml 1xTAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0) and by boiling. The solution was cooled to 40-50 ^{o}C before adding 15 µl of ethidium bromide (10 mg/ml). 10 µl of each PCR product was

mixed with 2 µl of 6xgel-loading dye. Then the samples and 5 µl of 1 kb DNA molecular weight marker (Fermentas) were loaded into the wells of agarose gel. The electrophoresis was performed for 1 h at 40 mA and 3 h at 60 mA in 1xTAE containing 0.02% ethidium bromide (10 mg/ml). The bands were visualised on an UV illuminator and recorded in a gel documentation system (Vilber Lourmat, France).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Isolation of *Bt* from Olive Tree-related Habitats

Two hundred and forty samples from different olive tree-related habitats in İzmir, Manisa, and Aydın regions were collected. Most of the samples were associated with olive. Soil, leaf residues, fresh leaves, animal faeces, insect cadavers were taken from olive groves; dust samples and olive pomace were taken from processing places in olive-oil factories. Both shaken-flask technique and sodium acetate selection methods were used for the isolation of *Bt.* Colonies on plates of nutrient agar that had similar colony morphology to *Bt* were selected (Figure 3.1). Isolates were named in the following order; sample number showing sampling site, *Bt*-like colony morphology with upper letter "Q" and a number to differentiate isolates obtained from the same sample. After observation by phase-contrast microscopy, one hundred isolates from different sources and regions were characterized as *Bt* based on the crystal protein production and other sporeforming *Bacillus* were eliminated. *Bt* isolates were found in 54 samples out of the 240 samples analysed (Table 3.1). The highest percentage of samples containing the bacterium was in soils. Out of 100 isolates, 63 (63%) isolates were obtained from soil whereas the lowest abundance was observed in samples of animal faeces yielding only two isolates (2%). Most isolates were derived from Eski Foça, Turgutlu, Kemalpaşa, Aliağa, Dikili, İzmir, and two olive-oil factories from Bergama. On the other hand, no *Bt* was recovered in samples from Kuşadası, Çeşme, Urla, and Manisa. The occurrence of *Bt* was the highest in samples from Eski Foça (29 %).

Table 3.1. *Bt* Isolation from Olive Tree-related Habitats

Sample material	**No.of samples analysed**	**No. of samples yielding Bt**	**% of *Bt* positive samples**	**No. of *Bt* isolates**
Soil	124	36	29.0	63
Olive leaf residue	31	6	19.4	13
Green olive leaves	19	5	26.3	10
Animal faeces	5	1	20.0	2
Dust	22	3	13.6	8
Olive pomace	38	3	7.9	4
Insect cadaver	1	0	-	-
Total	**240**	**54**		

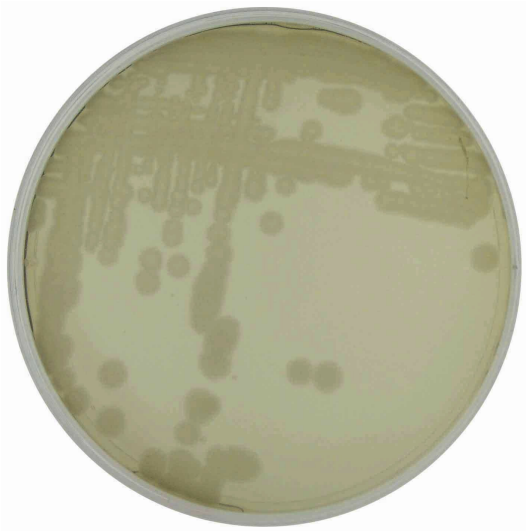

Figure 3.1. Colony morphology of the isolate of 41Q3

Table 3.2. Distribution of Isolates According to Source of Isolation

Source of Isolation	Isolates
Soil	70Q, 58Q2, 54Q2, 290Q1, 284Q, 50Q3, 53Q1, 59Q2, 55Q3, 55Q1, 52Q3, 50Q1, 284Q1, 41Q3, 41Q, 58Q1, 43Q, 184Q, 56Q2, 53Q3, 69Q1, 68Q, 58Q3, 74Q, 73Q, 37Q1, 65Q, 62Q, 37Q3, 37Q2, 52Q1, 60Q1, 44Q2, 184Q1, 67Q, 69Q, 75Q, 56Q1, 59Q1, 41Q2, 50Q2, 60Q2, 63Q, 44Q1, 90Q1, 290Q2, 183Q1, 71Q, 61Q, 37Q4, 48Q1, 55Q2, 57Q1, 125Q, 53Q2, 59Q3, 54Q1, 183Q, 71Q1, 72Q, 64Q1, 64Q, 189Q
Olive leaf residue	36Q1, 19Q, 39Q1, 19Q4, 19Q2, 21Q, 36Q2, 39Q, 19Q3, 19Q1, 5Q, 19Q5, 17Q
Green olive leaves	20Q1, 14Q1, 20Q3, 14Q4, 24Q, 14Q3, 14Q2, 14Q, 16Q, 25Q
Dust	249Q, 167Q1, 167Q2, 161Q5, 161Q3, 161Q4, 161Q2, 167Q
Olive pomace	169Q, 164Q1, 174Q, 164Q2
Animal faeces	45Q1, 45Q2

Table 3.3. Distribution of Isolates According to Sampling Sites

Sampling Site		Isolate
İzmir	Foça	58Q2, 58Q1, 58Q3, 48Q1, 64Q1, 64Q, 19Q, 19Q4, 19Q2, 19Q3, 19Q1, 19Q5, 17Q, 20Q1, 14Q1, 20Q3, 14Q4, 14Q3, 14Q2, 14Q, 16Q, 65Q, 62Q, 44Q2, 67Q, 63Q, 44Q1, 61Q, 5Q
	Güzelbahçe-Küçükkaya village	54Q2, 60Q1, 60Q2, 57Q1, 54Q1
	Konak-Tırazlı village	55Q3, 55Q1, 59Q2, 59Q1, 55Q2, 59Q3
	Kemalpaşa	290Q1, 284Q, 50Q3, 53Q1, 52Q3, 50Q1, 284Q1, 56Q2, 53Q3, 52Q1, 56Q1, 50Q2, 290Q2, 125Q, 53Q2, 45Q1, 45Q2
	Dikili	24Q, 25Q, 36Q1, 39Q1, 21Q, 36Q2, 39Q, 183Q, 189Q, 184Q1, 183Q1, 184Q
	Aliağa	41Q3, 41Q, 43Q, 37Q1, 37Q3, 37Q2, 37Q4, 41Q2
Manisa-Turgutlu	Dalbahçe village	70Q, 68Q, 71Q, 71Q1, 72Q
	Irlamaz village	69Q1, 74Q, 73Q, 69Q, 75Q, 90Q1
Olive Oil Factories (Bergama)	Tunçyağ olive oil factory Sulu olive oil factory	249Q,167Q1,167Q2,161Q5, 161Q3,161Q4,161Q2,167Q, 169Q, 164Q1, 174Q, 164Q2

3.2. Phenotypic Characterization

3.2.1. Crystal Protein Morphology of *Bt* Isolates

The phase-contrast microscopy results showed the presence of visible and refringent crystal proteins and spore position in isolates. Morphological features of vegetative cells were typical of the *Bt* and spore positions of all isolates were subterminal. In all cases, parasporal inclusions were produced outside of the endospore and were distinctly separated from it. Eleven different parasporal morphologies were observed by phase contrast microscopy. The most abundant combination belonged to spherical/cuboidal/irregular pointed group (24.2%). The differences in the crystal protein morphology distribution might be due to genetic variation caused by the difference in the environmental conditions or to habitat effects (Al-Momani *et al*. 2004). Typically, 59 isolates were found to produce more than one crystal in the sporulating cell. Crystal morphology of one isolate was not determined. The spherical inclusions were the most frequent crystals. Bipyramidal crystals were observed only in isolates from soil and olive leaf residue samples. Cuboidal crystals were present in all the samples except animal faeces. All types of samples were found to contain irregular pointed crystals.

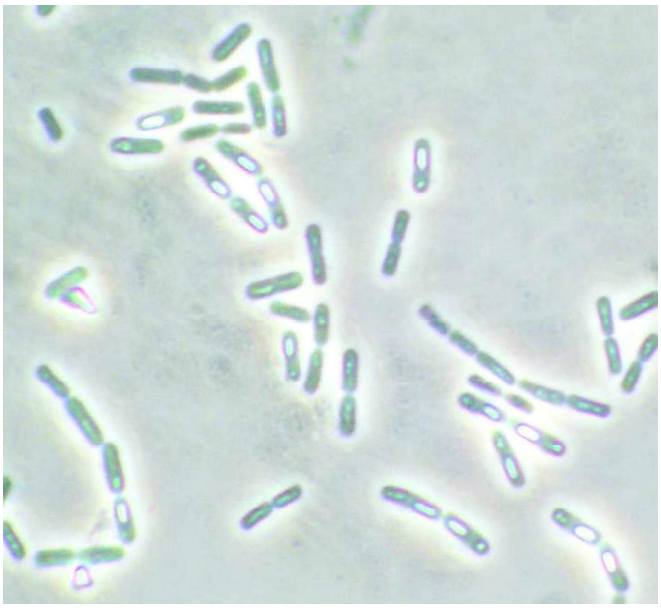

Figure 3.2. Phase contrast photograph of the isolate 19Q3

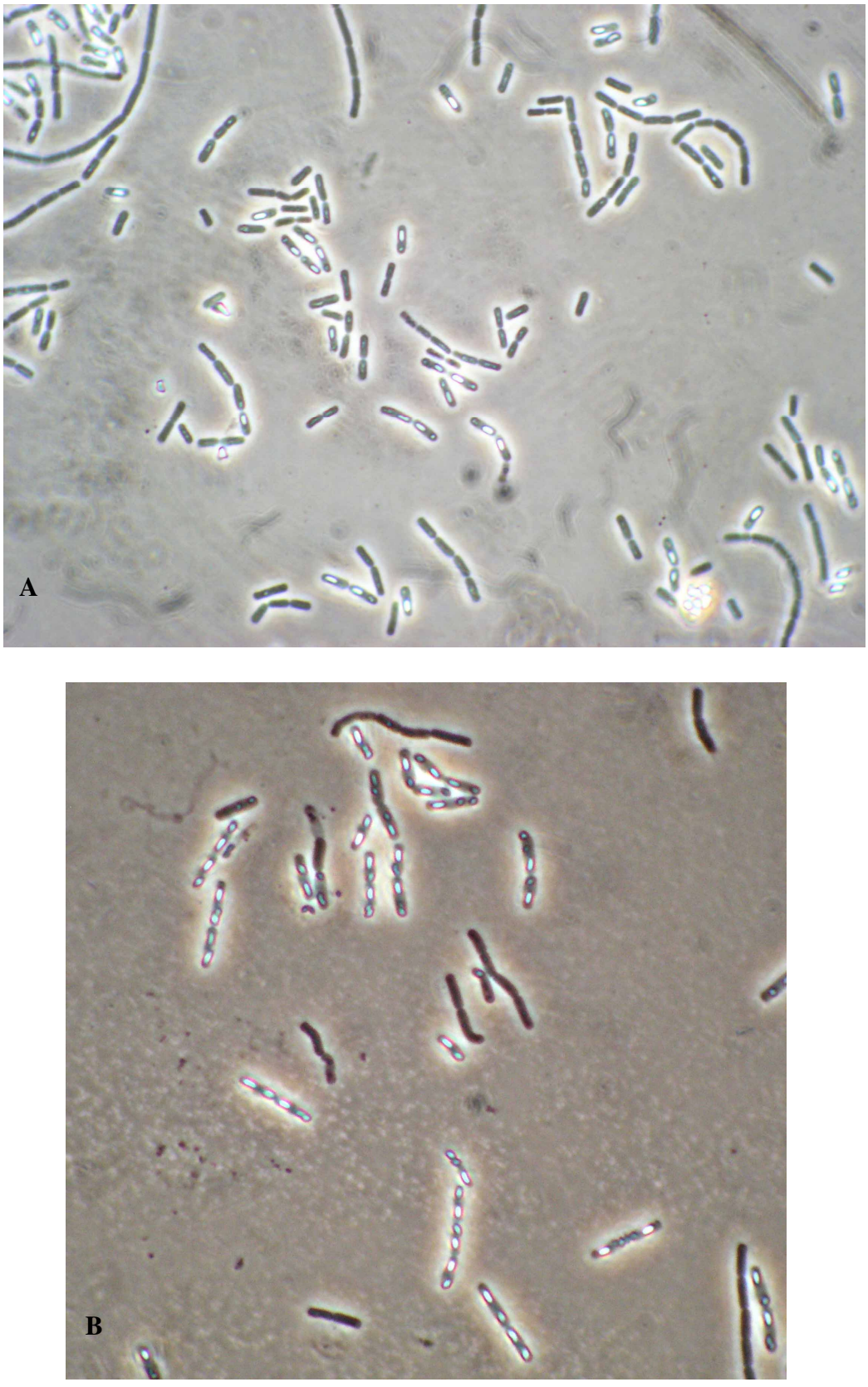

Figure 3.3. Phase contrast photograph of the isolate (A) 14Q1 and (B) 60Q2

Table 3.4. Distribution of Crsytal Protein Morphologies

Crystal Protein Shape Groups	Isolate Number	Isolate Names
Spherical	16	70Q, 58Q2, 290Q1, 50Q1, 284Q1, 69Q1, 52Q1, 59Q1, 90Q1, 290Q2, 5Q, 249Q, 14Q4, 16Q, 169Q, 45Q1
Cuboidal	17	54Q2, 284Q, 58Q3, 73Q, 37Q3, 67Q, 50Q2, 183Q1, 71Q, 54Q1, 71Q1, 39Q, 19Q3, 167Q1, 167Q2, 14Q3, 174Q
Irregular Pointed	3	184Q, 53Q3, 19Q
Bipyramidal	3	60Q1, 60Q2, 125Q
Cuboidal and Spherical	6	55Q1, 62Q, 183Q, 17Q, 14Q1, 25Q
Spherical and Irregular Pointed	19	59Q2, 41Q3, 43Q, 68Q, 74Q, 37Q1, 75Q, 63Q, 61Q, 55Q2, 57Q1, 59Q3, 64Q, 21Q, 36Q2, 161Q2, 24Q, 164Q1, 45Q2
Bipyramidal and Cuboidal	3	37Q4, 19Q2, 19Q1
Bipyramidal and Spherical	1	69Q
Spherical, Irregular Pointed and Cuboidal	24	50Q3, 53Q1, 55Q3, 52Q3, 41Q, 56Q2, 37Q2, 44Q2, 56Q1, 41Q2, 53Q2, 64Q1, 36Q1, 39Q1, 19Q4, 19Q5, 161Q5, 161Q4, 167Q, 20Q1, 20Q3, 14Q2, 14Q, 164Q2
Cuboidal and Irregular Pointed	6	58Q1, 184Q1, 44Q1, 48Q1, 189Q, 161Q3
Bipyramidal and Irregular Pointed	1	65Q

3.2.2. Catalase test

All the isolates exhibited catalase activity.

3.2.3. Proteolytic activity

Protease activity was different when performed with the gelatinase test (98%) than with caseinase (100%).

3.2.3.1. Hydrolysis of Casein

All the strains hydrolyzed casein (Table 3.5).

3.2.3.2. Hydrolysis of Gelatin

Ninty-eight (98%) isolates hydrolyzed gelatin. Among them, most gave positive reactions in 48 h but after further for 7 day incubation, 9 isolates out of 98 hydrolyzed gelatin (Table 3.5).

3.2.4. Lecithinase Activity

Ninety-one (91%) isolates were lecithinase-positive on egg yolk media. Only one isolate did not grow on this medium (Table 3.5).

3.2.5. Amylase Activity

In large-scale production, media containing starch are used as the main carbon source of the fermentation of *Bt* strains (Rossa *et al.* 1995).

Out of 100 isolates, 94 (94 %) formed zones of clearance indicating amylase activity (Table 3.6). Baumann (1984) found that 99 % of the 172 strains of *Bt* and *B. cereus* produced extracellular amylase. *B. cereus* strains which usually can not degrade starch produce emetic toxins (Kramer and Gilbert 1989, Agata *et al*. 1996). The isolates

that lack amylase activity could have emetic toxins. All reference strains except *B. thuringiensis* subsp. *finitimus* produced amylase on starch-nutrient agar.

3.2.6. DNase Activity

Sixteen (16 %) isolates showed a clear zone around the spot by the production of extracellular DNase. After the addition of 1 N HCl, some colonies were taken off from the inoculated area leaving clearance in the medium. Among reference strains only *B. thuringiensis* subsp. *finitimus* was found as a DNase producer (Table 3.5).

3.2.7. Urease Activity

The ammonia produced by the decomposition of urea was obtained in 6 (6 %) of the isolates. The tubes that were light pink were considered as negative after 24 h of incubation. *B. thuringiensis* subsp. *aizawai*, *B. thuringiensis* subsp. *thompsoni*, *B. thuringiensis* subsp. *kurstaki*, and *B. thuringiensis* subsp. *galleriae* exhibited urease activity (Table 3.6).

3.2.8. Esculin Hydrolysis

Only 14 (14 %) isolates formed black-brown colonies with a black halo due to esculin hydrolysis. Also, *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *finitimus* hydrolysed esculin among reference strains (Table 3.5).

3.2.9. Fermentation of Carbohydrates

3.2.9.1. Sucrose Hydrolysis

Forty-eight (48 %) strains were able to utilize sucrose and produced acid that turned the medium's color from purple to yellow (Table 3.5). *B. thuringiensis* subsp. *kurstaki, B. thuringiensis* subsp. *morrisoni*, *B. thuringiensis* subsp. *kumamtoensis,* and *B. thuringiensis* subsp. *finitimus* produced acid from sucrose but de Barjac showed that *B. thuringiensis* subsp. *thompsoni* fermented sucrose and *B. thuringiensis* subsp. *kurstaki* did not. These findings were in disagreement with our findings.

3.2.9.2. Salicin, Mannose, Cellobiose and Maltose Hydrolysis

Sixty-five (65%) of isolates fermented salicin. The least hydrolyzed carbohydrate was mannose, with 12 percent (Table 3.5). Most strains showed very weak cellobiose utilization reactions and changed the color of the fermentation broth from purple to olive green. Thirty-two (32%) isolates hydrolyzed cellobiose (Table 3.6). All strains were positive for maltose fermentation and produced acid in 24 h incubation. de Barjac showed *B. thuringiensis subsp. israelensis* as negative for mannose utilization but it was positive in our experiment.

3.2.10. Arginine Hydrolysis

Eighy-one (81%) isolates were capable of hydrolyzing the amino acid arginine (Table 3.6). Green reactions after 48 h of incubation were interpreted as negative. The test was considered as positive if the medium stayed purple or became purple-grey.

3.2.11. Voges-Proskauer and Methyl Red Tests

Acetyl-methyl-carbinol production by VP reaction was positive for 96 isolates (Table 3.6). Sixty-seven isolates were able to perform mixed-acid fermentation of glucose and produced stable acids by MR reaction. Two isolates were not determined by MR because of the orange color production although they were tested again after longer incubation in glucose-phosphate broth. Results of MR were shown in Appendix E.

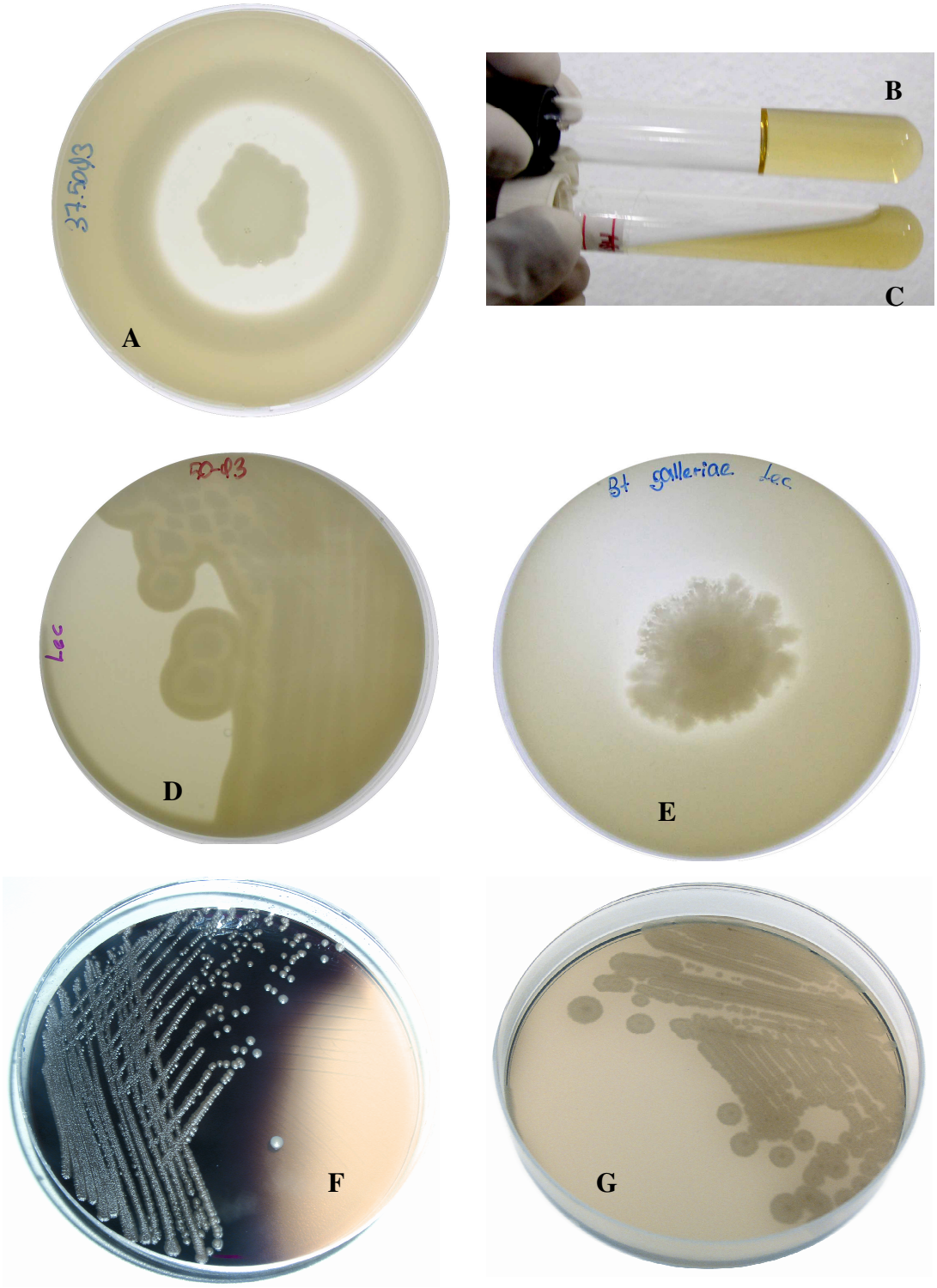

Figure 3.4. Detection of Caseinase, Gelatinase, Lecithinase, Esculinase Activities
A. Caseinase activity of isolate 37Q3, **B.** Gelatinase negative isolate, 41Q3,
C. Gelatinase activity of isolate 17Q, **D.** Lecithinase activity of isolate 50Q3,
E. Lecithinase negative reference strain *B. thuringiensis* subsp. *galleriae*,
F. Esculinase activity of 72Q, **G.** Esculinase negative isolate, 16Q

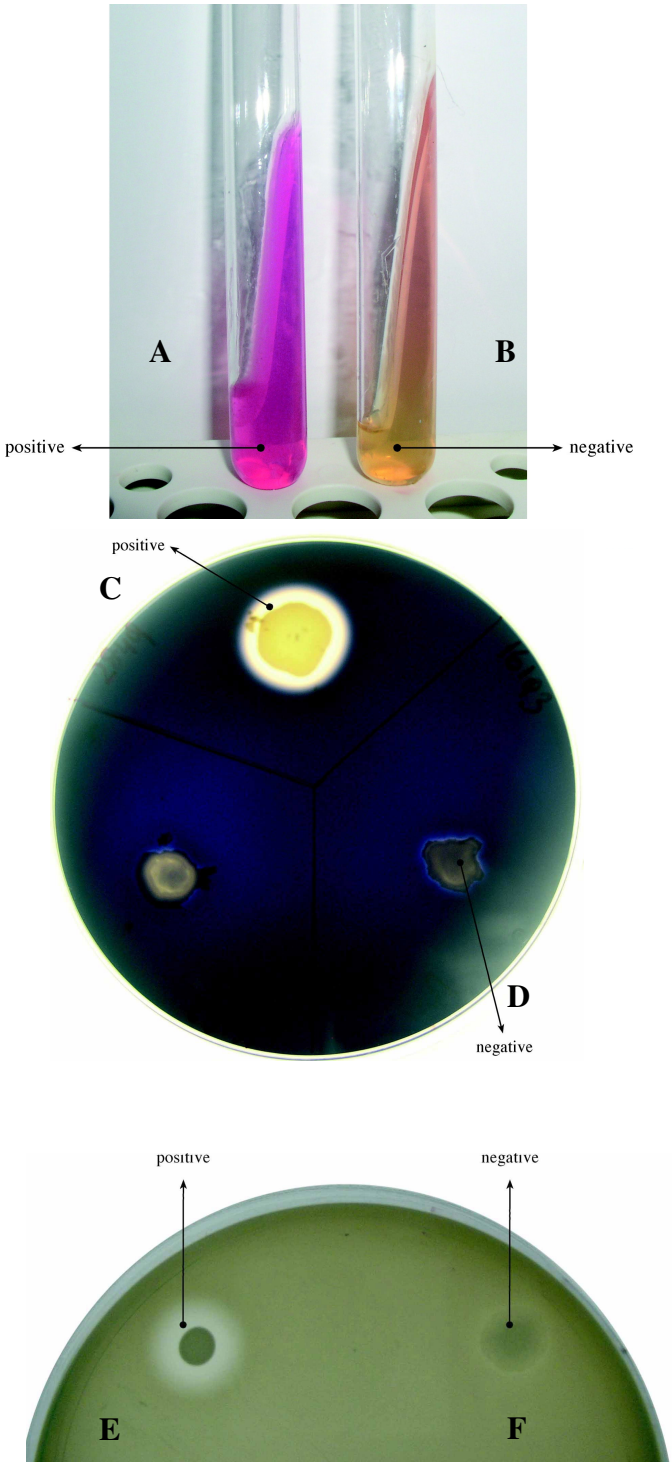

Figure 3.5. Detection of Urease, Amylase, DNase Activities. **A.** Urease activity of isolate 19Q2, **B.** Urease negative isolate, 21Q, **C.** Amylase activity of isolate 58Q2, **D.** Amylase negative isolate, 5Q, **E.** DNase activity of isolate 183Q **F.** DNase negative isolate, 25Q

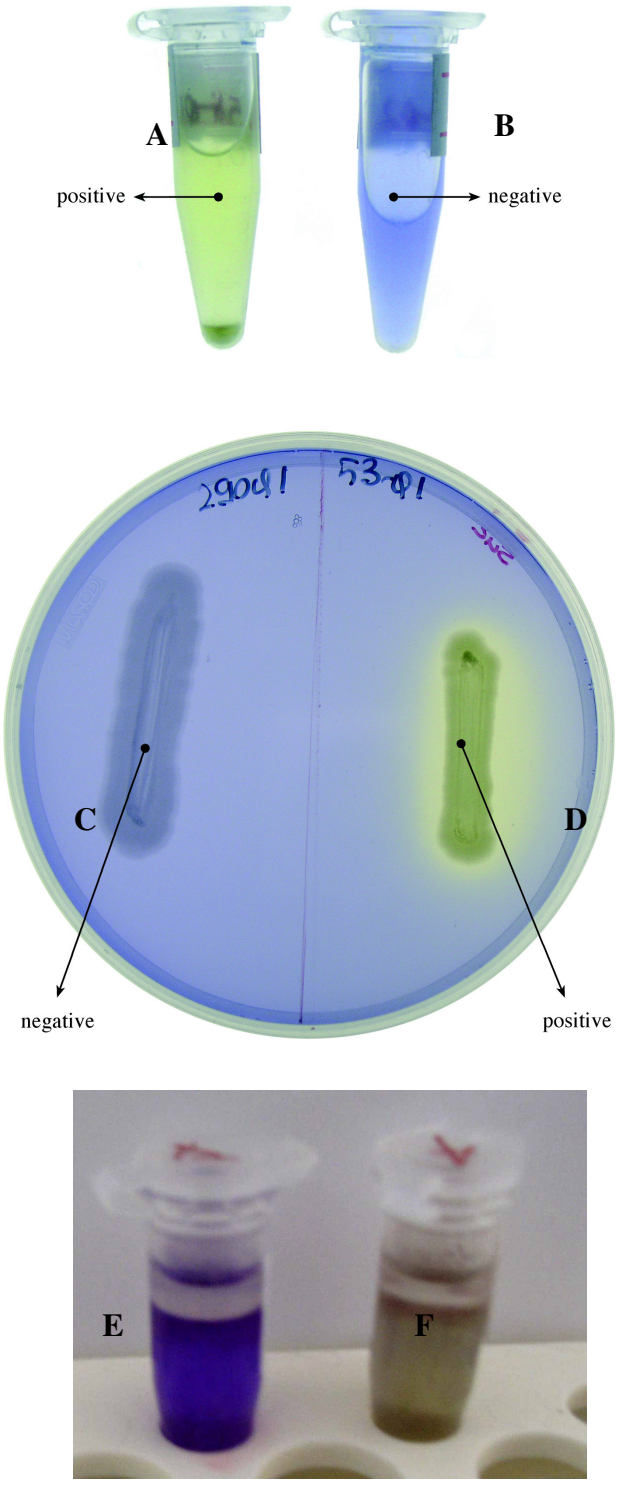

Figure 3.6. Detection of Salicin and Sucrose Hydrolysis, Arginine dihydrolyse Activity **A.** Salicin hydrolysis of isolate 24Q, **B.** Salicin hydrolysis-negative isolate, 19Q, **C.** Sucrose hydrolysis-positive isolate 53Q1, **D.** Sucrose hydrolysis-negative isolate, 290Q1, **E.** ADH-positive isolate 290Q2, **F.** ADH-negative isolate 167Q

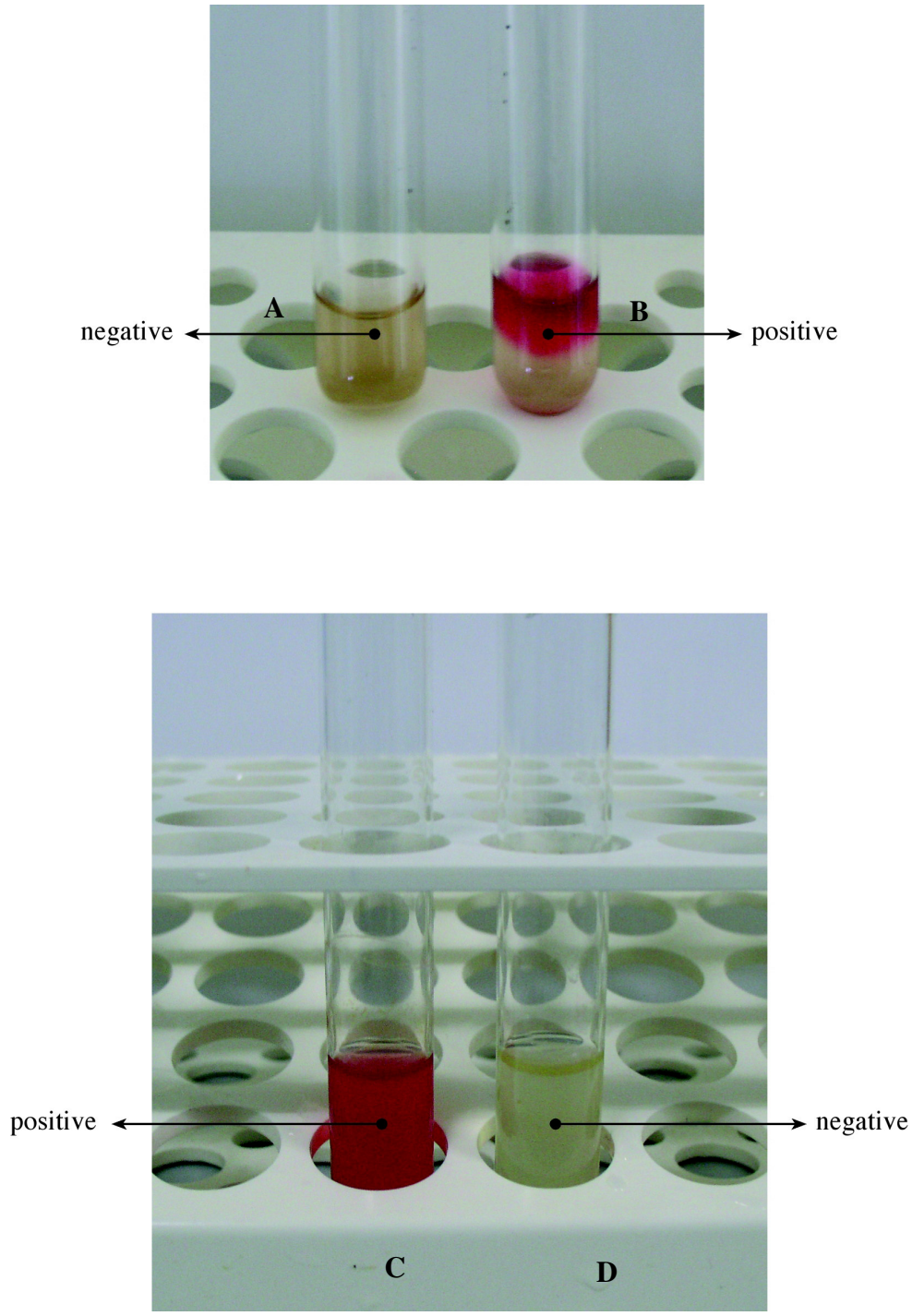

Figure 3.7. Results of Voges-Proskauer and Methyl Red Tests**. A.** VP-negative isolate 69Q1, **B.** VP-positive isolate 68Q, **C.** MR- positive 249Q, **D.** MR-negative isolate, 14Q1

3.2.12. Antibiotic Resistance

Penicillin resistance is widespread in *Bt* strains (Johnson and Bishop1996). Nighty-eight isolates were resistant to penicillin (10 μg/ml). All isolates except four were resistant to ampicillin (100 μg/ml) and 21 isolates had chloramphenicol (25 μg/ml) resistance (Appendix F).

3.3. Biotyping

The results of esculin, salicin, sucrose, and mannose hydrolysis, lecithinase and DNase production created different biochemical types. Ninghteen biochemical groups were obtained. The results were compared with reference strains used in this study. Most isolates belonged to the biochemical type 5. The other abundant biotypes among isolates were type 1 and type 2. The biochemical type1 consisting of 12 isolates also included *B. thuringiensis* subsp. *aizawai*. *B. thuringiensis* subsp. *alesti* showed similar characteristics to isolates belonging to type 2. Among reference strains, *B. thuringiensis* subsp. *finitimu*s and *galleria* showed distinct biochemical characteristics and formed different biotype groups (Table 3.5).

Biochemical tests except MR reaction were used to subdivide biotypes into different groups (Table 3.6). These included the urease, amylase, gelatinase, arginine dihydrolyse (ADH), acetyl-methyl-carbinol (AMC) production, and cellobiose hydrolysis. Some of the isolates belonging to the same biotype had the same patterns. All isolates in group 2, group 4, group 14, group 15, and group 17 produced amylase, gelatinase, ADH, and AMC but did not produce urease and not hydrolyze cellobiose.

Group 11 isolates produced amylase, gelatinase, ADH, AMC, and hydrolyze cellobiose but did not produce urease. Only one isolate corresponding to group 12 produced amylase, gelatinase, ADH and hydrolyzed cellobiose but did not produce urease and AMC. Isolate found in group 16 only produced urease.

Table 3.5. Biochemical Characteristics of Isolates

Biotype	Biochemical Groups	No	Isolate Names
Biotype 1	Lecithinase, Salicin Hydrolysis	12	16Q, 167Q2, 25Q, 19Q2, 62Q, 21Q, 290Q2, 58Q2, 290Q1, 41Q3, 41Q, 24Q, *B. thuringiensis* subsp. *aizawai*
Biotype 2	Lecithinase	11	14Q, 54Q1, 65Q, 52Q1, 44Q2, 63Q, 44Q1, 14Q2, 54Q2, 52Q3, 14Q1, *B. thuringiensis* subsp. *alesti*
Biotype 3	Lecithinase, Sucrose Hydrolysis, DNase	8	183Q1, 164Q1, 5Q, 174Q, 164Q, 183Q, 284Q, 284Q1
Biotype 4	Lecithinase, Sucrose Hydrolysis	5	67Q, 50Q2, 90Q1, 50Q3, 50Q1
Biotype 5	Lecithinase, Sucrose, and Salicin Hydrolysis	20	71Q, 37Q4, 55Q2, 57Q1, 53Q2, 59Q3, 71Q1, 53Q3, 74Q, 37Q3, 45Q2, 56Q1, 59Q1, 53Q1, 59Q2, 55Q3, 55Q1, 45Q1, 58Q1, 56Q2
Biotype 6	Esculinase, Lecithinase, Salicin Hydrolysis	9	61Q, 68Q, 37Q2, 69Q, 39Q, 14Q3, 167Q, 39Q1, 69Q1, *B. thuringiensis* subsp. *kurstaki*
Biotype 7	Sucrose Hydrolysis	6	125Q, 17Q, 60Q1, 60Q2, 20Q1, 14Q4, *B. thuringiensis* subsp. *morrisoni*
Biotype 8	Lecithinase, Salicin Hydrolysis, DNase	10	48Q1, 167Q1, 64Q, 161Q5, 161Q3, 161Q4, 161Q2, 58Q3, 41Q2, 43Q
Biotype 9	Lecithinase, Salicin and Mannose Hydrolysis	4	19Q5, 19Q3, 19Q1, 19Q4, *B. thuringiensis* subsp. *thompsoni*

(Cont. on next page)

Table 3.5. Biochemical Characteristics of Isolates (Cont.)

Biotype	Biochemical Characteristics	No	Isolate Names
Biotype 10	Lecithinase, Sucrose,Salicin and Mannose Hydrolysis	3	73Q, 184Q1, 184Q,*B. thuringiensis* subsp. *kumamtoensis*
Biotype 11	Esculinase, Lecithinase, Sucrose and Salicin Hydrolysis	3	64Q1, 37Q1, 75
Biotype 12	Esculinase, Lecithinase, Mannose and Salicin Hydrolysis	1	70Q
Biotype 13	Salicin Hydrolysis, DNase	2	189Q, 169Q
Biotype 14	Sucrose and Mannose Hydrolysis	1	19Q, *B. thuringiensis* biovar. *tenebrionis*
Biotype 15	Sucrose and Mannose Hydrolysis, DNase	1	20Q3
Biotype 16	Esculinase, Sucrose Hydrolysis No growth on egg yolk agar	1	72Q
Biotype 17	Lecithinase, Mannose Hydrolysis	2	36Q1, 36Q2, *B. thuringiensis* subsp. *israelensis*
Biotype 18	Salicin Hydrolysis	1	*B. thuringiensis* subsp. *galleriae*
Biotype 19	Esculinase, Lecithinase, Sucrose and Salicin Hydrolysis, DNase	1	*B. thuringiensis* subsp. *finitimus*

Table 3.6. Subgroups of Biochemical Types of Isolates

Subgroup	Urease	Amylase	Gelatinase	ADH	Cellobiose Hydrolysis	AMC	Isolates
B1	-	+	+	+	+	+	16Q, 21Q, 290Q2, 58Q2
	-	+	+	+	-	+	62Q, 290Q1, 41Q, 24Q
	-	+	-	+	-	-	41Q3
	+	+	+	+	-	+	19Q2
	-	+	+	-	+	+	167Q2
	-	+	+	-	-	+	25Q
B 2	-	+	+	-	-	+	174Q, 284Q, 284Q1
	-	+	+	+	-	+	183Q1, 164Q1, 164Q, 183Q
	-	-	+	-	-	+	5Q
B 3	-	+	+	+	+	+	37Q4, 57Q1, 53Q2, 74Q, 45Q2, 56Q1, 59Q1, 53Q1, 55Q3
	-	+	+	+	-	+	71Q, 55Q2, 59Q3, 71Q1, 53Q3,37Q3, 59Q2, 55Q1, 45Q1,56Q2, 58Q1
B 4	-	+	+	-	-	+	167Q
	-	+	+	+	-	+	68Q, 39Q1
	-	+	+	+	+	-	69Q1
	-	+	+	+	+	+	61Q, 37Q2, 69Q, 39Q, 14Q3

(Con. on next page)

Table 3.6. Subgroups of Biochemical Types of Isolates (Cont.)

Subgroup	Urease	Amylase	Gelatinase	ADH	Cellobiose Hydrolysis	AMC	Isolates
B 7	-	+	+	+	-	+	17Q, 20Q1
	-	+	+	-	-	+	125Q, 60Q1, 60Q2
	-	+	+	+	+	+	14Q4
B 8	-	+	+	+	-	+	64Q, 58Q3, 41Q2, 43Q
	-	+	+	-	-	+	48Q1
	-	+	+	-	+	+	167Q1
	-	-	+	-	+	+	161Q5, 161Q3, 161Q4
	-	-	+	-	-	+	161Q2
B 9	+	+	+	+	-	+	19Q4, 19Q3, 19Q1
	+	+	+	+	+	+	19Q5
B 10	-	+	+	+	-	+	184Q1, 184Q
	-	+	+	+	+	+	73Q
B 13	-	+	+	-	+	+	169Q
	-	+	+	-	-	+	189Q

3.4. Investigation of *cry* Genes

The *cry* gene types of the isolates were determined by PCR using universal primers for *cry1, cry2, cry4, cry9,* and *cry11* and a specific primer for *cry13* gene. The *cry* genotype is defined as the set of *cry* genes found within a given *Bt* isolate (Maduell *et al*. 2002).

After PCR identification, 21 groups of *cry* genes (genotypes) were found (Table 3.7). The most abundant *cry* gene was *cry1*–type genes and present in 68 of the 100 *Bt* isolates (68%). About 20% of the isolates contained *cry2* gene. The gene *cry4* was also present in 57 samples (57%). Twenty-six of the isolates carried *cry9* genes, and only 20 isolates (20%) carried *cry11* genes. None of the isolates appeared to contain *cry13* genes.

The *cry1* genes are the most commonly found in *Bt*, the second most frequent gene family is *cry2* genes, whereas *cry9* genes have the lowest frequency (Wang *et al*. 2003). The results did not directly correlate with these data except the *cry1* genes. The second most frequent gene family was *cry4* genes; third group was *cry9* gene family. The lowest frequency was found for *cry2* and *cry11* genes.

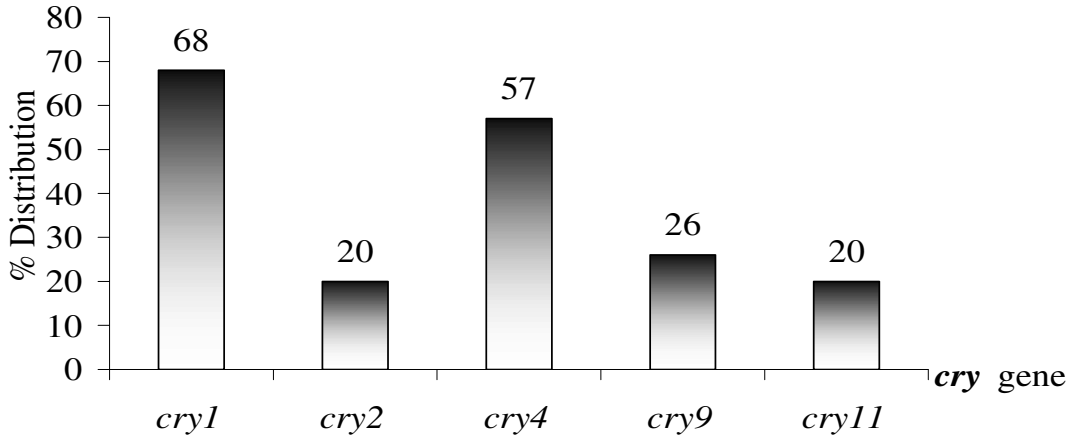

Figure 3.8. *cry* Gene Distribution among Isolates

The number of *cry* gene in isolates varied from one to five genes and 58 out of the 100 isolates possessed more than one type of *cry* gene examined. Ten (10%) strains did not contain any of the genes searched. The isolates with no amplification products may belong to other *cry* gene classes or represent a novel *cry* gene. It was found that the isolates containing the common genes (*cry1* and *cry4*) were isolated from soil samples.

3.4.1. *cry1* Gene Analysis

The *cry1* gene was present in 68 out of the 100 isolates (68%). The presence of *cry1* gene could be considered as an indicator of the Lepidopteran activity.

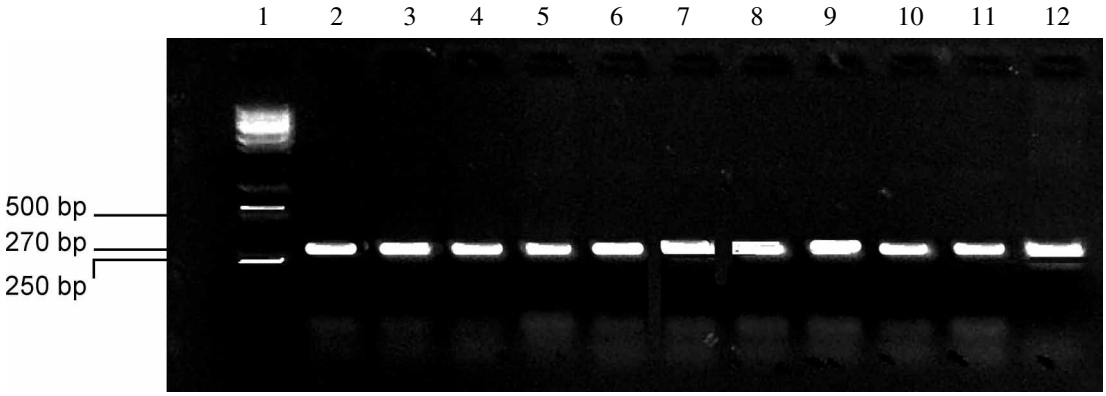

Figure 3.9. *cry1* PCR products of Isolates. **Lanes1.** 1 kb DNA MW marker; **2.** 54Q2; **3.** 53Q1; **4.** 41Q3; **5.** 14Q; **6**. 48Q1; **7**. 59Q2; **8.** 290Q2; **9.** 167Q1; **10.** 53Q2; **11.** 16Q; **12.** *B. thuringiensis* subsp. *kurstaki*

3.4.2. *cry2* Gene Analysis

Twenty isolates (20%) were found to carry *cry2* genes. These isolates may be active against both Lepidopteran and Dipteran insects.

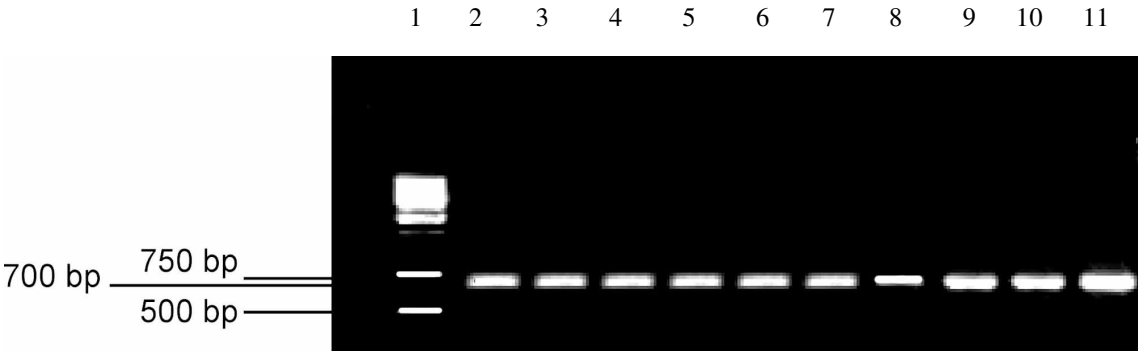

Figure 3.10. *cry2* PCR products of Isolates. **Lanes1.** 1 kb DNA MW marker; **2.** 36Q1; **3.** 53Q1; **4.** 167Q; **5.** 67Q; **6**. 19Q2; **7**. 61Q; **8.** 53Q2; **9.** 39Q; **10.**125Q; **11.** *B. thuringiensis* subsp. *kurstaki*

3.4.3. *cry11* Gene Analysis

Only 20 isolates (20%) seemed to carry *cry11* genes. The Cry11 proteins are known to be effective against only Dipterans. *B. thuringiensis* subsp. *israelensis,* which is being produced as a commercial biopesticide to kill Dipteran insects, also contains *cry11* gene besides other Dipteran-active genes.

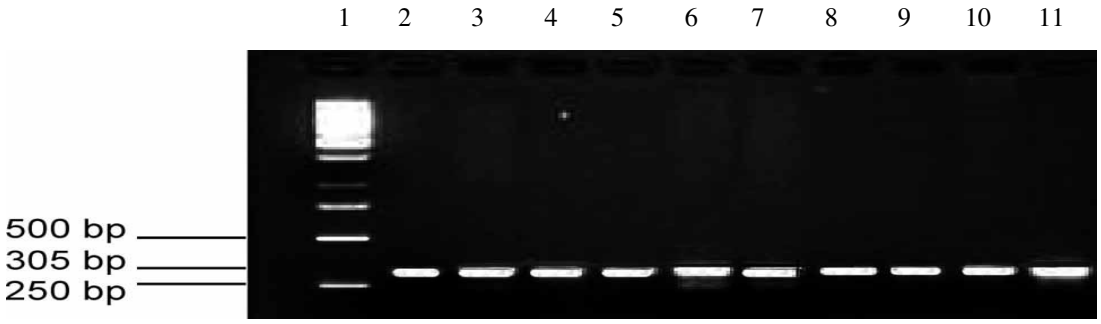

Figure 3.11. *cry11* PCR products of Isolates. **Lanes1.** 1 kb DNA MW marker; **2.** 41Q3; **3.** 58Q3; **4.** 74Q; **5.** 37Q1; **6**. 69Q; **7**. 36Q2; 8**.** 56Q1; **9.** 41Q2; **10.** 14Q; **11.** *B. thuringiensis* subsp. *israelensis*

3.4.4. *Cry4* Gene Analysis

The second abundant *cry* gene was *cry4*-type gene and it was obtained in 57 isolates (57%). Fifty of these isolates also contained cytolytic genes (*cyt1* and *cyt2*). Seventy percent of *cry4*-containing isolates were isolated from soil samples.

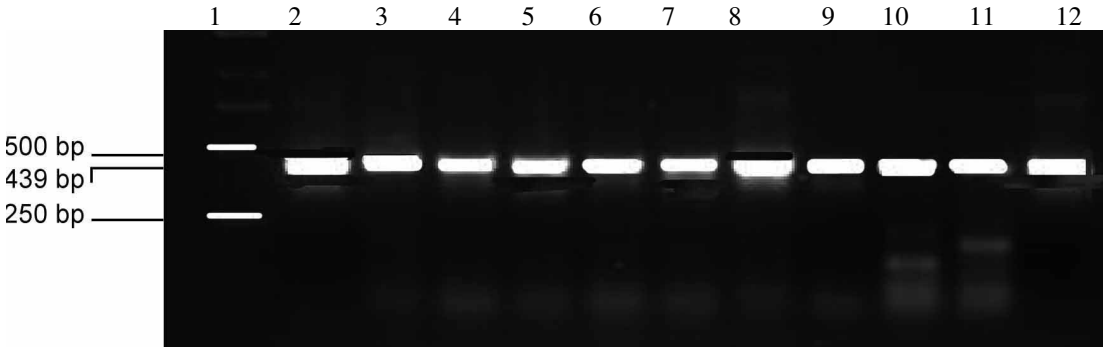

Figure 3.12. *cry4* PCR products of Isolates. **Lanes1.** 1 kb DNA MW marker; **2.** 74Q; **3.** 184Q ; **4.** 58Q3; **5.** 56Q2; **6**. 37Q1; **7**. 41Q2; **8.** 73Q; **9.** 69Q; **10.** 55Q1; **11.** 55Q2; **12.** *B.thuringiensis* subsp. *israelensis*

3.4.5. *Cry9* Gene Analysis

Twenty-six isolates (26%) were positive for *gral-cry9* primers. Seventeen of these isolates were isolated from soil samples collected from olive groves.

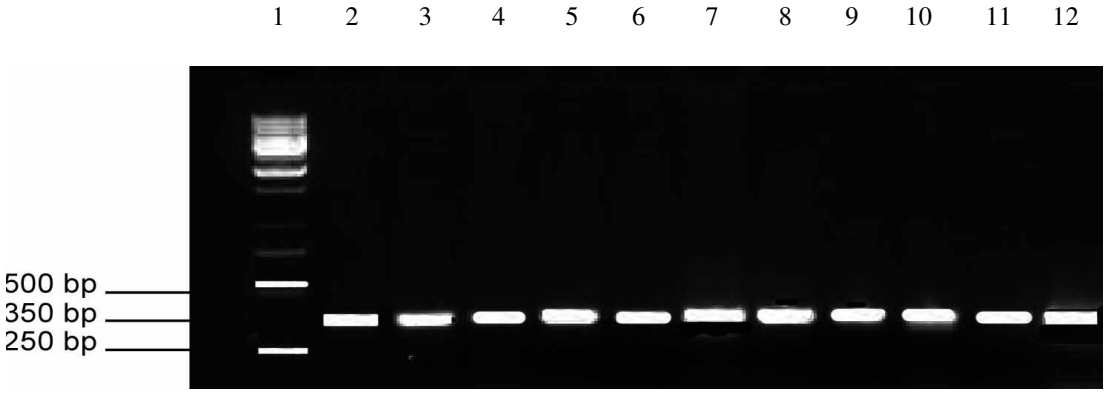

Figure 3.13. *cry9* PCR products of Isolates. **Lanes1.** 1 kb DNA MW marker; **2.** 59Q2; **3.** 41Q3; **4.** 39Q1; **5.** 24Q; **6**. 74Q; **7**.19Q2; **8.** 37Q1; **9.** 56Q2; **10.** 14Q; **11.** 71Q; **12.** *B. thuringiensis* subsp. *aizawai*

3.4.6. *Cry13* Gene Analysis

None of the isolates contained *cry13* genes.

3.5. Prediction of Insecticidal Activity

Predictions of insecticidal activity were made on the basis of the *cry* gene content of the isolates determined by PCR analysis (Table 3.7). Fourteen isolates were predicted to be active only against Lepidopteran, whereas 13 isolates were thought to be toxic to Dipteran. The rest might be active against both Lepidopteran and Dipteran insects. In the insect bioassays, isolates and target insects will be chosen in the lights of these finding.

Table 3.7. *cry* Genotype and Predicted Insecticidal Activity of Isolates by PCR

***cry* genotype**	**Isolate**	**Predicted insecticidal activity**
cry1	58Q2, 50Q3, 14Q1, 43Q, 90Q1, 290Q2, 174Q, 16Q, 54Q1	L
cry2	19Q, 52Q1, 75Q, 60Q2	L&D
cry4	284Q1, 41Q, 69Q1, 68Q, 65Q, 62Q, 37Q3, 37Q2, 44Q2, 14Q3, 183Q1, 164Q2	D
cry1, cry2	36Q1, 284Q, 53Q1, 167Q, 19Q4, 67Q, 19Q3, 19Q1, 19Q5	L&D
cry1, cry4	48Q1, 55Q2, 164Q1, 57Q1, 167Q1, 5Q, 59Q3, 249Q, 70Q, 54Q2, 55Q1, 45Q1, 50Q1, 20Q1, 169Q, 73Q, 63Q, 44Q1, 37Q4, 167Q2, 183Q	L&D
cry1, cry9	59Q2, 55Q3, 20Q3, 184Q1, 71Q	L
cry1, cry11	36Q2, 161Q5, 161Q2	L&D
cry4, cry9	24Q, 71Q1	L&D
cry4, cry11	58Q3	D
cry1, cry4, cry9	52Q3, 39Q1, 56Q1	L&D
cry1, cry2, cry9	19Q2, 61Q	L&D
cry1, cry2, cry11	60Q1	L&D
cry1, cry2, cry4	184Q, 39Q1, 14Q2, 125Q	L&D
cry1, cry4, cry11	56Q1	L&D
cry1, cry9, cry11	161Q3.161Q4	L&D
cry2, cry4, cry11	41Q2	L&D
cry4, cry9, cry11	37Q1, 69Q, 25Q	L&D
cry1, cry4, cry9, cry11	41Q3, 74Q, 14Q, 189Q	L&D
cry2, cry4, cry9, cry11	64Q1	L&D
cry1, cry2, cry4, cry9	53Q2	L&D
cry1, cry2,cry4,cry9,cry11	72Q	L&D

Abbreviations: L: Lepidopteran and D: Dipteran

3.6. *cry* Gene Distribution Frequency

The percentage of the *Bt* isolates containing a *cry* gene among all the isolates from that origin is defined as the distribution frequency of a *cry* gene in *Bt* strains (Wang *et al*. 2003). Table 3.8 represents the *cry* gene distibution frequency.

Table 3.8. *cry* Gene Distribution Frequency

Location	**Total isolate no.**	***cry* gene distribution frequency (%)**				
		cry1	***cry2***	***cry4***	***cry9***	***cry11***
Foça	29	68.96	34.48	51.72	24.13	17.24
Küçükkaya Village	5	80.0	40.0	40.0	-	20.0
Tırazlı Village	6	83.33	-	50.0	33.33	-
Dalbahçe Village	5	60.0	20.0	80.0	60.0	20.0
Irlamaz Village	6	50.0	16.66	66.66	33.33	33.33
Kemalpaşa	17	64.7	23.52	52.94	17.64	5.88
Dikili	12	66.66	25.0	66.66	41.66	25.0
Aliağa	8	37.5	12.5	87.5	25.0	37.5
Olive oil Factories (Bergama)	12	91.66	8.33	50.0	16.66	33.33

3.7. Investigation of *cyt* Genes Content

Table 3.9 represents the *cyt* gene content of the isolates. Also, the gene contents of the isolates in the combinations of *cyt* and *cry* genes were listed in Appendix G.

3.7.1. Investigation of *cyt 1* Gene

It was found that 40 isolates (40%) contained *cyt-1* related genes in addition to other *cry* genes. Among the *cyt1* gene containing isolates, 25 isolates (62.5%) also harbored a *cyt2* gene. Also, 10 reference strains were positive for *cyt1* genes. *cyt1* genes were the most abundant in isolates containing *cry1* and *cry4* genes.

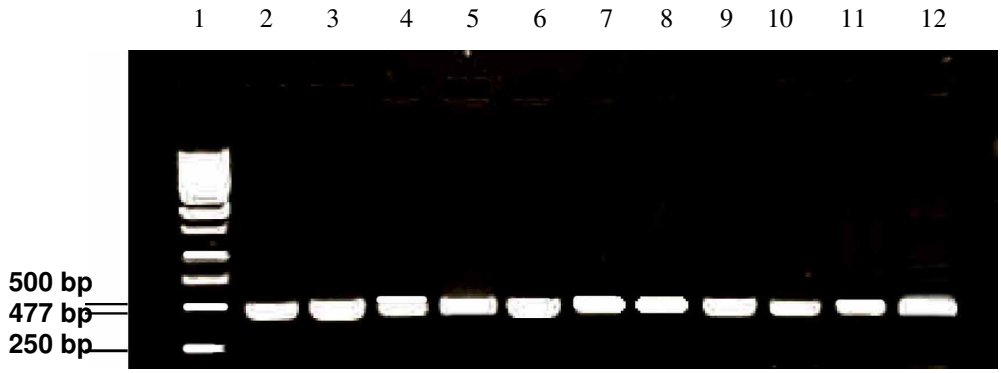

Figure 3.14. *cyt1* PCR products of Isolates. **Lanes1.** 1 kb DNA MW marker; **2.** 59Q3; **3.** 19Q3; **4.** 39Q; **5.** 174Q; **6**. 74Q; **7**.169Q; **8.** 60Q1; **9.** 167Q2; **10.** 17Q; **11.** 70Q; **12.** *B. thuringiensis* subsp. *israelensis*

3.7.2. Investigation of *cyt 2* Gene

PCR amplification results revealed that the most abundant *cyt* gene was *cyt2*. Eighty isolates (80%) produced *cyt-2* fragments with universal primers. Twenty-five of these isolates (31.2%) also contained *cyt1* genes. Most of the *cyt2* genes were found together with *cry1* genes.

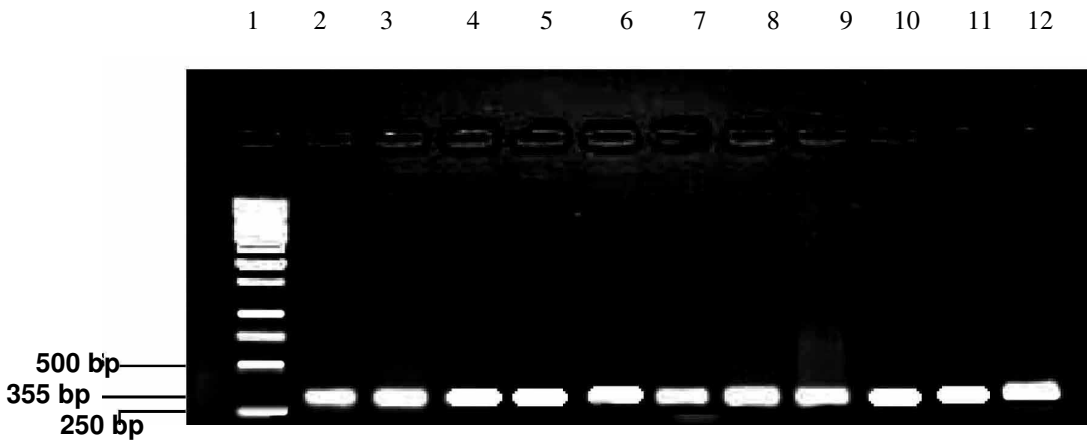

Figure 3.15. *cyt 2* PCR products of Isolates. **Lanes1.** 1 kb DNA MW marker; **2.** 58Q2; **3.** 50Q3; **4.** 59Q1; **5.** 61Q; **6**. 63Q; **7**.19Q2; **8.** 56Q1; **9.** 44Q2; **10.** 14Q4; **11.** 37Q3; **12.** *B. thuringiensis* subsp. *israelensis*

Table 3.9. *cyt* Gene Content of the Isolates

***cyt* gene content**	**Isolate name**
cyt1	70Q, 45Q1, 169Q, 284Q1, 24Q, 74Q, 60Q1, 184Q1, 14Q2, 5Q, 174Q, 167Q2, 183Q, 71Q1, 64Q
cyt2	58Q2, 290Q1, 284Q, 50Q3, 53Q1, 59Q2, 55Q1, 167Q, 50Q1, 20Q1, 14Q1, 19Q, 20Q3, 41Q3, 41Q, 58Q1, 39Q1, 43Q, 19Q4, 184Q, 56Q2, 14Q4, 53Q3, 69Q1, 19Q2, 73Q, 37Q1, 65Q, 37Q3, 52Q1, 44Q2, 21Q, 45Q2, 67Q, 56Q1, 59Q1, 41Q2, 50Q2, 60Q2, 63Q, 19Q1, 44Q1, 90Q1, 290Q2, 14Q3, 14Q, 71Q, 61Q, 55Q2, 164Q2, 189Q, 161Q5, 161Q3, 161Q4, 161Q2
cyt1, cyt2	55Q3,52Q2,68Q,58Q3,62Q,37Q2,69Q,36Q2,39Q,75Q,19Q3,37Q4, 48Q1,164Q1,167Q1,125Q,16Q,53Q2,59Q3,44Q1,19Q5,25Q,72Q, 17Q,64Q1

3.8. Plasmid Profiles of *Bt* Isolates

Plasmid DNA was extracted by the method of O'Sullivan and Haenhammer (1993), their numbers and sizes were determined by gel electrophoresis. Plasmid DNA analysis revealed that the content of plasmids varied from one to eight in number, and from 3.952 kb to 40.0 kb in size. On the other hand, PCR analysis using plasmid DNA as template showed that most of the isolates harbored *cry* and *cyt* genes on their plasmids which were not larger than 40 kb. On some of the plasmids, no amplification products were obtained. This could suggest that the *cry* or *cyt* genes resided in the chromosome.

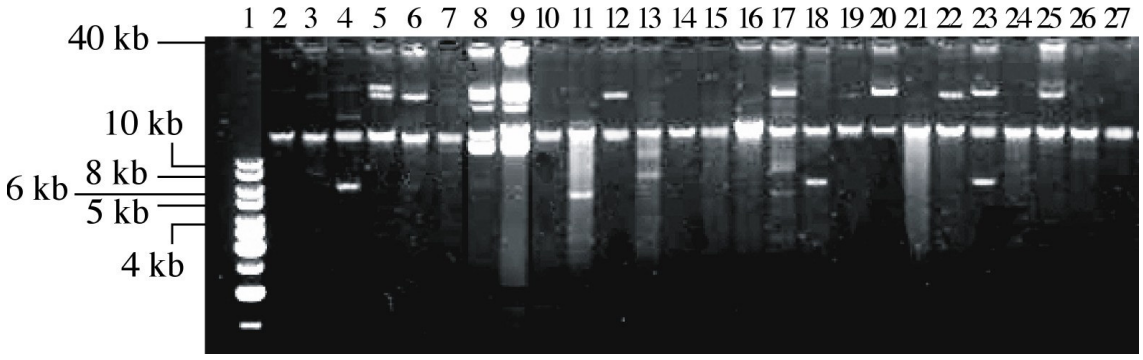

Figure 3.16. Plasmid Profiles of a Set of Isolates by Agarose Gel Electrophoresis.
Lanes1. 1 kb DNA MW marker; **2.** 19Q1; **3.** 70Q ; **4**. 44Q1 ; **5.** 90Q1 **6.** 290Q2 ; **7.** 14Q3 ; **8.** 14Q2 ; **9.** 14Q ; **10.** 183Q1 ; **11.** 71Q ; **12.** 61Q **13.** 37Q4 ; **14.** 48Q1 ; **15.** 56Q1 ; **16.** 59Q1 ; **17.** *B. thuringiensis* subsp. *aizawai* ; **18.** 58Q2 ; **19.** 55Q2 ; **20.** 164Q1 ; **21.** 57Q1 ; **22.** 167Q1 ; **23.** 249Q ; **24.** 54Q2 ; **25.** 53Q2 ; **26.** 55Q1 ; **27**. 5Q

3.9. 16S-ITS rDNA RFLP

3.9.1. Genomic DNA Isolation from *Bt*

Fourteen isolates that showed different biochemical characteristics were chosen and analysed on the basis 16S-ITS rDNA RFLP. First, genomic DNA was isolated by boiling method. The PCR amplification of template DNA resulted in several nonspecific banding products. Cell compounds may inhibit polymerase activity when

lysed cells are used as template (Wilson 1997). Then a new procedure was used for genomic DNA isolation by combining two different methods (Ausubel *et al.* 1994, Cardinal *et al.* 1997). This provided the efficient isolation of pure genomic DNA in sufficient quantity.

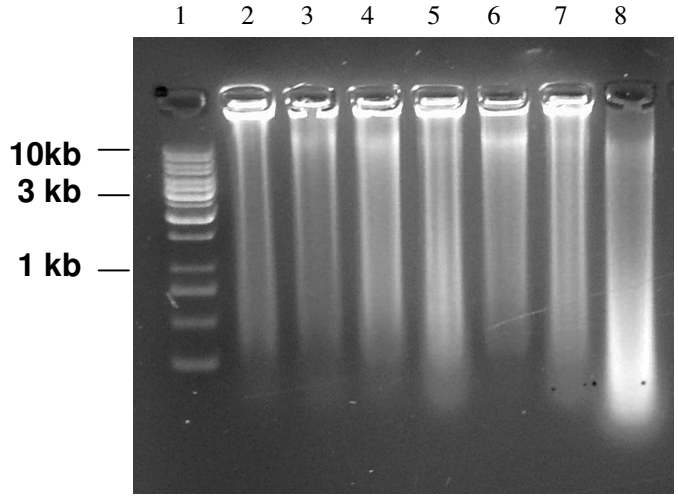

Figure 3.17. Genomic DNA Profiles of a Set of Isolates by Agarorose Gel Electrophoresis **Lanes1.** 1 kb DNA MW marker; **2.** 167Q; **3.** 169Q.**4.** 184Q; **5.** 189Q; **6**. 61Q; **7.** 19Q4; **8.** 37Q1

3.9.2. 16S-ITS rDNA RFLP

After the amplification by using Ege1 and L1 specific primers for 16S-ITS region, four cutter endonucleases, *Taq*I and *Hae*III, were used for the restriction reactions. The amplicon size was approximately 2000 bp (Figure 3.18). The amplicons were digested with either of the enzymes and together.

A long electrophoresis run was applied in order to obtain a better resolution of restriction fragments. *Taq*I digestion resulted in 4 distinct homology groups while profiles created by *Hae*III digestion could not discriminate any of the isolates. When the two enzymes used together, *Taq*I specific profiles were obtained again. Groups obtained by *Taq*I restriction analysis were presented in Table 3.10. Some of the isolates produced different banding patterns from those of reference strains. The gel profiles were analyzed by the program BIO-ID++. Dendogram of reference strains and representative isolates were performed using 12% homology coefficient (Figure 3.20).

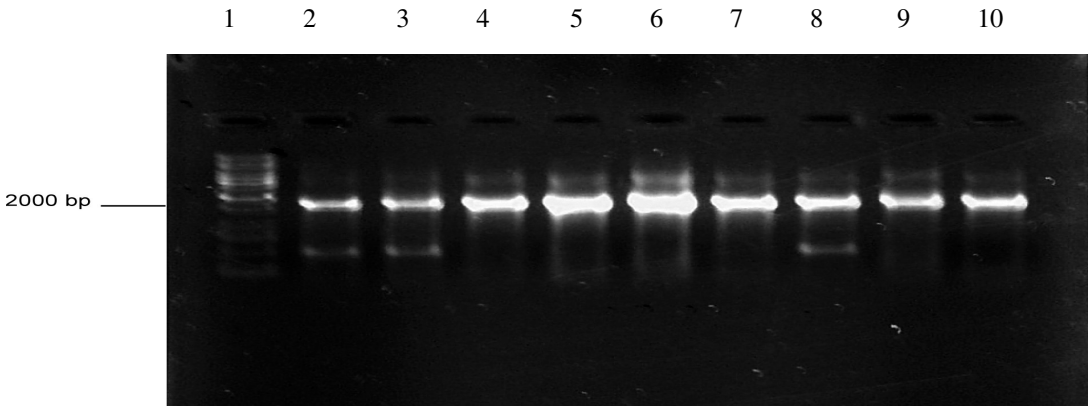

Figure 3.18. 16S-ITS rDNA of isolates. **Lanes1.** 1 kb DNA MW marker; **2.** 167Q; **3.**169Q; **4.** 184Q; **5.** 61Q; **6**. 189Q; **7**. 64Q1; **8.** 54Q2; **9.** 73Q; **10.** *B. thuringiensis* subsp. *thompson*i

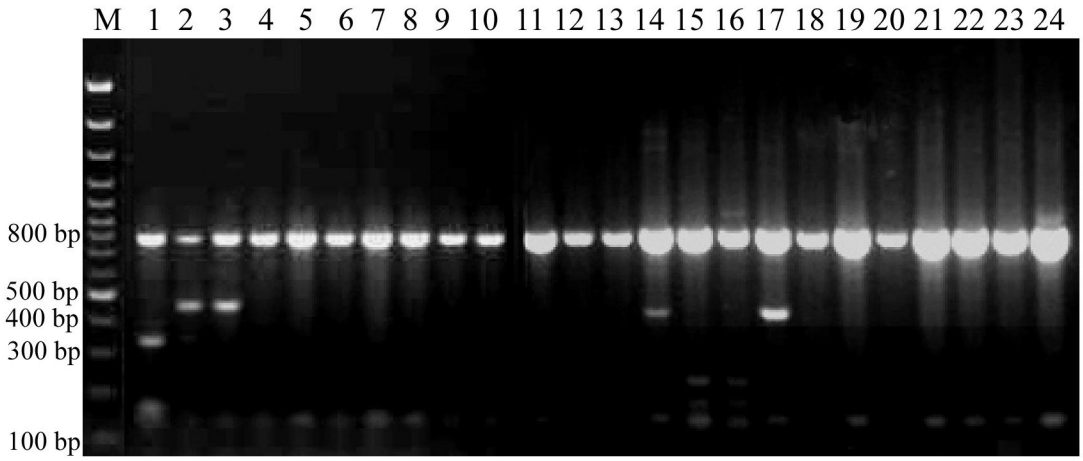

Figure 3.19. *Taq*I Digests of 16S-ITS rDNA Fragments.
Lanes1. 100 bp DNA MW marker; **2.** *B. thuringiensis* subsp. *finitimus*; **3.** *B. thuringiensis* subsp. *israelensis*; **4.** *B. thuringiensis* subsp. *alesti*; **5.** *B. thuringiensis* biovar. *tenebrionis*; **6**. *B. thuringiensis* subsp. *morrisoni*; **7**. *B. thuringiensis* subsp. *kumamtoensis*; **8.** *B. thuringiensis* subsp. *aizawai*; **9.** *B. thuringiensis* subsp. *galleriae*; **10.** *B. thuringiensis* subsp. *kurstaki*; **11.** *B. thuringiensis* subsp. *thompson*i; **12.** 125Q; **13.** 61Q; **14.** 21Q; **15.** 36Q1; **16.** 167Q; **17**. 169Q; **18**. 54Q2; **19.** 20Q3; **20.** 64Q1; **21.** 189Q; **22.** 37Q1; **23**. 184Q; **24**. 73Q; **25**. 19Q4

Table 3.10. Groups Obtained by *Taq*I Restriction Analysis of 16S-ITS rDNA

***Taq*I Genotypic Groups**	**Isolates**
T1	*B. thuringiensis* subsp. *finitimus*
T4	167Q, 169Q
T3	*B. thuringiensis* subsp. *israelensis*, *B. thuringiensis* subsp.*alesti*, 36Q1, 54Q2
T2	*B. thuringiensis* subsp. *aizawai*, *B. thuringiensis* subsp. *thompsoni*, *B. thuringiensis* subsp. *kumamtoensis*, *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *galleriae*, *B. thuringiensis* biovar. *tenebrionis*, *B. thuringiensis* subsp. *morrisoni*, 125Q, 61Q, 21Q, 20Q3, 64Q1, 189Q, 37Q1, 19Q4, 184Q, 73Q

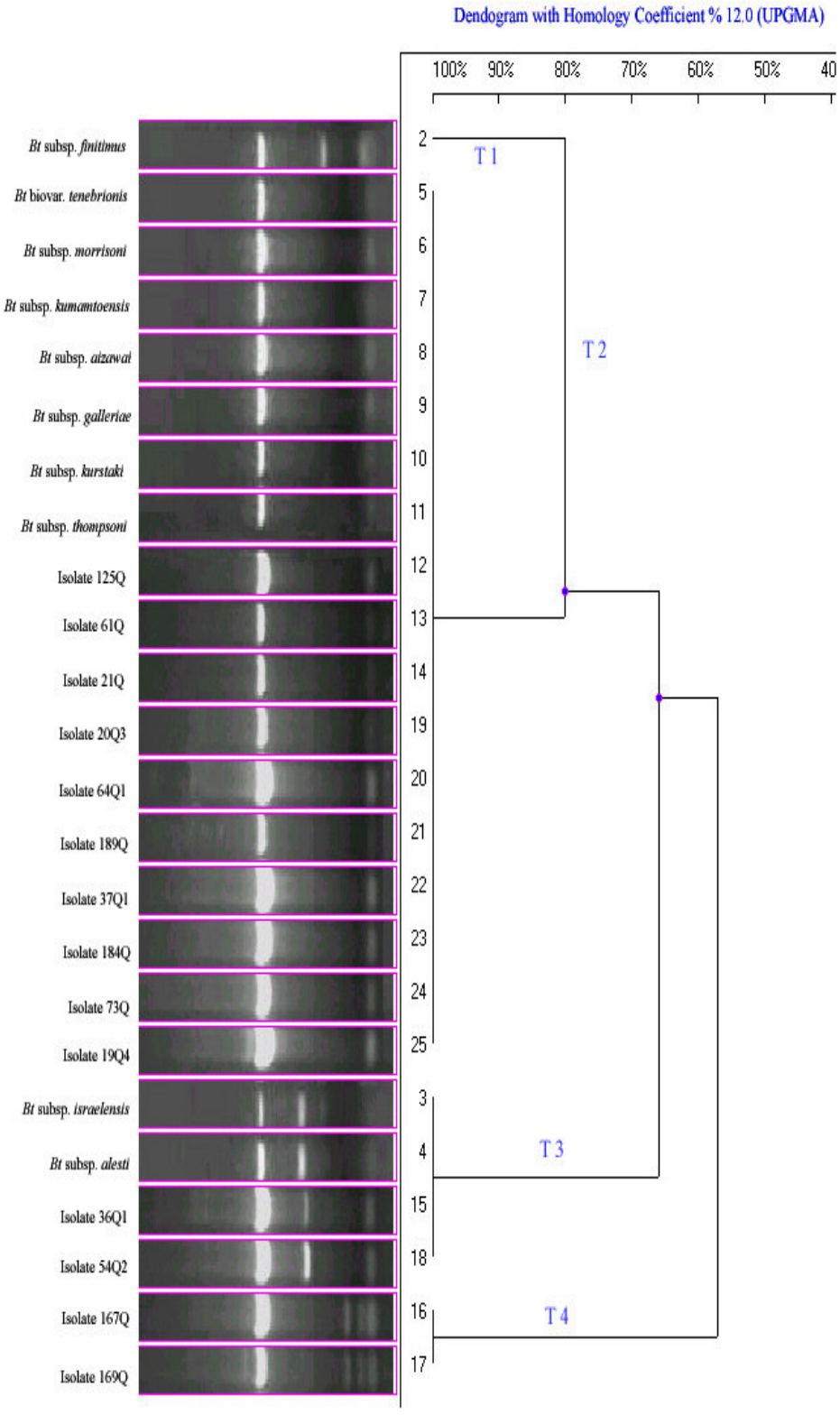

Figure 3.20. Dendogram of Representative *Taq*I RFLP of Isolates and Reference Strains.

3.10. Analysis of the Genotypic and Phenotypic Groups

An analysis was carried out to find out a correlation between genotypic and biochemical groups.

3.10.1. Analysis of the Genotypic and Phenotypic Groups of Reference Strains

B. thuringiensis subsp. *finitimus* was differentiated into genotypic group T1, while both *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *alesti* were clustered into genotypic group T3. The rest of the reference strains belonged to T2 containing most of the representative isolates.

B. thuringiensis subsp. *finitimus* differed from other reference strains in terms of genotypic and biochemical characteristics and constituted a distinct group (Table 3.5 and Table 3.10).

B. thuringiensis subsp. *israelensis* and *B. thuringiensis* subsp. *alesti* belonged to biotype 17 and biotype 2, respectively. These two subspecies showed almost identical biochemical characters except mannose hydrolysis.

Reference strains of T2 were all DNase negative. *B. thuringiensis* subsp. *aizawai* and *B. thuringiensis* subsp. *kurstaki* differed from each other only in esculinase production.

B. thuringiensis subsp. *morrisoni* and *B. thuringiensis* biovar. *tenebrionis* were grouped in distinct biotypes because of the difference in mannose hydrolysis. Besides, they are the subspecies of *morrisoni* but belonged to different serotypes.

B. thuringiensis subsp. *thompsoni* differed from *B. thuringiensis* subsp. *aizawai* because it was able to hydrolyze mannose.

The only difference between *B. thuringiensis* subsp. *kumamtoensis* and *B. thuringiensis* subsp. *thompsoni* was in the ability of sucrose hydrolysis.

B. thuringiensis subsp. *galleriae* could not hydrolyze salicin which replaced this strain into a different biochemical group and differentiated from *B. thuringiensis* subsp. *thompsoni.*

3.10.2. Analysis of the Genotypic and Phenotypic Groups of Isolates

Isolates within the same genotypic groups showed different phenotypic profiles. Two genotypic groups T2 and T4 displayed approximately 66% similarity level by *Taq*I. Isolates belonging to the same biotype were in different genotypic groups. Also isolates in the same genotypic group showed distinct biochemical characters. For example, strains 61Q and 167Q were clustered into T2 and T4, respectively but they found in the same biochemical group. These strains might be different.

Similarly, 169Q and 189Q could be distinct showing the same biochemical profiles, they were classified into different genotypic groups.

CHAPTER 4

CONCLUSION AND FUTURE EXPERIMENTS

Bt is a Gram-positive, spore-forming soil bacterium that produces insecticidal crystal proteins during sporulation. *Bt* is the most widely used microbial control agent all over the world. Biological pesticides based on *Bt* are becoming increasingly important in pest management programs, accounting for 80-90% of all biological pest control agents worldwide and 2% of all insecticides used. Screening samples from different sources and habitats may be useful to obtain *Bt* strains with broader host ranges and novel crystal proteins.

A total of 54 samples out of 240 samples collected from olive tree-related habitats yielded *Bt*. A hundred isolates were obtained by using two different isolation methods; skaken-flask and sodium acetate-selection methods. Most isolates were derived from soil samples collected from olive groves. Out of 100 isolates, 63 (63%) isolates were obtained from soil. The result of this study showed that *Bt* can be recovered fom olive tree-related habitats.

In the crystal protein morphology, the spherical inclusions were the most frequent crystals in the isolates. The results of esculin, salicin, sucrose, and mannose hydrolysis, also lecithinase and DNase production were used to divide isolates and reference strains into different biochemical types. Nighteen biochemical groups were obtained. According to the biotyping, most isolates belonged to the biochemical type5 (sucrose and salicin hydrolyzers and lecithinase producers). The other tests of biochemical characterization except MR reaction were used to subdivide biotypes into different groups. The *cry* and *cyt* gene composition of the isolates was determined by using universal primers for *cry1*, *cry2*, *cry4*, *cry9*, *cry11*, *cyt1*, *cyt2*, and a specific primer for *cry13* gene. 68% of the isolates amplified *cry1* gene; 57% amplified *cry4*; 20% amplified *cry11*; 26% amplified *cry9*; 20% amplified *cry2* genes, and none of the isolates harbored *cry13* gene. *Cyt1* gene was found in 40% of the isolates while *cyt2* gene was present in 80% of the isolates. The most abundant genotype of *cry* genes was *cry1* and *cry4*. Most of the isolates (58%) possessed more than one *cry* gene. *Bt* biopesticides containing multiple endotoxins that interact with different membrane receptors may eliminate resistant insect populations (Chambers *et al*. 1991). A new

method, 16S-ITS rDNA RFLP was used to find out a correlation between the biotypes and RFLP profiles. For RFLP analysis, 16S rDNA and ITS regions were used as a single amplicon. The use of *Taq*I restriction enzyme provided the division of representative isolates and reference strains into groups.

The synergistic effect of extracellular DNase produced by isolates on the activation of Cry proteins may be examined by toxicity tests. Amylase-negative *B. cereus* strains have been reported to produce emetic toxins (Kramer and Gilbert 1989, Agata *et al.*1996). A correlation between the production of amylase and emetic toxins may be searched in the isolates.

In light of the PCR results**,** most isolates could be active against both Lepidopteran and Dipteran insects. However, bioactivity tests should be performed to find out active strains against different insects. First, isolates will be examined against the Mediterranean fruit fly (Diptera: Tephritidae) because of the ease of propagation in the laboratory. This insect also causes severe damage to many fruit species in the Mediterranean countries. Serological studies should also be carried out using antigens of known serotypes to identify new serotypes. Other universal and specific primers should be used to identify other possible *cry* and *cyt* genes. The presence of enterotoxin and β-exotoxin encoding genes may be determined by PCR analysis to identify strains that are not harmful to humans. This approach may be useful for the determination of strains that will be produced in large scale as biopesticides.

REFERENCES

Adang, M.J. 1991. "*Bacillus thuringiensis* Insecticidal Crystal Proteins: Gene Structure, Action, and Utilization", in *Biotechnology for Biological Contol of Pests and Vectors*, edited by K. Maramorosch (CRC Press, Boston), pp.3-24.

Adang, M.J., Brody, M.S., Cardineau, G., Roush, R.t., Shewmaker, C.K., Jones, A., Oakes, J.V. and McBride, K.E. 1993. "The Reconstruction and Expression of a *Bacillus thuringiensis cryIIA* Gene in Protoplasts and Potato Plants," *Plant. Mol. Biology*. Vol.21, pp. 1131-1145.

Agaisse, H. and Lereclus, D. 1995. "How Does *Bacillus thuringiensis* Produce So Much Insecticidal Crystal Protein?,"*Journal of Bacteriology*. Vol.177, pp. 6027-6032.

Akhurst, R.J., Lyness, E.W., Zhang, Q.Y., Cooper, D.J. and Pinnock, D.E. 1997. "A 16S rRNA Gene Oligonucleotide Probe for Identification of *Bacillus* Isolates," *Journal of Invertebrate Pathology*. Vol. 69, pp. 24-30.

Akiba, Y. 1986. "Microbial Ecology of *Bacillus thuringiensis.* IV. Germination of *Bacillus thuringiensis* Spores in the Soil," *Appl. Ent. Zoology*. Vol. 21, pp. 76-80.

Alcaide, E.M. and Nefzaoui, A. 1996. "Recycling of Olive Oil by-products: Possibilities of Utilization in Animal Nutrition," *Int. Biodeterior. Biodegradation*. pp. 227-235.

Amann, R., Ludwig, W., Schwippl, I., Beisker, W., Wallner, G. and Fuchs, B.M. 1998. "Flow Cytometric Analysis of the in situ Accessibility of *E.coli* 16S rRNA for Fluorescently Labelled Oligonucleotide Probes," *Applied and Environmental Microbiology*. Vol. 64, pp. 4973-4982.

Ammons, D. and Rampersad, J. 2002. "Usefulness of Staining Parasporal Bodies When Screening for *Bacillus thuringiensis,*" *Journal of Invertebrate Pathology*. Vol. 79, pp. 203-204.

Anagnou-Veroniki, M. 1996. “Pathogenesis of Three Virus Types on the Olive Fruit Family *Bactrocera* (*Dacus oleae*),” *Hellenic Virology*. Vol. 1, pp. 42-45.

Angst, B.D., Marozzi, C. and Magee, A.J. 2001. “The Cadherin-superfamily,”*Journal of Cellular Science*. Vol. 114, pp. 625-626.

Arambourg, Y. 1986. **“**Traite d'Entomologie Oleicole”, Conseil Oleicole International, Madrid, pp. 1-360.

Aronson, A. I., Beckmann, W. and Dunn, P. 1986. “ *Bacillus thuringiensis* and Related Insect Pathogens,” *Microbiological Reviews*. Vol. 50, pp.1-24.

Asano, S.-I., Yamashita, C., Iuzika, T., Takeuchi, K., Yamanaka, S., Cerf, D. and Yamamoto, T. 2003. “A Strain *Bacillus thuringiensis* subsp. *galleriae* Containing a Novel *cry8* Gene Highly Toxic to *Anomala cuprea* (Coleoptera: Scarabaeidae),” *Biological Control*. Vol. 28, pp. 191-196.

Bardin, P.G., Van Eeden, S.F., Moolman, J.A., Foden, A.P. and Joubert, J.R. 1994. “Organophosphate and Carbamate Poisoning,” *Arch. Intern. Med*. Vol. 154, pp. 1433-1444.

Barkay, T., Chatteragee, D., Cuskey, S., Watter, R., Genthren, F. and Bourquin, A. 1989. “Bacteria and the Environment”, in *A Revolution in Biotechnology*, edited by J.L. Marx (Cambridge University Press, New York), pp. 94-102.

Baum, J.A. and Malvar, T. 1995. “ Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*,” *Mol. Microbiology*. Vol. 18, pp.1-12.

Baum, J.A., Johnson, T.B. and Carlton, B.C. 1999. “*Bacillus thuringiensis*: Natural and Recombinant Biopesticide Products”, in *Biopesticides: Use and Delivery*, edited by F.R. Hall and J.J. Menn (Humana Press, Totowa), pp. 189-210.

Baumann, I., Okamoto, K., Unterman, M.B., Lynch, J.M. and Baumann, P. 1984. "Phenotypic Characterization of *Bacillus thuringiensis* and *Bacillus cereus*," *Journal of Invertebrate Pathology*. Vol. 44, pp. 329-341.

Ben-Dov, E., Zaritsky, A., Dahan, E., Barak, Z., Sınal, R., Manasherob, R., Khamraev, A., Troitskaya, E., Dubitsky, A., Berezina, N. and Margalith, Y. 1997. "Extended Screening by PCR for Seven *cry*-group Genes from Field- collected Strains of *Bacillus thuringiensis*,"*Applied and Environmental Microbiology.* Vol. 63, pp. 4883- 4890.

Bjurlin, M.A., Bloomer, S. and Nelson, C.J. 2002. "Characterization of Proteolytic Activity of Proteases,"*Biotechnology Letters*. Vol. 24, pp. 191-195.

Bohorova, N., Cabrera, M., Abarca, C., Quintero, R., Maciel, A.M., Brito, R.M., Hoisington, D. and Bravo, A. 1997. "Suspectibility of Four Tropical Lepidopteran Maize Pests to *Bacillus thuringiensis* CryI-type Insecticidal Toxins,"*Journal of Economic Entomology*. Vol. 90, pp. 412-415.

Bonnefoi, A. and Beguin, S. 1959. "Resherces sur l'action des cristaux de *Bacillus thuringiensis* Souche,"Entomophaga. Vol. 4, pp. 193-199.

Borovsky, D.1986." Proteolytic Enzymes and Blood Digestion in the Mosquito, *Culex nigripalpus*,"*Arch. Insect Biochem. Physiology*. Vol. 3, pp.147-160.

Borror, D.J., Triplehorn, C.A. and Johnson, N.F. 1989. "An introduction to the Study of Insects", (Saunders College Publishing, Newyork)

Bravo, A., Sarabia, S., Lopez, L., Ontiveros, H., Abarca, C., Ortiz, A., Ortiz, M., Lina, L., Villalobos, V., Pena, G., Nunez-Valdez, M., Soberon, M. and Quintero, R. 1998. "Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain Collection,"*Applied and Environmental Microbiology.* Vol. 64, No.2, pp. 4965-4972.

Burton, S.L., Ellar, D.J., Li, J. and Derbyshire, D.J. 1999. "N-acetylgalactosamine on the Putative Insect Receptor Aminopeptidase N is Recognised by A Site on the Domain III Lectin-like Fold of A *Bacillus thuringiensis* Insecticidal Toxin,"*Journal of Molecular biology*. Vol. 287, pp. 1011-1022.

Butko, P., Huang, F., Pusztai-Carey, M. and Surewicz, K.W. 1996. "Interaction of the Delta-endotoxin CytA from *Bacillus thuringiensis* var. *israelensis* with Lipid Membranes,"*Biochemistry*. Vol. 36, pp. 12862-12868.

Butko, P., Huang, F., Pusztai-Carey, M. and Surewicz, K.W. 1997. "Membrane Permeabilization Induced by Cytolytic Delta-endotoxin CytA from *Bacillus thuringiensis* var. *israelensis* With Lipid Membranes,"*Biochemistry*. Vol. 35, pp. 11355-11360.

Cabras. P., Anglont, A., Mehs, M., Minelli, E.V. and Pirisi, F.M. 1997. "Simplified Multiresidue Method for the Determination of Organophosphorus Insecticides in Olive Oil,"*Journal of Chromatography*. Vol. 761, pp. 327-331.

Cantwell, G.E., Dougherty, E. and Cantelo, W.W. 1983. "Activity of the β-exotoxin of *Bacillus thuringiensis* var. *thuringiensis* Against the Colorado Potato Beetle and the Ames Test," *Environ. Entomology*. Vol. 12, No.5, pp. 1424-1427.

Carlton, B.C. and Gonzalez, J.M. 1985. "The Genetics and Molecular Biology of *Bacillus thuringiensis*", in *The Molecular Biology of the Bacilli*, edited by D.A. Dubnau (Academic Pres, New York), pp. 211-249.

Carozzi, N.B., Kramer, V.C., Warren, G.W., Evola, S. and Koziel, G.M. 1991. "Prediction of Insecticidal Activity of *Bacillus thuringiensis* Strains by Polymerase Chain Reaction Product Profiles,"*Applied and Environmental* Microbiology. Vol. 353, pp. 3057-3061.

Charles, J.F., Delécluse A. and Nielsen-LeRoux, C. 2000. *Entomopathogenic bacteria*: *From laboratory to field application*, (Kluwer Academic), pp. 524.

Chen, M.L. and Tsen, H.Y. 2002. "Discrimination of *Bacillus cereus* and *Bacillus thuringiensis* with 16S rRNA and *gyrB* Gene Based PCR Primers and Sequencing of Their Annealing Sites,"*Journal of Applied Microbiology*. Vol. 92, pp. 912-919.

Cheriff, A., Ouzari, H., Daffonchio, D., Cherif, H., Ben Slama, K., Hassen, A. and Jaoua, S. 2001. "Thuricin 7: A Novel Bacteriocin Produced by *Bacillus thuringiensis* BMG1.7, A New Strain Isolated from Soil,"*Letters of Applied Microbiology*. Vol.32, pp. 243-247.

Choma, C.T., Surewickz, W.K., Carey, P.R., Raynor, M. and Kaplan, T., 1990. "Unusual Proteolysis of the Protoxin and Toxin from *Bacillus thuringiensis*,"Struct. *Implicat*. Vol. 189, pp. 523-527.

Cirio, U. 1997. "Agrichemicals and Environmental Impact in Olive Farming,"*Olivae*. Vol. 65, pp. 32-39.

Clairmont, F.R., Milne, R.E., Pham, V.T., Carriere, M.B. and Kaplan, H. 1998. "Role of DNA in the Activation of the Cry1A Insecticidal Crystal Protein from *Bacillus thuringiensis*," *J. Biol. Chemistry*. Vol. 273, pp. 9292-9296.

Collins, C.H., Lyne, P.M. and Grange, J.M. 1995. *Collins and Lyne's Microbiological Methods*, (Oxford University Press, London).

Copping, L.G.1998. *The Biopesticide Manual*, (The British Crop Protection Council, UK).

Crickmore, N., Bone, E.J., Williams, J.A. and Ellar, D.J. 1995. "Contribution of the Individual Components of the δ-endotoxin Crystal to the Mosquitocidal Activity of *Bacillus thuringiensis* subsp. *israelensis*,"*FEMS Microbiol. Letters*. Vol. 131, pp. 249-254.

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van-Rie, J., Lereclus, D., Baum, J. and Dean, D.H. 1998. "Revision of Nomenclature for the *Bacillus thuringiensis* Pesticidal Crystal Proteins,"*Microbiology and Molecular Biology Reviews*. Vol. 62, pp. 807-813.

Crickmore, N. 2000. "The Diversity of *Bacillus thuringiensis* δ-endotoxins", in *Entomopathogenic Bacteria: From laboratory to field application*, edited by J.F. Charles, A. Delécluse, and C. Nielsen-LeRoux (Kluwer Academic, Dordrecht), pp. 65-79.

Daffonchio, D., Borin, S., Consolandi, Mora, D., Manachini, P.L. and Sorlini, C. 1998. "16S–23S rRNA Internal Transcribed Spacers as Molecular Markers for the Species of the 16S rRNA Group 1 of the Genus *Bacillus*," *FEMS Microbiology Letters*. Vol. 163, pp. 229-236.

Dai, S-M. and Gill, S. 1993. "*In vitro* and *in vivo* Proteolysis of the *Bacillus thuringiensis* subsp. *israelensis* CryIVD Protein by *Culex quinquefasciatus* Larval Midgut Proteases,"*Insect Biochem. Mol. Biology*. Vol. 23, pp. 273-283.

Damgaard, P.H. 1995. "Diarrhoeal Enterotoxin Production by Strains of *Bacillus thuringiensis* Isolated from Commercial *Bacillus thuringiensis*-based Insecticides,"*FEMS Immunol. Med. Microbiology*. Vol.12, pp. 245-250.

Davidson, E.W. 1992. "Development of Insect Resistance to Biopesticides,"*Pesqui. Agropecu. Bras*. Vol. 27, pp. 47-57.

de Barjac, H. 1981. "Identification of H-serotypes of *Bacillus thuringiensis*," in *Microbial control of pests and plant diseases*, edited by H.D. Burges (London:Academic Press), pp.35-43

Delrio, G. 1992. "Integrated control in olive groves", Biological Control and Integrated Crop Protection: Towards Environmentally Dafer Agriculture, Proc. Int. Conf. IOBC/WPRS, Veldhohen, Netherlands (8-13 September, 1991), pp. 67-76.

DeLucca, A.J., Simonson, J.G. and Larson, A.D. 1981. "*Bacillus thuringiensis* Distibution in Soils of the United States,"Can. J. *Microbiology*. Vol. 27, pp. 865-870.

DeLucca, AJ II., Palmgran, MS. and Ciegler, A. 1982. "*Bacillus thuringiensis* in Grain Elevator Dusts,"*Can. J. Microbiology*. Vol. 28, pp. 452-456.

De Maagd, R.A., Bravo, A. and Crickmore, N. 2001. "How *Bacillus thuringiensis* has Evolved Specific Toxins to Colonize the Insect World," *Trends in Genetics,* Vol.17**,** pp**.**193-199.

Des Rosier, J.P. and Lara, J.C. 1981. "Isolation and Properties of Pili from Spores of *Bacillus cereus*,"*Journal of Bacteriology*. Vol. 145, pp. 613-619.

Dich, J., Zahm, S.H., Hanberg, A. and Adami, H., 1997. "Pesticides and Cancer,"*Cancer Causes and Control*, Vol. 8, pp. 420-443.

Dominguez, E. 1993. "Dipteros: La mosca del olivo *Dacus oleae* Rossi", in *Plagas y enfermedades de las plantas cultivadas*, (Mundi-Prensa, Madrid), pp. 679-685.

Du, J., Knowles, B.H., Li, J. and Ellar, D.J., 1999. "Biochemical Characterization of *Bacillus thuringiensis* Cytolytic Toxins in Association with Phospholipids Bilayer," *Biochemical Journal*. Vol. 338, pp. 185-193.

Dugo, G., Di Bella, G., La Torre, L. and Saitta, M. 2005. "Rapid GC-FPD Determination of Organophosphorus Pesticides Residues in Sicilian and Apulian Olive Oil,"Food Control. Vol. 16, pp. 435–438.

Dulmage, H.T. 1981. "Insecticidal Activity of Strains of *Bacillus thuringiensis* and Their Potential for Pest Control", in *Microbial Control of Pests and Plant Diseases*, edited by H.D. Burges (Academic Press, London), pp. 193-222.

Ellar, D.J. 1997. "The Structure and Function of *Bacillus thuringiensis* δ-endotoxins and Prospects for Biopesticide Improvement", Microbial Insecticides: Novelty or Necessity?, Br. Crop Protect. Corp. Symp. Proc., Univ. Of Warwick, Coventry (16-18 April 1997), No.68, pp. 83-100.

Ellis, R.T., Stockhoff, B.A., Stamp, L., Schnepf, H.E., Schwab, G.E., Knuth, M., Russell, J., Cardineau, G.A. and Narva, K.E. 2002. "Novel *Bacillus thuringiensis* Binary Insecticidal Crystal Proteins Active on Western Corn Rootworm, *Diabrotica virgifera virgifera* Le Conte," *Applied and Environvironmental Microbiol*ogy. Vol. 68, pp. 1137-1145.

Entwistle, P.F., Lory, J.S., Baily, M.J. and Higgs, S. 1993. "*Bacillus thuringiensis*, An Environmental Biopesticide", (John Wiley, New York).

EPA, 1988. "Guidance for the Registration of Pesticide Products Containing *Bacillus thuringiensis* as the Active Ingredient", (Registration Standard 540/RS-89-023, December, 1998).

EPA, 1998. "*Bacillus thuringiensis*", RED Facts, EPA-738-F-98-001.

Estruch, J., Warren, G.W., Mullins, M.A., Nye, G.J., Craig, J.A. and Koziel, H.G. 1996. "Vip3A, A Novel *Bacillus thuringiensis* Vegetative Insecticidal Protein with A Wide Spectrum of Activities Against Lepidopteran Insects", *Proc. Natl. Acad. Sci. USA*, Vol. 93, pp. 5389-5394.

Farkas, J., Sebesta, K., Horská, Samek, K., Dolejs, L. and Sorm, F. 1969. "The Structure of Exotoxin *Bacillus thuringiensis* var. *gelechiae*," *Collect. Czech. Chem. Commun*. Vol. 34, pp. 1118-1120.

Favret, M. and Younsten, A. 1989. "Thuricin: the Bacteriocin Produced by *B. thuringiensis*," *Journal of Invertebrebrate Pathology*. Vol. 53, pp. 206-216.

Feitelson, J.S. 1993. "The *Bacillus thuringiensis* Family Tree", in *Advanced Engineered Pesticides*, edited by L. Kim, (Marcel Dekker, Inc., New York), pp. 63-72.

Fimiani, P., 1989. "Mediterranean Region", in *World crop pests, fruit flies, their biology, natural enemies and control*, edited by A. Robinson, and G. Hooper, (Elsevier, Amsterdam), pp. 39-50.

Fischhoff, D.A., Bowdisch, K.S., Perlak, F.J., Marrone, P.G., McCormick, S.H., Nieder, J.G., Dean, D.A., Kusano-Kretzmer, K., Mayer, E.J., Rochester, D.E., Rogers, S.G. and Fraley, R.T., 1987. "Insect tolerant transgenic tomato plants,"*Bio/Technology*. Vol. 5, pp. 807-813.

Flexner, J.L. and Belnavis, D.L. 1999. "Microbial insecticides", in *Biological and Biotechnological Control of Insect Pests*, edited by J.E. Rechcigland N.A. Rechcigl (Lewis Publishers), pp. 35-62

Gill, S.S., Cowles, E.A. and Pietrantonio, P.V. 1992. "The mode of action of *Bacillus thuringiensis* endotoxins", *Annu. Rev. Entomology*. Vol. 37, pp. 615-636.

Glare, T.R. and O'Callaghan M. 2000."*Bacillus thuringiensis*", in *Biology, Ecology and Safety* (John Wiley, Chichester), p. 423

González, J.M., JR., Dulmage, T.H. and Carlton, C.B. 1981. "Correlation between Specific Plasmids and δ-endotoxin Production in *Bacillus thuringiensis*,"*Plasmid*. Vol. 5, pp. 351-365.

González, J.M., Jr., Brown, J.B. and Carlton, C.B. 1982. "Transfer of *Bacillus thuringiensis* Plasmids Coding for δ-endotoxin Among Strains of *B.thuringiensis* and *B.cereus*," *Proc. Natl. Acad. Sci. USA*, Vol. 79, pp. 6951-6955.

Gordon, R., Haynes, W. and Pang, C. 1973. "The Genus *Bacillus*", in *US Dept. of Agricultural Handbook*, No.427.

Grochulski, P., Mason, L., Borisova, S., Puzstai-Carey, M., Schwartz, J.-L., Brousseau, R. and Cygler, M. 1995. "*Bacillus thuringiensis* CryIA(a) Insecticidal Toxin: Crystal Structure and Channel Formation,"*Journal of Molecular Biology*. Vol. 254, pp. 447-464.

Guerchicoff, A., Delecluse, A. and Rubinstein, CP., 2001. "The *Bacillus thuringiensis cyt* Genes for Haemolytic Endotoxins Constitute A Gene Family," *Applied and Environmental Microbiology*. Vol. 67, pp. 1090-1096.

Gurbuz, S., Kiran-Ciliz, N. and Yenigun, O. 2004. "Cleaner Production Implementation through Process Modifications for Selected SMEs in Turkish Olive Oil Production," *Journal of Cleaner Production*. Vol.12, pp. 613-621.

Gürtler, V. and Stanisich, V.A. 1996. "New Approaches to Typing and Identification of Bacteria Using 16S-23S rDNA Spacer Region," *Microbiology*. Vol. 142, pp. 3-16.

Gürtler, V. 1999. "The Role of Recombination and Mutation in 16S-23S rDNA Spacer Rearrangement,"*Gene*. Vol. 238, pp. 241-252.

Hansen, B.M., Damgaard, P.H., Eilenberg, J. and Pedersen, J.C. 1998. "Molecular and Phenotypic Characterization of *Bacillus thuringiensis* Isolated from Leaves and Insects,"*Journal of Invertebrate Pathology*. Vol. 71, pp. 106-114

Heim, G. 1984. "Effect of Insecticidal Sprays on Predators and Indifferent Arthropods Found on Olive Trees in the North of Lebanon", in *Integrated Pest Control in Olive Groves*, edited by R. Cavalloro and A. Crovetti (A.A. Balkema, Rotterdam), pp. 456-465.

Heimpel, A.M. 1954. "Investigations of the Mode of Action of Strains of *Bacillus cereus* Frankland and Frankland Pathogenic for the Larch Sawfly, Doctoral dissertion, (Queen's University, Canada),

Heimpel, A.M. 1967. "A Critical Review of *Bacillus thuringiensis* var. *thuringiensis* Berliner and Other Crystalliferous Bacteria," *Annual Review of Entomology*. Vol. 12, pp. 287-322.

Helgason, E., Okstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A. and Mock M. 2000. "*Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis*-one species on the Basis of Genetic Evidence," *Applied and Environmental Microbiol*ogy. Vol.66, pp. 2627-2630.

Higuchi, K., Saitoh, H., Mizuki, E. and Ohba, M. 1998. "Similarity in Moth-fly Specific Larvicidal Activity between to Serologically Unrelated *Bacillus thuringiensis* Strains," *FEMS Microbiology Letters*. Vol. 169, pp. 213-218.

Höfte, H. and Whiteley, H.R. 1989. "Insecticidal Crystal Proteins of *Bacillus thuringiensis*," *MicrobiologyReviews*. Vol. 53, pp. 242-255.

Ichimatsu, T., Mizuki, E., Nishimura, K., Higuchi, K., Saitoh, H. and Ohba, M. 1998. " *Bacillus thuringiensis* Populations Naturally Occurring in Fresh Waters in Japan", Abstracts of the VIIth International Colloquium on Invertebrate Pathology and Microbial Control, Sapporo, (1998), No.43.

Jensen, M.A., Webster, J.A. and Straus, N. 1993. "Rapid Identification of Bacteria on the basis of Polymerase Chain Reaction-amplified Ribosomal DNA," *Applied and Environmental Microbiology*. Vol. 59, pp. 945-952.

Johnson, C. and Bishop, A.H. 1996. "A technique for the Enrichment and Isolation of *Bacillus thuringiensis*," *FEMS Microbiology Letters*. Vol. 142, pp. 173-177.

Kapatos, E.T. 1991. "Integrated Pest Management Systems of *Dacus oleae*", in *World Crop Pests. Fruit flies. Their biology, natural enemies and control*, edited by A.S. Robinson and G. Hooper (Elsevier Science Publishers, Amsterdam), pp. 391-398.

Knowles, B.H. and Ellar, D.J. 1987. "Colloid-osmotic Lysis Is A General Feature of the Mechanism of Action of *Bacillus thuringiensis* δ-endotoxins With Different Insect Specificities," *Biochim. Biophys. Acta*. Vol. 924, pp. 509-518.

Knowles, B.H., Blatt, M.R., Tester, M., Horsnell, J.M., Carroll, J., Menestrina, G. and Ellar, D.J. 1989. "A cytolytic δ-endotoxin from *Bacillus thuringiensis* var. *israelensis* Forms Cation-selective Channels in Planar Lipid Bilayers," *FEBS Letters*. Vol. 244, pp. 259-262.

Kondo, S., Mizuki, E., Akao, T. and Ohba, M. 2002. "Screening of *Bacillus thuringiensis* Parasporal Inclusion Proteins for Antitrichomonal Activity," *ITE Lett. Batt. New Technol. Med*. Vol. 3, pp. 97-100.

Koni, P.A. and Ellar, J.D. 1994. "Biochemical Characterization of *Bacillus thuringiensis* Cytolytic δ-endotoxins," *Microbiology*.Vol. 140, pp. 1869-1880.

Kramer, J.M. and Gilbert, R.J. 1989. "*Bacillus cereus* and Other *Bacillus* species", in *Foodborne Bacterial Pathogens*, edited by P.M. Doyle (Marcel Dekker, New York), pp. 21-70.

Krieg, A. and Lysenko, O. 1979. "Toxine und Enzyme bei einigen Bacillus-Arten unter besondere Berücksichtigung der *Bacillus cereus-thuringiensis* Gruppe," *Zentralblatt für* Ba*cteriologie-Naturwissenschaften*, Vol. 134, pp. 70-88.

Krieg, A. and Langenbruch, A.G. 1981. "Suspectibility of Arthropod Species to *Bacillus thuringiensis*", in *Microbial Control of Pests and Plant Diseases*, edited by H.D. Burges (Academic Press, London), pp. 837-896.

Kumar, P.A., Sharma, R.P. and Malik, V.S. 1996. "The Insecticidal Proteins of *Bacillus thuringiensis*," *Advances in Applied Microbiology*. Vol.42, pp. 1-43.

Lacey, L.A. and Siegel, J.P. 2000. "Safety and Ecotoxicology of Entomopathogenic Bacteria", in *Entomopatgenic Bacteria: From Laboratory to Field Application*, edited by J.F. Charles, A. Delecluse and C. Nielsen (Kluwer Academic, Dordrecht), pp. 253-273.

Lambert, B. and Peferoen, M. 1992. "Insecticidal Promise of *Bacillus thuringiensis*. Facts and Mysteries about A Succesful Biopesticide," *Bioscience*. Vol. 42, pp. 112-122.

Leonard, C., Yahua, C. and Mahilion J. 1997. "Diversity and Distribution of IS231, IS232, and IS240 among *B. cereus, B. thuringiensis*, and *B. mycoides*," *Microbiology*. Vol. 143, pp. 2537-2547.

Lereclus, D., Bourgouin, C., Lecadet, M.-M., Killer, A. and Rapoport, G. 1989. "Role, Structure, and Molecular Organizaton of the Genes Coding for the Parasporal δ-endotoxins of *Bacillus thuringiensis*", in *Regulation of prokaryotic development* edited by I. Smith, A.R. Slepeceky and P. Setlow (American Society for Microbiology), pp. 255-276.

Lereclus, D., Delecluse, A. and Lecadet, M.-M. 1993. "Diversity of *Bacillus thuringiensis* Toxins and Genes", in *Bacillus thuringiensis An Environmental Biopesticide: Theory and Practice*, edited by P. Entwistle, S.J. Cory, J.M. Bailey, and S. Higss (John Wiley , New York), pp.37-70.

Lereclus, D. 1996. "Views on the Ecology and the Virulence of *Bacillus thuringiensis*", 29 th. Annual Meeting and IIIrd International Colloquim on *Bacillus thuringiensis*, Spain.

Li, R.S., Jarrett, P. and Burges, H.D. 1987. "Importance of Spores, Crystals and δ-endotoxins in the Pathogenicity of Different Varieties of *Bacillus thuringiensis* in *Galleria mellonella* and *Pieris brassicae*," *Journal of Invertebrate Patho*logy. Vol. 50, pp. 277-284.

Li, J., Carroll, J. and Ellar, D.J. 1991. "Crystal Structure of Insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 A° Resolution," *Nature*. Vol. 353, pp. 81-821.

Lin, Y. and Xiong, G., 2004. "Molecular Cloning and Sequence Analysis of Chitinase Gene from *Bacillus thuringiensis* serovar. *alesti*", *Biotechnology Letters*. Vol. 26, pp. 635-639.

Lopez-Villatta, L.C., 1998. *The Olive Tree, the Oil, the Olive* (International Olive Oil Council Publications, Madrid).

Lotti, M. 1995. "Cholinesterase Inhibition. Complexities in Interpretation," *Clin. Chemistry*. Vol.41, No. 12, pp. 1814-1818.

Maduell, A., Callejas, R., Cabrera, K.R., Armengol, G. and Orduz, S. 2002. "Distribution and Characterization of *Bacillus thuringiensis* on the Phylloplane of Species of Piper (*Piperaceae*) in Three Altitudinal Levels," *Microbial Ecology*. Vol. 44, pp. 144-153.

Maeda, M., Mizuki, E., Nakamura, Y., Hatano, T., Ohba, M. 2000. " Recovery of *Bacillus thuringiensis* from Marine Sediments of Japan," *Current Microbiology.* Vol. 40, pp. 413-422.

Martin, P.A.W. and Travers, R.S. 1989. "Worlwide abundance and distribution of *Bacillus thuringiensis* isolates," *Applied and Environmental Microbiology*. Vol. 55, pp. 2437-2442.

McClintock, J.T., Schaffer, C.R. and Sjoblad, R.D. 1995. "A Comparative Review of the Mammalian Toxicity of *Bacillus thuringiensis*-based Pesticides," *Pesticide Science*. Vol. 45, pp. 95-105.

Meadows, M.P., Ellis, D.J., Butt, J., Jarrett, P., Burges, H.D. 1992. "Distribution, Frequency and Diversity of *Bacillus thuringiensis* in An Animal Feed Mill," *Applied and Environmental Microbiology*. Vol. 58, pp. 1344-1350.

Meadows, M.P. 1993. "*Bacillus thuringiensis* in the Environment: Ecology and Risk Assessment, in *Bacillus thuringiensis*, *An Environmental Biopesticide: Theory and practice*, edited by P.F. Entwistle, J.S. Cory, M.J. Bailey and S. Higgs (John Wiley, New York).

Mikkola, A.R., Calberg, G.A., Vaara, T. and Gyllenberg, H.G. 1982. "Comparison of Inclusions in Different *Bacillus thuringiensis* Strains. An Electron Microscope Study," *FEMS Microbiology Letters*. Vol.13, pp. 401-408.

Milne, R.E. and Kaplan, H. 1993. "Purification and Characterization of A Trypsin-like Digestive Enzyme from Spruce Budworm (*Choristoneura fumiferana*) Responsible for the Activation of δ-endotoxin from *Bacillus thuringiensis*," *Insect Biochem. Mol. Biology*. Vol. 23, pp. 663-673.

Mizuki, E., Ohba, M., Ichimatsu, T., Hwang, S., Higuchi, K., Saitoh, H. and Akao, T., 1998. "Unique Appendages Associated with Spores of *Bacillus cereus* Isolates," *Journal of Basic Microbiology*. Vol. 38, pp. 33-39.

Mizuki, E., Ichimatsu, T., Hwang, S.H., Park, Y.S., Saitoh, H., Higuchi, K. and Ohba, M. 1999a. "Ubiquity of *Bacillus thuringiensis* on Phylloplanes of Arboreous and Herbaceous Plants in Japan,"*Journal of Applied Microbiology*. Vol. 86, pp. 979-984.

Mizuki, E., Ohba, M., Akao, T., Yamashita, S., Saitoh, H. and Park, Y.S. 1999b. "Unique Activity Associated With Non-insecticidal *Bacillus thuringiensis* Parasporal Inclusions: *in vitro* Cell-killing Action on Human Cancer Cells,"*Journal of Applied Microbiology*. Vol. 86, pp. 477-486.

Mizuki, E., Park, Y.S., Saitoh, H., Yamashita, S., Akao, T., Higuchi, K. and Ohba, M. 2000. "Parasporin, A Human Leukemic Cell-recognizing Parasporal Protein of *Bacillus thuringiensis*," *Clin. Diagn. Lab. Immunology*. Vol. 7, pp. 625-634.

Mohd-Salleh, M.B. and Lewis, L.C. 1982. "Feeding Deterrent Response of Corn Insects to β-exotoxin of *Bacillus thuringiensis*,"*Journal of Invertebrate Pathology*. Vol. 39, pp. 323-328.

Moir, A. 1992. "Spore Germination", in *Biology of Bacilli. Applications to Industry*, edited by R.H. Doi and M. McGloughlin (United States of America: Butterworth-Heinemann. ISBN 0-7506-9122-0), pp. 23-38.

Nault, B.A. and Kennedy, G.G.1999. "Influence of Foliar Applied *Bacillus thuringiensis* subsp. *tenebrionis* and An Early Potato Harvest on Abundance and Overwinter Survival of Colorado Potato Beetles (Coleoptera: Chrysomelidae) in North Carolina," *Journal of Economic Entomology*. Vol. 92, pp. 1165-1171.

Navon, A. 2000. "*Bacillus thuringiensis* Insecticides in Crop Protection-reality and Prospects," *Crop Protection*. Vol. 19, pp. 669-676.

Navrodizis, E.I., Vasara, E., Karamanlidou, G., Salpiggidis, G.K. and Koliais, S.I. 2000. "Biological control of *Bactrocera oleae* (Diptera: Tephritidae) Using a Greek *Bacillus thuringiensis* Isolate," *Biological and Microbial Control*. Vol. 93, No. 6, pp. 1657-1661.

Nayak, P., Basu, D., Das, S., Basu, A., Ghosh, D., Ramakrishnan, N.A., Ghosh, M. and Sen, S.K. 1997. "Transgenic Elite Rice Plants Expressing CryIAc delta-endotoxin of *Bacillus thuringiensis* Are Resistant Against Yellow Stem borer (*Scirpophaga incertulas*)," *Proc. Natl. Acad. Sci. USA*. Vol. 94, pp. 2111-2116.

Ohba, M. and Aizawai, K. 1986. "Distribution of *Bacillus thuringiensis* in Soils of Japan,"*Journal of Invertabrate Pathology*. Vol. 47, pp. 277-282.

Ohba, M. and Aratake, Y. 1994. "Comparative Study of the Frequency and Flagellar Serotype Flora of *Bacillus thuringiensis* in Soils and Silkworm-breeding Environments,",*Journal of Applied Bacteriology*. Vol. 76, pp. 203-209.

Ohba, M. 1996. "*Bacillus thuringiensis* Populations Naturally Occurring on Mulberry Leaves: A Possible Source of the Populations Associated With Silkworm-rearing Insectaries," *Journal of Applied Bacteriology*. Vol. 80, pp. 56-64.

Olgun, H., Kıran, N., Çoban, T., Tıns, M. and Akgün, F. 2001. "Feasibility Report for Pomace Thermolysis Processes,"TUBITAK-MRRC, Kocaeli.

O'Sullivan, D.J. and Klaenhammer, T.R. 1993. "Rapid Mini-prep. Isolation of High-Quality Plasmid DNA from Lactococcus and Lactobacillus spp.," *Applied and Environmental Microbiology*. Vol. 59**,** pp. 2730-2733.

Payne, J., Narva, K.E., Uyeda, K., Stalder, C.J. and Michaels, T.E. 1995. "*Bacillus thuringiensis* Isolate PS201T6 Toxin," U.S. Patent, No. 5,436,002.

Peňa, J.E. and Schaffer, B.A. 1997. "Intraplant Distribution of the Citrus Leafminer (Lepidoptera: Gracillariidae) on Lime," *Journal of Economic Entomology*. Vol. 90, pp. 458-464.

Perlak, F.J., Deaton, R.W., Armstrong, A.T., Fuchs, L.R., Sims, R.S., Greenplate, T.J. and Fischhoff, A.D. 1990. "Insect Resistance Cotton Plants,"*Bio/Technology*. Vol. 8, pp. 939-943.

Poncet, S., Delecluse, A., Anello, G., Klier, A. and Rapoport, 1994. "Evaluation of Synergistic Interactions among CryIVA, CryIVB, and CryIVD Toxic Components of *Bacillus thuringiensis* subsp. *israelensis* Crystals," *Journal of Invertebrate Pathology*. Vol. 66, pp. 131-135.

Porcar, M. and Caballero, P., 2000. "Molecular and Insecticidal Characterization of a *Bacillus thuringiensis* Strain Isolated during A Natural Epizootic Event," *Journal of Applied Microbiology*. Vol. 89, pp. 309-316.

Porcar, M. and Juarez-Perez,V. 2002. "PCR-based Identification of *Bacillus thuringiensis* Pesticidal Crystal Genes," *FEMS Microbiology Reviews*. Vol. 757, pp. 1-4.

Priest, F.G. 1993. "Systematics and Ecology of *Bacillus*", in *Bacillus subtilis and other Gram-positive bacteria*, edited by A.L. Sonenshein and H.R. Losik (American Society for Microbiology, Washington), pp. 3-16.

Priest, F.G. 2000. "Biodiversity of the Entomopathogenic, Endospore-forming Bacteria", in *Entomopathogenic Bacteria: From laboratory to field application*, edited by J.F. Charles, A. Delécluse and C. Nielsen-LeRoux (Kluwer Academic, Dordrecht), pp. 1-22.

Rastrelli, L., Totaro, K. and DeSimone, F. 2002. "Determination of Organophosphorus Residues in Cilento (Campania, Italy) Virgin Olive Oil by Capillary Gas Chromatography,"*Food Chemistry*. Vol. 79, pp. 303-305.

Regev, A., Keller, M., Strizhov, N., Sneh, B., Prudovsky, E., Chet, I., Ginzbug, I., Kalman, Z.K., Koncz, C. and Schell, J. 1996. "Synergistic Activity of *Bacillus thuringiensis* δ-Endotoxin and A Bacterial Endochitinase against *Spodoptera littoralis* Larvae," *Applied and Environmental Microbiology*. Vol.62, pp. 3581-3586.

Robacker, D.C., Martinez, A.J., Garcia, J.A., Diaz, M. and Romero, C. 1996. "Toxicity of *Bacillus thuringiensis* to Mexican Fruit Fly (Diptera: Tephritidae)," *Journal of Economic Entomology*. Vol. 89, pp. 104-110.

Sankaranarayanan, R., Sekar, K., Banerjee, R., Sharma, V., Surolia, A. and Vijayan, M. 1996. "A Novel Mode of Carbohydrate Recognition in Jacalin, A *Moraceae* Plant Lectin with A β- prism Fold,"*Nat. Struct. Biology*. Vol. 3, pp. 596-603.

Schnepf, H.E. and Whiteley, H.R. 1981. "Cloning and Expression of the *Bacillus thuringiensis* Crystal Protein Gene *Escherichia coli*," *Proc. Natl. Acad. Sci.* USA. Vol. 78, pp. 2893-2897.

Schnepf, H.E., Tomnczak, K., Ortega, J.P. and Whiteley, H.R. 1990. "Specificity-Determining Regions of A Lepidopteran-specific Insectcidal Protein Produced by *Bacillus thuringiensis*," *J. Biol. Chemistry*. Vol. 265, pp. 20923-20930.

Schnepf, E., Crickmore, N., Van-Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H. 1998. "*Bacillus thuringiensis* and Its Insecticidal Proteins," *Microbiology and Molecular Biology* Reviews. Vol. 62, pp. 774-806.

Sebesta, K. and Sternbach, H. 1970. “The specificity of Inhibition of DNA-dependent RNA Polymerase by *Bacillus thuringiensis* Exotoxin,”*FEBS Letters*. Vol. 8, pp. 223-235.

Sebesta, K. and Horská, K. 1970. “Mechanism of Inhibition of DNA-dependent RNA Polymerase by Exotoxin of *Bacillus thuringiensis*,” *Biochem. Biophys. Acta*. Vol. 209, pp. 357-376.

Sela, S., Schickler, H., Chet, I. and Spiegel, Y. 1988. “Purification and Characterization of *Bacillus cereus* Collagenolytic/proteolytic Enzyme and Its Effect on *Meloidogyne javanica* Cuticular Proteins,”*European Journal of Plant Pathology*. Vol. 104, pp. 59-67.

Shimuzi, T. and Morikawa, K., 1996. “The ß–prism: A New Folding Motifi,” *Trend Biochem. Science*. Vol. 21, pp. 3-6.

Shisa, N., Wasano, N., Ohgushi, A., Lee, D. and Ohba, M., 2001. “Extremely High Frequency of Common Flagellar Antigens between *Bacillus thuringiensis* and *Bacillus cereus*,”*FEMS Microbiology Letters*. Vol. 213, pp. 93-96.

Singsit, C., Adang, M.J., Lynch, R.E., Anderson, W.F., Wang, A., Cardineau, G. and Ozias Akins, P. 1997. “Expression of A *Bacillus thuringiensis cry1A(c)* Gene in Transgenic Peanut Plants and Its Efficacy against Lesser Cornstalk Borer,” *Transgenic Res*. Vol. 6, pp. 169-176.

Slatin, S., Abrams, C.K. and English, L. 1990. “Delta-endotoxins Form Cation-selective Channels in Planar Lipid Bilayers,”*Biochem. Biophys. Res. Commun*. Vol. 169, pp. 765-772.

Slepecky, R.A. and Leadbetter, E.R. 1994. “Ecology and Relationships of Endospore-forming Bacteria: Changing Perspectives”, in *Regulation of Bacterial Differentiation*, edited by P.J. Piggot, C.P.JR. Moran and P. Youngman (American Society for Microbiology, Washington, ISBN 1-55581-066-7), pp. 195-206.

Smirnoff, W.A. and Berlinquet, L. 1966. "A Substance in Some Commercial Preparations of *Bacillus thuringiensis* var. *thuringiensis* Toxic to Sawfly Larvae," *Journal* of *Invertebrate Pathology*. Vol. 8, p. 376.

Smirnoff, W.A. 1973. "Results of Tests with *Bacillus thuringiensis* and Chitinase on Larvae of the Spruce Budworm", *Journal of Invertebrebrate Pathology*, Vol.21, p. 116

Smirnova, T.A., Kulinich, L.I., Galperin, M.Y. and Azizbekyan, R.R. 1991. "Subspecies-Specific Haemagglunitiation Patterns of Fimbriated *Bacillus thuringiensis* Spores,"*FEMS Microbiology Letters*. Vol. 90, pp. 1-4.

Smith, R.A. and Couche, G.A. 1991. "The phylloplane as A Source of *Bacillus thuringiensis* Variants," *Applied and Environmental Microbiology*. Vol. 57, pp. 311-315.

Sneath, P.H.A. 1986. "Endospore-forming Gram-positive Rods and Cocci", in *Bergey's Manual of Systematic Bacteriology*, edited by P.J. Butler (Williams & Wilkins, Baltimore), pp. 1104-1207.

Spiro-Kern, A. 1974. "Untersuchungen uber die proteasen bei *Culex pipiens*,"*J.Comp. Physiology*. Vol. 90, pp. 53-70.

Steinhaus, E.A. 1960. "Insect Pathology: Challenge, Achievements and Promise," *Bull. Ent. Soc. Am*. Vol.6, pp. 9-16.

Tailor, R., Tippett, J., Gibb, G., Pells, S., Pike, D., Jordan, L. and Ely, S. 1992. "Identification and Characterization of A Novel *Bacillus thuringiensis (kurstaki)* Delta-endotoxin Entomocidal to Coleopteran and Lepidopteran Larvae," *Mol. Microbiology*. Vol. 6, pp. 1211-1217.

Te Giffel, M.C., Beumer, R.R., Granum, P.E. and Rombouts, F.M. 1997. "Isolation and Characterization of *Bacillus* cereus from Pasteurized Milk in Household Refrigerators in the Netherlands," *Journal of Food Microbiology*. Vol. 34, pp. 307-318.

Thapinta, A. and Hudak, P.F. 2000. "Pesticide Use and Residual Occurrence in Thailand," *Environmental Monitoring and Assessment*. Vol. 60, pp. 103-114.

Thiery, I. and Frachon, E. 1997. "Identification, Isolation, Culture and Preservation of Entomopathogenic Bacteria", in *Manual of Techniques in Insect Pathology*, edited by L. A. Lacey (NY: Academic Press, New York), pp. 54-77.

Thomas, W.E. and Ellar, D.J. 1983. "*Bacillus thuringiensis* var. *israelensis* Crystal δ-endotoxin: Effects on Insect and Mammalian Cells *in vitro* and *in vivo*,"*J. Cell Science*. Vol. 60, pp. 181-197.

Thorne, C.B. 1993. "*Bacillus anthracis*", in *Bacillus subtilis and Other Gram-positive Bacteria*, edited by A.L. Sonenshein, J.A. Hoch and R. Losick (American Society for Microbiology, Washington), pp. 113-124.

Toumanoff, C. and Vago, C. 1951. "L'agent pathogène de la flacherie des vers à soie endémique dans la règion des Cévennes : *Bacillus cereus* var. *alesti* var. nov. ," *C.R. Acad. Science*. Vol. 233, pp. 1504-1506.

Travers, R.S. 1982. "Ph.D. dissertion, University of Maryland, College Park.

Travers, R.S., Martin, P.A.W. and Reichelderfer, C.F. 1987. "Selective Process for Efficient Ssolation of soil *Bacillus* sp.,"*Applied and Environmental Microbiology*. Vol. 53, pp.1263-1266.

Trindade, G.E., Vilas Boas, L., Lereclus, D. and Arantes, O.N. 1996. "*Bacillus thuringiensis* Conjugation in Environmental Conditions", 29th Annual Meeting and IIIrd International Colloquium on *Bacillus thuringiensis*, Spain.

Vaeck, M., Reybnaerts, A., Hofte, J., Jansens, S., DeBeuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M. and Leemans, J. 1987. "Transgenic Plants Protected from Insect Attack,"*Nature*. Vol. 328, pp. 33-37.

Van der Salm, T., Bosch, D., Honee, G., Feng, L., Munstermann, E., Bakker, P., Stiekama, J.W., and Visser, B. 1994. "Insect resistance of transgenic plants that express modified *Bacillus thuringiensis cry1A(b)* and *cry1C* genes: a resistance management strategy", *Plant Mol. Biology*, Vol. 26**,** pp. 51-59.

Van Rie, J., Jansens, S., Höfte, H., Degheele, D. and Van Mellaert, H. 1990. "Receptors on the Brush Border Membrane of the Insect Midgut as Determinants of the Specificity of *Bacillus thuringiensis* Delta-endotoxins," *Applied and Environmental Microbiology*. Vol. 57, pp. 1650-1655.

Wang, J., Boets, A., Van Rie, J. and Ren, G. 2003. "Characterization of *cry1*, *cry2* and *cry9* Genes in *Bacillus thuringiensis* Isolates from China," *Journal of Invertebrate Pathology*. Vol. 82, pp. 63-71.

Web_1, 2004. Statistical World Pesticide consumption, 15/04/2005. http://faostat.fao.org/faostat/formcollection

Web_2, 2005. *Bacillus thuringiensis* toxin nomenclature, 20/03/2005. http://www.biols.susx.ac.uk/Home/NeilCrickmore/Bt/index.html.

Wirth, M.C., Walton, W.E. and Federicci, B.A. 2000. "Cyt1A from *Bacillus thuringiensis* Restores Toxicity of *Bacillus sphaericus* against Resistant *Culex quinquefasciatus* (Diptera: Culicidae),"*J. Med. Entomology*. Vol.37, No. 3, pp. 401-407.

Yang, Y.J., Davies, D.M. 1971. "Digestive Enzymes in the Excreta of *Aedes aegypti* Larvae", *Journal of Insect Physiology*. Vol.17, pp. 2119-2123.

Yavuz, E., Gunes, H., Bulut, C., Harsa, S. and Yenidunya, A.F., 2004. "RFLP of 16S-ITS rDNA Region to Differentiate Lactobacilli at Species Level," *World Journal of Microbiology*. Vol. 20, pp. 535-537.

Yu, C-G., Mullins, M.A., Warren, G.W., Koziel, M.G. and Estruch, J.J. 1997. "The *Bacillus thuringiensis* Vegetative Insecticidal Protein Vip3A Lyses Midgut Epithelium Cells of Suspectible Insects," *Applied and Environmental Microbiology*. Vol. 63, pp. 532-536.

Zelansky, B., Stephan, D. and Hamacher, J. 1994. "Irregular Crystal Formation in Some Isolates of *Bacillus thuringiensis*,"*Journal of Invertebrate Pathol*ogy. Vol. 63, pp. 229-234.

Zeren, O., Dikmen, N. and Taga, S. 2002. "Measuring the Exposure to Organophosphorus Insecticides of People Involved in Crop Protection in Turkey," *Agriculture, Ecosystems and Environment*. Vol. 93, pp. 447-448.

Zhang, H.Y., Yu, Z.N., Deng, W.X. 2000. "Isolation, Distribution and Toxicity of *Bacillus thuringiensis* from Warehouses in China," *Crop Protection*. Vol. 19, pp. 24

APPENDIX A

CHEMICALS USED IN EXPERIMENTS

CHEMICAL	CODE
Agar-Agar	Applichem A0949
Peptone from Casein	Applichem A2210
D (+)-Glucose	Applichem A3666
Yeast Extract	Merck 1.03753
Glycerol	Applichem A2926
Sodium chloride	Applichem A2942
Potassium phosphate	Applichem A2945
$MgSO_4.7H_2O$	Merck 1.05886
Soluble starch	Merck 1.01252
Ammonium sulphate	Applichem A3585
Immersion oil	Applichem A0699
Cetyltrimethylammonium bromide	Applichem A0805
Nutrient broth	Applichem A3714
Potassium iodide	Sigma P8256
Tris Base	Sigma T6066
EDTA	Applichem A2937
Isopropanol	Applichem A3928
Proteinase K	Applichem A3830
Ethidium bromide	Applichem A1151
Ethanol	Applichem A1151
Taq DNA polymerase	MBI, Fermentas EP0401
dNTP set	MBI, Fermentas R0181
*Taq*I	Fermentas ER0671
*Hae*III	Fermentas ER0151
Agarose (Standard)	Applichem A2114
Lysozyme	Applichem A3711
Proteinase K	Applichem A3830
Chloroform	Applichem A3830

Isoamyl alcohol	Applichem A2610
Bromophenol blue	Merck 1.08122
Sodium dodecyl sulphate	Applichem A2263
Sodium hydroxide	Merck 1.06498
Boric acid	Applichem A2940
Hydrochloric acid (HCl)	Merck 1.00317
Ammonium acetate	Applichem A2936
Phenol	Applichem A1594
Ribonuclease A	Applichem A3832
1 kb DNA Ladder Gene RulerTM	Fermentas SM0313
D (+)-Sucrose	Applichem A3935
Urea agar base	Oxoid CM53
Urea	Applichem A1049
L-Arginine monohydrochlorid	Merck K398043
DNase agar	Oxoid CM321
Bromcresol purple	Merck 1.03025
Methyl- red	Riedel 059120
Potassium hydroxide	Amresco 1073B60
α-Napthol	Merck 6221
D (+)-Mannose	DIFCO 0171-13
D (-)-Salicin	Fluka 84150
D-Maltose	Merck Art5911
D-Cellobiose	Sigma C-7252
Listeria Selective Agar base	Mast Diagnostics DM256D
Gelatin	Applichem A1693
Sodium acetate	Sigma S-2889
Iodine	Sigma I-3380
Potassium iodide	Applichem A1040

APPENDIX B

MEDIA

B.1. Nutrient Agar Medium Used for Isolation

	g/l
Nutrient Broth	13
Agar	15

Ingredients are dissolved in distilled water by stirring with gentle heating. Medium is sterilised by autoclaving at 121°C for 15 min.

B.2. *Bacillus thuringiensis* Medium Used for Sporulation

	g/l
Yeast extract	2
$MgSO_4.7H_2O$	0.2
$K_2HPO_4.\ 3H_2O$	0.5
Glucose	3
$(NH_4)_2SO_4$	2
$CaCl_2.\ 2H_2O$	0.08
$MnSO_4.\ 4H_2O$	0.05
Agar	15

Components are added into distilled water and mixed thoroughly. The pH is brought to 7.3. Medium is sterilised by autoclaving at 121°C for 15 min.

B.3. Milk Glucose Agar

	g/l
Glucose	20
Agar	20
UHT light milk	333 ml

Ingredients except milk are added into 667 ml of distilled water, mixed well. Medium is autoclaved at 121°C for 15 min. Milk is added into the medium aseptically.

B.4. Nutrient Gelatin

	g/l
Nutrient broth	13
Gelatin	150

Ingredients are dissolved in water with constant stirring. Medium is boiled to dissolve gelatin and then sterilised at 121°C for 15 min.

B.5. Starch Nutrient Agar

	g/l
Nutrient broth	13
Soluble Starch	4
Agar	15

Ingredients are added into distilled water and dissolved by heating and stirring. Medium is sterilised at 121°C for 15 min.

B.6. Bromcresol Purple Sucrose Nutrient Agar

	g/l
Nutrient broth	13
Sucrose	10
Bromcresol purple solution (1%)	2.5 ml
Agar	15

Ingredients are dissolved in distilled water and the volume is completed to 1000 ml. The pH is brought to 7.2. Medium is sterilised at 121°C for 15 min.

B.7. Bromcresol Purple Carbohydrate Broth

	g/l
Nutrient broth	13
Bromcresol purple solution (1%)	2.5 ml
Carbohydrate solution	100

Components except carbohydrate and bromcresol purple solutions are added to the distilled water and dissolved by steaming. After adjusting the pH to 7.2, the indicator is added and the volume is completed to 900 ml with distilled water. Medium is sterilised at 121°C for 15 min. After cooling, carbohydrate solution that has been filtered-sterilised is added to the medium aseptically.

B.8. Peptone Water

	g/l
Peptone	10
NaCl	5

Ingredients are dissolved in distilled water and the pH is brought to 7.2. Medium is sterilised at 121°C for 15 min.

B.9. DNase Agar (Oxoid CM321)

	g/l
Tryptose	20
DNA	2
NaCl	5
Agar	12

39 g of powder is suspended in distilled water and brought to the boil to dissolve completely. Medium is sterilised at 121 ^{o}C for 15 min.

B.10. Listeria Selective Agar Base (Mast Diagnostics DM256 D)

	g/l
Peptone mixture	15.2
Yeast extract	2
Casein hydrolysate-enzymic	4
NaCl	5
Glucose	0.5
K_2HPO_4	0.8
Starch	1.0
Lithium chloride	15
Aesculin	1
Ferric ammonium citrate	0.5
Agar A	12

57 g of powder is suspended in distilled water by swirling and medium is sterilised at 121°C for 15 min.

B.11. Glucose Phosphate Broth

	g/l
Glucose	5
Peptone	5
K_2HPO_4	5

Ingredients are dissolved in distilled water and the pH is adjusted to 7.5. Medium is sterilised by autoclaving at 121°C for 15 min.

B.12. Luria-Bertani Broth

	g/l
Yeast extract	5
Peptone	10
NaCl	10

Ingredients are dissolved in distilled water and the pH is adjusted to 7.0. Medium is sterilised by autoclaving at 121°C for 15 min.

B.13. Urease Slant Agar (Oxoid CM53)

	g/l
Peptone	1
Glucose	1
NaCl	5
Disodium phosphate	1.2
K_2HPO_4	0.8
Phenol red	0.012
Agar	15

24 g of urea agar base is suspended in 950 ml of distilled water. Medium is brought to the boil to dissolve completely and sterilised by autoclaving at 115 °C for 20 min. After cooling to 50 °C, 50 ml of sterile urea solution is introduced into the medium to give a

final concentration of 2%. Medium is mixed well and distributed 5 ml amounts into sterile tubes. Tubes are allowed to set in the sloped position.

B.14. Egg Yolk Nutrient Agar

	g/l
Nutrient broth	13
Agar	18
Egg yolk emulsion	100 ml

Ingredients except egg yolk emulsion are dissolved in 900 ml of distilled water. Medium is sterilised by autoclaving at 121°C for 15 min. After cooling medium to 50°C, sterile egg yolk emulsion is added aseptically to the medium.

APPENDIX C

REAGENTS AND SOLUTIONS

C.1. Bromcresol Purple Solution

Bromcresol purple	1 g
Distilled water	100 ml

Bromcresol purple is added into the water and solution is mixed thoroughly.

C.2. Carbohydrate Stock Solution

Carbohydrate	10 g
Distilled water	100 ml

All carbohydrates except salicin are prepared as 10% solutions but salicin is prepared as 5% solutions because of low solubility. Carbohydrates are dissolved in distilled water by stirring and solution is sterilised by passing through filter.

C.3. Gram's Iodine Solution

Iodine	1 g
Potassium iodide	2 g
Distilled water	300 ml

Ingredients are grinded in a mortar and dissolved by adding water slowly. The prepared solution is mixed well by stirring.

C.4. KOH Solution (16%)

KOH	16 g
Distilled water	100 ml

C.5. α-Napthol Solution (5%)

α-Napthol	5 g
Ethanol (95%)	100 ml

C.6. Methyl Red Solution

Methyl red	0.1 g
Ethanol (95%)	300 ml
Distilled water	200 ml

Methyl red is dissolved in the ethanol (95%) by constant stirring and the volume is made up to 500 ml with water.

C.7. Egg Yolk Emulsion

NaCl	0.85 g
Distilled water	100 ml
Egg yolk	100 ml

The yolks from the whites of the eggs are separated aseptically by sterile pipette and drained in a sterile falcon. 0.85 g of NaCl is dissolved in 100 ml water and sterilised at 121 °C for 15 min. Equal volume of egg yolk and sterile saline solution is transferred to a sterile bottle aseptically. Solution is stirred until the egg yolk is completely dispersed.

APPENDIX D

BUFFERS AND STOCK SOLUTIONS

D.1. 50 X TAE

242 g of Tris base is dissolved in deionised water. 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) are added. Volume is adjusted to 1000 ml with deionised water.

D.2. 1XTAE

20 ml of 50X TAE buffer is taken and 980 ml of deionised water is added to obtain 1X TAE buffer.

D.3. 10 X TBE

108 g of Tris base and 55 g boric acid are mixed and dissolved in 800 ml of deionised water. 40 ml of 0.5 M EDTA (pH 8.0) is added. The volume is brought to 1000 ml with deionised water.

D.4. 1M Tris-HCl (pH 7.2/ pH 8.0)

121.1 g of Tris base is dissolved in 800 ml of deionised water. The desired pH is obtained by adding concentrated HCl. The volume of the solution is brought to 1000 ml with deionised water.

D.5. 1xTE (pH 8.0)

10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0) is mixed.

D.6. 0.5 M EDTA pH 8.0

186.1g of EDTA are added to 800 ml of deionised water. Solution is stirred vigorously and the pH is adjusted to 8.0 with NaOH pellets (~ 20 g). Volume is completed to 1000 ml with deionised water. Solution is dispensed into aliquots and sterilised by autoclaving.

D.7. 3 M SODIUM ACETATE (pH 5.2)

408.1 g sodium acetate.$3H_2O$ is dissolved in 800 ml of deionised water and the pH is adjusted to 5.2 with glacial acetic acid. The volume is brought to 1000 ml. Solution is sterilised by autoclaving.

D.8. ETHIDIUM BROMIDE STOCK SOLUTION (10 mg/ml)

1 g of ethidium bromide is dissolved in 100 ml of deionised water by stirring on a magnetic stirrer to dissolve the dye completely. Solution is transferred to a dark bottle and stored at room temperature.

D.9. CHLOROFORM-ISOAMYL ALCOHOL SOLUTION

96 ml of chloroform is mixed with 4 ml of isoamyl alcohol.

D.10. PHENOL

Phenol is allowed to warm at room temperature, and melted at 68 ^{o}C. Equal volume of buffer (usually 0.5 M Tris.Cl pH 8.0, at room temperature) is added to the melted phenol. The mixture is stirred for 15 minutes. When the two phases have separated, the aqueous (upper) phase is removed using a separation funnel. Equal volume of 0.1 M Tris.Cl pH 8.0 is then added to the phenol. The mixture is again stirred for 15 minutes. The aqueous phase is removed as described below. The extractions are repeated until the pH of the phenolic phase is > 7.8. The pH is measured by using pH paper slips. After the phenol is equilibrated, the mixture is divided into aliquots and they are stored under 100 mm Tris.Cl (pH 8.0) at -20 ^{o}C. When needed, the phenol is melted at room

temperature. Hydroxyquinoline and β-mercaptoethanol are added to a final concentration of 0.1% and 0.2 %, respectively. The phenol solution can be stored in this form at 4 ^{o}C.

D.11. CTAB/NaCl SOLUTION

4.1 g NaCl is dissolved in 80 ml water. 10 g CTAB is added slowly while heating and stirring. To increase dissolution, the solution is heat to 65 ^{o}C. The final volume is adjusted to 100 ml.

D.12. 10 M AMMONIUM ACETATE

770 g of ammonium acetate is dissolved in 800 ml of distilled water and the volume is adjusted to 1000 ml. Solution is filtered-sterilised.

D.13. 10% SODIUM DODECYL SULFATE (SDS)

100 g of SDS is dissolved in 900 ml of deionised water. Solution is heat to 68 ^{o}C to dissolve. The pH is adjusted to 7.2 by adding a few drops of concentrated HCl. The volume is brought to 1000 ml with water.

D.14. GEL-LOADING DYE (6X)

2 ml of 10xTBE, 6 ml of glycerol is mixed in a falcon and the volume is adjusted to 20 ml with sterile deionised water. Bromophenol blue is added until the adequate colour is obtained.

D.15. dNTP (10X)

20 µl of each 100mM dATP, dCTP, dGTP and dTTP are taken and mixed in an eppendorf tube. 920 µl of sterile deionised water is added to dilute the solution to a final concentration of 2 mM. Solution is mixed gently and stored at –20 °C until its needed.

APPENDIX E

RESULTS OF METHYL RED REACTION

Isolate	MR	Isolate	MR
249Q	+	284Q1	-
36Q1	+	20Q3	+
70Q	+	41Q3	+
58Q2	+	41Q	+
54Q2	+	58Q1	+
290Q1	-	39Q1	-
284Q	-/+	43Q	+
50Q3	+	19Q4	-
53Q1	-	184Q	+
59Q2	+	56Q2	-
55Q3	+	14Q4	+
55Q1	-	53Q3	+
52Q3	+	69Q1	+
167Q	+	24Q	-
45Q1	-	68Q	-
50Q1	+	58Q3	-/+
20Q1	+	74Q	+
169Q	-	19Q2	+
14Q1	-	73Q	+
19Q	-	37Q1	+
65Q	+	60Q1	+
62Q	-	44Q2	-
37Q3	+	21Q	-
37Q2	-	45Q2	+
52Q1	+	184Q1	+

Isolate	MR	Isolate	MR
67Q	+	164Q1	+
69Q	+	57Q1	+
36Q2	+	167Q1	+
39Q	+	5Q	+
75Q	+	125Q	+
56Q1	+	174Q	+
19Q3	-	16Q	+
59Q1	+	53Q2	-
41Q2	+	59Q3	+
50Q2	+	164Q2	-
60Q2	-	167Q2	+
63Q	-	54Q1	+
19Q1	-	183Q	-
44Q1	+	71Q1	+
90Q1	+	19Q5	-
290Q2	+	25Q	+
14Q3	+	72Q	-
14Q2	-	17Q	+
14Q	-	64Q1	+
183Q1	+	64Q	+
71Q	+	189Q	+
61Q	+	161Q5	-
37Q4	+	161Q3	+
48Q1	+	161Q4	+
55Q2	-	161Q2	-

APPENDIX F

ANTIBIOTIC RESISTANCE OF ISOLATES

Isolate	Amp.	Pen.	Chloram.	Isolate	Amp.	Pen.	Chloram.
249Q	+	+	-	39Q1	+	+	-
36Q1	+	+	-	43Q	+	+	-
70Q	+	+	-	19Q4	+	+	-
58Q2	+	+	+	184Q	+	+	-
54Q2	+	+	-	56Q2	+	+	-
290Q1	+	+	-	14Q4	+	+	-
284Q	+	+	-	53Q3	+	+	+
50Q3	+	+	-	69Q1	+	+	-
53Q1	+	+	-	24Q	+	+	-
59Q2	+	+	-	68Q	+	+	-
55Q3	+	+	-	58Q3	+	+	-
55Q1	+	+	-	74Q	+	+	+
52Q3	+	+	-	19Q2	+	+	+
167Q	+	+	+	73Q	+	+	-
45Q1	+	+	-	37Q1	+	+	-
50Q1	+	+	-	65Q	+	+	-
20Q1	+	+	-	62Q	+	+	-
169Q	+	+	+	37Q3	+	+	-
14Q1	+	+	-	37Q2	+	+	-
19Q	+	+	-	52Q1	+	+	-
284Q1	+	+	-	60Q1	+	+	-
20Q3	+	+	-	44Q2	+	+	-
41Q3	+	+	-	21Q	-	+	+
41Q	+	+	-	45Q2	+	+	+
58Q1	+	+	-	184Q1	+	+	-

Isolate	Amp.	Pen.	Chloram.	Isolate	Amp.	Pen.	Chloram.
67Q	+	+	-	164Q1	+	+	+
69Q	+	+	-	57Q1	+	+	-
36Q2	+	+	-	167Q1	+	+	-
39Q	+	+	-	5Q	+	+	-
75Q	+	+	-	125Q	+	+	-
56Q1	+	+	-	174Q	+	+	-
19Q3	+	+	-	16Q	-	-	+
59Q1	+	+	+	53Q2	+	+	-
41Q2	+	+	-	59Q3	+	+	-
50Q2	+	+	-	164Q2	+	+	+
60Q2	+	+	-	167Q2	+	+	+
63Q	+	+	-	54Q1	+	+	-
19Q1	+	+	-	183Q	+	+	+
44Q1	+	+	-	71Q1	+	+	-
90Q1	+	+	-	19Q5	+	+	-
290Q2	+	+	-	25Q	-	+	+
14Q3	+	+	-	72Q	-	+	+
14Q2	+	+	-	17Q	+	-	-
14Q	+	+	-	64Q1	+	+	+
183Q1	+	+	-	64Q	+	+	-
71Q	+	+	-	189Q	+	+	-
61Q	+	+	-	161Q5	+	+	+
37Q4	+	+	-	161Q3	+	+	+
48Q1	+	+	-	161Q4	+	+	+
55Q2	+	+	-	161Q2	+	+	+

(Abbreviations: Amp. ampicillin; Pen. penicillin; Chloram. Chloramphenicol)

APPENDIX G

RESULTS OF GENE CONTENT BY PCR

Gene content	Isolate name
cry1, cry4	249Q, 54Q2, 57Q1
cry1, cry2	36Q1
cry1, cry4, cyt1	70Q, 45Q1, 169Q, 5Q, 167Q2, 183Q
cry1, cyt2	290Q1, 58Q2, 50Q3, 14Q1, 43Q, 90Q1
cyt2	59Q1, 50Q2, 290Q1, 58Q1, 14Q4, 53Q3, 21Q, 45Q2
cry1, cry2, cyt2	19Q1, 284Q, 53Q1, 167Q, 19Q4, 67Q
cry1, cry9, cyt1, cyt2	55Q3
cry1, cry4, cyt2	55Q1, 50Q1, 20Q1, 73Q, 63Q, 44Q1, 55Q2
cry1, cry4, cry9, cyt1, cyt2	52Q3
cry2, cyt2	19Q, 52Q1, 60Q2
cry4, cyt1	284Q1
cry1, cry9, cyt2	59Q2, 20Q3, 71Q
cry1, cry4, cry9, cry11, cyt2	41Q3, 14Q, 189Q
cry4, cyt2	164Q2, 41Q, 69Q1, 65Q, 37Q3, 44Q2, 14Q3
cry1, cry4, cry9, cyt2	39Q1, 56Q2
cry4, cry9, cyt1	24Q, 71Q1
cry4, cyt1, cyt2	68Q, 62Q, 37Q2
cry4, cry11, cyt1, cyt2	58Q3
cry1, cry4, cry9, cry11, cyt1	74Q
cry1, cry2, cry9, cyt2	19Q2, 61Q
cry1, cry2, cry4, cry9, cyt1, cyt2	53Q2

Gene content	Isolate name
cry4, cry9, cry11, cyt2	37Q1
cry1, cry2, cry11, cyt1	60Q1
cry1, cry9, cyt1	184Q1
cry4, cry9, cry11, cyt1, cyt2	69Q, 25Q
cry1, cry11, cyt1, cyt2	36Q2
cry1, cry2, cry4, cyt1, cyt2	39Q, 125Q
cry2, cyt1, cyt2	75Q
cry1, cry4, cry11, cyt2	56Q1
cry1, cry2, cyt1, cyt2	19Q3, 19Q5
cry2, cry4, cry11, cyt2	41Q2
cry1, cry2, cry4, cyt1	14Q2
cry4	183Q1
cry1, cry4, cyt1, cyt2	37Q4, 48Q1, 164Q1, 167Q1, 59Q3
cry1, cyt1	174Q
cry1, cyt1, cyt2	16Q, 54Q1
cry1,cry2,cry4, cry9, cry11, cyt1,cyt2	72Q
cry2, cry4, cry9, cry11, cyt1, cyt2	64
cry1, cry11, cyt2	161Q5, 161Q2
cry1, cry9, cry11, cyt2	161Q3, 161Q4
cry1, cry2, cry4, cyt2	184Q