CHAPTER-I

1. INTRODUCTION

Bacillus thuringiensis is a Gram positive, spore-forming, rod-shaped bacteria belonging to the family Bacillaceae (Schnepf *et al.,* 1998). It produces parasporal crystal proteins that exhibit specific insecticidal and nematicidal activities (Khyami-Horani *et al.,* 1996; Carneiro *et al*., 1998; Al-Banna and Khyami-Horani, 2004). Thus, *B. thuringiensis* could offer a remarkable alternative to chemical pesticides since it is benign to the environment and could be genetically engineered into crops to provide constant protection (Marroquin *et al*., 2000). Microbial insecticides are especially valuable because their toxicity to non-target animals and human is extremely low. Compared to other commonly used insecticides they are safe for both the pesticides user and consumers of treated foods (Neppl, 2000). The soil bacterium *B. thuringiensis* fulfills the requisites of a microbiological control agent against agricultural pest and vectors of disease that led to its wide spread commercial applications (Ben-Dov *et al*., 1999).

More than 50,000 strains isolated from several environments such as insects, stored products, plants, soil and marine environments are currently recognized (Sanchis *et al*., 1996). However, investigators have shown that these *B. thuringiensis* strains varied in their pesticidal activities (Sharma, 1994; Leyns *et al*., 1995; Khyami-Horani *et al*., 1996; Carneiro *et al*., 1998; Al-Banna and Khyami-Horani, 2004). The main target pest of *B. thuringiensis* insecticides include various lepidopterous (butterfly), dipterous (flies and mosquitoes), and individual coelopterous (Beatle) species. Some strains have also been found to kill off nematodes (Edward *et al*., 1998).

Differentiation and grouping of such a huge number of strains are absolutely necessary to infer their microbial activity and to enable the tracking of strains dispersed in the

environment. The taxonomy of *B. thuringiensis* is based on morphological and biochemical characteristics. Whereas flagellin H-antigen serotyping has been used for classification and identification of *B. thuringiensis* varieties (de Barjac and Bonefoi, 1962; Xu and Cote, 2006), isolates, within the same serotype vary in their biochemical characteristics, plasmid patterns and microbial activities. Moreover, the "auto-agglutinate" *B. thuringiensis* strains and non-motile strains are not typable by this H-serotyping method (Sadder *et al*., 2006). Furthermore, pulsed field gel electrophoresis (PFGE) is another method used for genotyping different bacterial strains of a specific species as in the case of *B. thuringiensis* strains (Gaviria Rivera and Priest, 2003b).

However, as PFGE requires special equipment and chemicals, it is not possible to be conducted in many laboratories. Currently, molecular typing methods such as ribosomal RNA gene fragment length polymorphism (Priest *et al*., 1994), rRNA gene intergenic spacer sequences comparison (Borque *et al*., 1995), and randomly amplified polymorphic DNA (RAPD) analysis (Hansen *et al*., 1998) have been applied to differentiate isolates of *B. thuringiensis* strains. The latter (RAPD) is widely used to genotype different species of bacilli, e.g. *B. anthracis* (Levy *et al*., 2005), *B. subtilis, B. thuringiensis* (Matarante *et al*., 2004), and thermophilic bacilli (Ruckert *et al*., 2004).

RAPD analysis produces reproducible and often distinctive sets of DNA fragments by subjecting genomic DNA to PCR primed by short (10-25 base) oligonucleotide primers of arbitrary sequences (Welsh *et al*., 1990; Williams *et al*., 1990).
This approach has been applied to detect genomic diversity among plants (Rafalski *et al*., 1993; Wolff *et al*., 1993), animals (Rothuizen *et al*., 1994), and microbial organisms (Akopyanz *et al*., 1992; Kauppinen *et a*l., 1994; Mazurier *et al*., 1992). The attractiveness of this method is that no knowledge of the sequence of the target organism is required and a very large number of arbitrary primers can be tested to identify those that might be suited to a particular application. It is fast and simple.

Several *B. thuringiensis* have been isolated and characterized from different habitats of Nepal and their insect toxicity had also been tested (Subedi, 1999; Bhattarai, 2002; Shrestha *et al*., 2006). Oligonucleotide primers that are capable of recognizing polymorphisms among *B. thuringiensis* isolates from Khumbu region had also been identified (Sahukhal *et al*., 2006). This study was conducted to assess the DNA polymorphism within subspecies and between subspecies of *B. thuringiensis* isolated from two different regions (Khumbu base camp and Biratnagar).

CHAPTER-II

2. OBJECTIVES

2.1 GENERAL OBJECTIVE

- To study the genetic similarities among *Bacillus thuringiensis* isolates from Tangboche of Khumbu region and Biratnagar of Terai region by RAPD-PCR

2.2 SPECIFIC OBJECTIVES

i. To isolate *B. thuringiensis* from soil samples of Tangboche and Biratnagar
ii. To identify and characterize *B. thuringiensis* isolates biochemically
iii. To extract the total genomic DNA from *B. thuringiensis* isolates
iv. To perform RAPD-PCR of the isolates
v. To assess the inter-subspecies genetic similarities of the isolates
vi. To assess the intra-subspecies genetic similarities of the isolates from different regions

CHAPTER - III

3. LITERATURE REVIEW

3. 1. *BACILLUS THURINGIENSIS*

Bacillus thuringiensis is a ubiquitous gram positive, spore forming bacterium that forms a parasporal crystal during the stationary phase of its growth cycle. *B. thuringiensis* was initially characterized as insect pathogen, and its insecticidal activity was attributed largely or completely (depending upon the insects) to the parasporal crystals. This observation leads to the development of bioinsecticides based on *B. thuringiensis* for the control of certain insect species among the order Lepidoptera, Diptera, and Coleoptera. There are more recent reports of *B. thuringiensis* isolates active against other insects of order (Hymenoptera, Homoptera, Orthoptera, and Mallophaga) and against Nematodes, mites and protozoa. *B. thuringiensis* is already a useful alternative or supplement to the synthetic chemical pesticide application in commercial agriculture for pest management and mosquito control. It is also a key source of genes for transgenic expression to provide pest resistant in plants (Schnepf *et al.*, 1998).

3.2. ECOLOGY AND PREVALENCE

B. thuringiensis seems to be indigenous to many environments. Strains have been isolated worldwide from many habitats, including soil, insects, dead insects, sericulture environments, forests and cultivated soils, stored products dust, and coniferous and deciduous leaves. Because of the economic importance of *B. thuringiensis*, many researchers have investigated the distribution of *B. thuringiensis* in various geographical regions and in different sources (Schnepf *et al.*, 1998).

The diversity in flagellar H-antigen agglutination reaction is one indication of enormous genetic diversity among *B.thuringiensis* isolates. The Pasteur Institute has catalogued 55 different flagellar serotypes and 8 non-flagellar biotypes (Schnepf *et al.*, 1998).

3.3. MORPHOLOGY AND PHYSIOLOGY

B. thuringiensis has dimensions ranging from 3-5 µm in length and 1-1.2 um in width. It is an entomopathogenic, spore forming *Bacillus* whose spore is ellipsoidal to cylindrical in shape with a thin spore wall enclosed by two envelops, the endo- and exosporium. Spore are central to terminal in position without distentention of the mother cell, and inclusion body inside the cell *B. thuringiensis* produces crystalline parasporal protein body, rarely two or three, in the cell during the phase of spore formation. The protein body stains like other cell material and is found outside the exosporium and separates readily from the liberated spore. Motility could be seen in some but not in all strains (Subedi, 1999 and Bhattarai, 2002).

Different strains of *B. thuringiensis* show a slight variation in their physiological characteristics such as production of acetoin, lecithinase, proteolytic enzymes, urease and fermentation of sucrose and salicin. These properties have been utilized for the recognition of strains; their association with particular serotypes has diminished as the number of isolates increases (Subedi, 1999 and Bhattarai, 2002). *B. thuringiensis* is able to ferment carbohydrates to produce acid from sugars like sucrose, glucose, mannose, salicin, esculin etc. but do not produce gas. It can as well as grow in aerobic and anaerobic conditions provide sufficient nutrients (Subedi, 1999 and Bhattarai, 2002).

Chemical tests shows that *B. thuringiensis* is able to reduce nitrate to nitrite or free nitrogen. It is catalase positive. It can utilize citrate as a sole source of carbon metabolism. Most strains give VP test and urease positive (Subedi, 1999 and Bhattarai, 2002). *B. thuringiensis* can hydrolyze gelatin, starch and skimmed milk and lecithin. Sporulation and crystal production occur best with vigorous aeration of liquid cultures or on solid media where the surface growth is exposed to atmospheric oxygen (Subedi, 1999 and Bhattarai, 2002). *B.thuringiensis* is unable to grow at 5ºC. However 11-89% is able to grow at 10ºC. It grows fully at various temperatures from 30-40ºC (Sneath, 1986) but unable to grow at and above 50ºC. *B.thuringiensis* shows pH ranging towards

the neutral condition is more preferable for the production of crystal proteins (Subedi, 1999 and Bhattarai, 2002).

3.4 HISTORY OF *B.THURINGIENSIS*

This common soil bacterium, most abundantly found in grain dust from silos and other grain storage facilities, was discovered first in Japan in 1901 by Ishawata and then in 1911 in Germany by Berliner. Chehsire and Cheyne (1885) studied foul wood disease of the honeybee and Ishwata (1902) isolated a spore forming aerobic bacteria from the diseased silkworm. In 1915, Berliner isolated *Bacillus* from the diseased larvae of the Mediterranean flour moth Anagasta (ephestia) in Thuringin; Germany. Later, Mates (1927) isolated the *Bacillus* from the same insect host, which Berliner had found earlier. A Canadian worker Hannay while studying sporulation in *B. thuringiensis* observed the inclusion bodies besides the spores, as Berliner. He suggested that these inclusions might be involved in the insect disease caused by these bacteria. Another Canadian worker T. Angus (1954-1956) confirmed the work of Aoki and Chigasaki as well as Hannay's suggestions of relationship between pathogenicity and the crystals (Neppl, 2000).

Subsequently there are thousands of strains of *B. thuringiensis* exist. Each strain produces its own unique insecticidal crystal protein, or δ-endotoxin, which is encoded by a single gene on a plasmid in the bacterium. *B. thuringiensis* toxins are biodegradable and do not persist in the environment (Neppl, 2000). *B. thuringiensis* first became available as a commercial insecticide in France in 1938 and in the 1950s entered commercial use in the United States. In 1987 came the first reports of insertion of genes encoding for *B. thuringiensis* δ-endotoxins into plants. The first transgenic plants to express *B. thuringiensis* toxins were tobacco and tomato plants. Although using *B. thuringiensis* in the form of transgenic crops is now very common, the more traditional spray form of *B. thuringiensis* is still widely used (Neppl, 2000).

3.5. CLASSIFICATION AND NOMENCLATURE

Since the first cloning of an insecticidal crystal protein gene from *B. thuringiensis* many other such genes have been isolated. Initially, each newly characterized gene or protein received an arbitrary designation from its discoverers: *icp*, *cry*, *kurhd1,* Bta, bt1, bt2 etc.type B and type C, and 4.5 kb, 5.3 kb, and 6.6 k*B.* The first systematic attempt to organize the genetic nomenclature relied on the insecticidal activities of crystal proteins for the primary ranking of their corresponding genes. The *cry* I genes encoded proteins toxic to lepidopterans; *cry*II genes encoded proteins toxic to both lepidopterans and dipterans; *cry*III genes encoded proteins toxic to coleopterans; and *cry*IV genes encoded proteins toxic to dipterans alone (Crickmore *et al.*, 1998)

This system provided a useful framework for classifying the ever-expanding set of known genes. Inconsistencies existed in the original scheme, however, due to attempts to accommodate genes that were highly homologous to known genes but did not encode a toxin with a similar insecticidal spectrum. The *cry*IIB gene, for example, received a place in the lepidopteran dipteran class with *cry*IIA, even though toxicity against dipterans could not be demonstrated for the toxin designated CryIIB. Other anomalies arose after the nomenclature was established. The protein named CryIC, for example, was reported to be toxic to both dipterans and lepidopterans, while the protein designated CryIB was reported to be toxic to both lepidopterans and coleopterans. Because the nomenclature system provided no central committee or database to maintain standardization, new genes encoding a diverse set of proteins without a common insecticidal activity each received the name *cry*V, based on the next available Roman numeral (Crickmore *et al.*, 1998). The known *cry* and Cyt gene sequences with revised nomenclature assignments and Phylogram demonstrating amino acid sequence identity between Cry and Cyt proteins as proposed by Crickmore *et al.*, 1998 (Appendix-X).

Currently 45 different serotypes of *B. thuringiensis* have been classified as 58 serovars (Bravo *et al.*, 1998). Many Cry protein genes have been cloned, sequenced, and named

cry and *cyt* genes. To date, over 100 *cry* gene sequences have been determined and classified in 22 groups and different subgroups with regard to their amino acid similarity. The proteins toxic for lepidopteran insects belong to the Cry1, Cry9, and Cry2 groups; toxins active against coleopteran insects are the Cry3, Cry7, and Cry8 proteins as well as the Cry1B and Cry1I proteins, which have dual activity. The Cry5, Cry12, Cry13, and Cry14 proteins are nematicidal, and the Cry2, Cry4, Cry10, Cry11, Cry16, Cry17, Cry19, and Cyt proteins are toxic for dipterans insects (Bravo, 1997).

3.6. *B. THURINGIENSIS* GENOME

B. thuringiensis strains have a genome size of 2.4 to 5.7 million bp. Most *B. thuringiensis* isolates have several extrachromosomal elements, some of them circular and others linear. The proteins comprising the parasporal crystal are encoded by large plasmids. Sequences hybridizing to *cry* gene probes occur among *B. thuringiensis* chromosomes as well, although it is unclear to what degree this chromosomal homolog contributes to production of the crystal (Schnepf *et al.*, 1998).

3.7. TOXIN STRUCTURE

To date, the structures of three crystal proteins—Cry3A, Cry1Aa, and Cyt2A have been solved by X-ray crystallography. Cry3A and Cry1Aa have about 36% amino acid sequence identity. This similarity is reflected in their three-dimensional structures; the corresponding domains can virtually be superimposed. Cyt2A, however, shows less than 20% amino acid sequence identity with Cry1Aa and Cry3A, and a similar alignment score would be obtained if the Cyt2A sequence were randomized. The structure of Cyt2A is radically different from the other two structures. The structures of Cry1Aa, Cry3A, and Cyt2A are compared in Fig. 1. The Cyt toxins, unlike the Cry δ-endotoxins, are able to lyse a wide range of cell types in-*vitro*. Cyt2A consists of a single domain in which two outer layers of α-helix wrap around a mixed β-sheet. Cyt1A is believed to have a similar structure. Cry3A and Cry1Aa, in contrast to Cyt2A, both possess three domains. Domain I consists of a bundle of seven antiparallel α-helices in

which helix 5 is encircled by the remaining helices. Domain II consists of three antiparallel β-sheets joined in a typical "Greek key" topology, arranged in a so-called β-prism fold (330, 343). Domain III consists of two twisted, antiparallel β-sheets forming a β-sandwich with a "jelly roll" topology (Schnepf *et al.*, 1998).

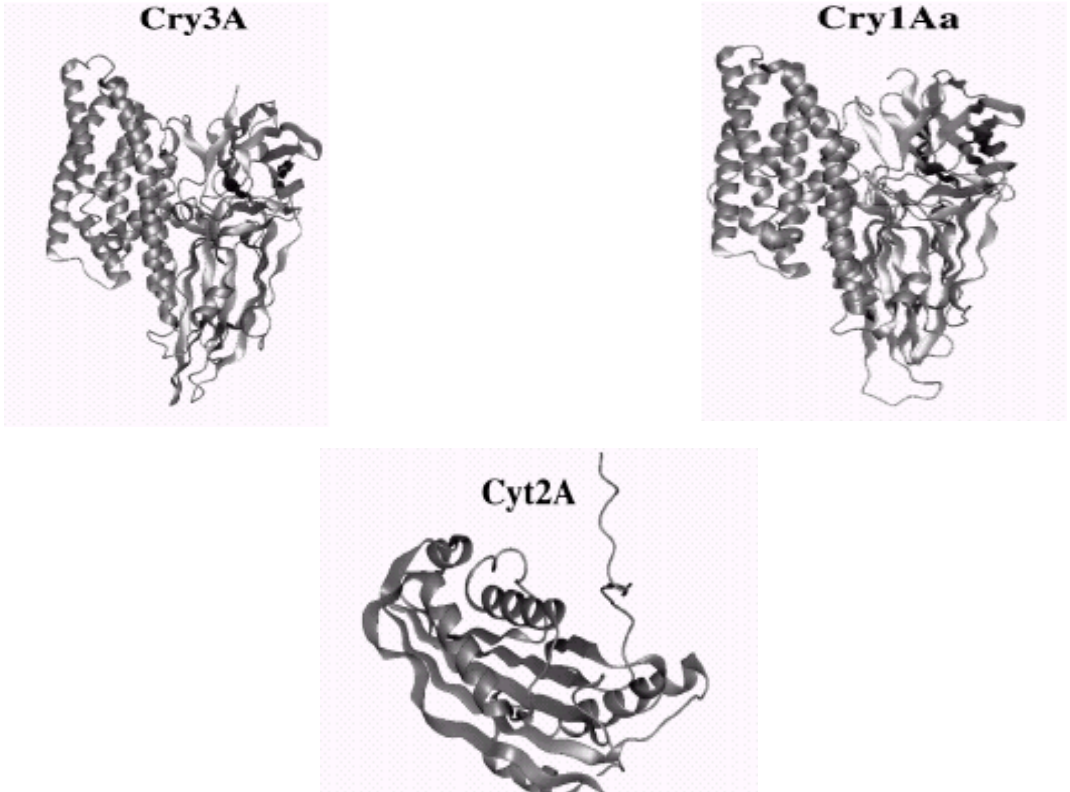


Figure 1: Three dimensional structures of Cry1A, Cry3A and Cyt2A

3.8. MECHANISM OF ACTION OF TOXIN (INSECTICIDAL ACTIVITY)

The mechanism of action of *B. thuringiensis* crystal proteins involves solubilization of the crystal in the insect midgut, proteolytic processing of the protoxin by midgut proteases, binding of the Cry-toxin to mid gut receptors and insertion of the toxin into the apical membrane to create ion channels or pores (Schnepf *et al.*, 1998). After solubilization, many protoxins must be processed by insect midgut proteases to become activated toxins. The major proteases of the lepidopteran insect midgut are trypsin-like or chymotrypsin-like. The Cry1A protoxins are digested to a 65-kDa toxin protein in a processive manner starting at the C terminus and proceeding toward the 55- to 65-kDa

toxic core. The carboxy terminal end of the protoxin, which initially appears to be wound around the toxin in an escargot-like manner, is clipped off processively in 10-kDa sections during processing of the protoxin. DNA is intimately associated with the crystal and appears to play a role in proteolytic processing. The mature Cry1A toxin is cleaved at R28 at the amino-terminal end; Cry1Ac, at least, is cleaved at K623 on the carboxy-terminal end. Two stages of processing have been detected for Cry1Ia with trypsin or *Ostrinia nubilalis* midgut proteases: a fully toxic intermediate, with an N terminus at protoxin residue 45 and a C terminus at residue 655 or 659, is further processed to a partially toxic core, with an N terminus clipped to residue 156 (Schnepf *et al.*, 1998).

Activated *cry* toxins have two known functions, receptor binding and ion channel activity. The activated toxin binds readily to specific receptors on the apical brush border of the midgut microvilli of susceptible insects. Binding is a two-stage process involving reversible and irreversible steps. The latter steps may involve a tight binding between the toxin and receptor, insertion of the toxin into the apical membrane, or both. It has been generally assumed that irreversible binding is exclusively associated with membrane insertion. The truncated Cry1Ab molecules containing only domains II and III can still bind to midgut receptors, but only reversibly, supports the notion that irreversible binding requires the insertion of domain I. Tight binding of Cry1Aa and Cry1Ab to purified *Manduca sexta* aminopeptidase N (APN) has been observed, and Cry1Ac may also show some degree of irreversible binding to *M. sexta* APN. There are likewise indications of irreversible binding for Cry1Ac to purified *Lymantria dispar* APN. Finally, Vadlamudi *et al.* calculated similar binding constants when toxin bound to brush border membrane vesicles (BBMV) and to nitrocellulose-immobilized receptor (i.e., a ligand blot). In *M. sexta,* the Cry1Ab receptor is believed to be a cadherin- like 210-kDa membrane protein, while the Cry1Ac and Cry1C receptors have been identified as APN proteins with molecular masses of 120 and 106 kDa, respectively. Incorporation of purified 120-kDa APN into planar lipid bilayers catalyzed channel formation by Cry1Aa, Cry1Ac, and Cry1C. There is also some evidence that domain II

from either Cry1Ab or Cry1Ac can promote binding to the larger protein, while domain III of Cry1Ac promotes binding to the presumed APN. Alkaline phosphatase has also been proposed to be a Cry1Ac receptor. In *Heliothis virescens*, three aminopeptidases bound to Cry1Ac on toxin affinity columns. One of them, a 170-kDa APN, bound Cry1Aa, Cry1Ab, and Cry1Ac, but not Cry1C or Cry1E. *N*-Acetylgalactosamine inhibited the binding of Cry1Ac but not that of Cry1Aa or Cry1A*B*. The three Cry1A toxins each recognized a high-affinity and a low- affinity binding site on this 170-kDa APN. In gypsy moth (*L. dispar*), the Cry1Ac receptor also seems to be APN, while Cry1Aa and Cry1Ab bind to a 210-kDa brush border membrane vesicle (BBMV) protein. In *Plutella xylostella* and *Bombyx mori* as well, APN appears to function as a Cry1Ac binding protein. An *M. sexta* gene encoding a Cry1Ab-binding APN has also been cloned, as has its *P. xylostella* homolog. Insertion into the apical membrane of the columnar epithelial cells follows the initial receptor-mediated binding, rendering the toxin insensitive to proteases and monoclonal antibodies and inducing ion channels or nonspecific pores in the target membrane (Schnepf *et al.*, 1998). The nature of the ion channel or pore-forming activity of *cry* toxins in the insect is still controversial. It is alternatively described as a large lytic pore that is not specific for particular ions or as an ion-specific channel that disrupts the membrane potential but does not necessarily lyse midgut epithelial cells (Schnepf *et al.*, 1998).

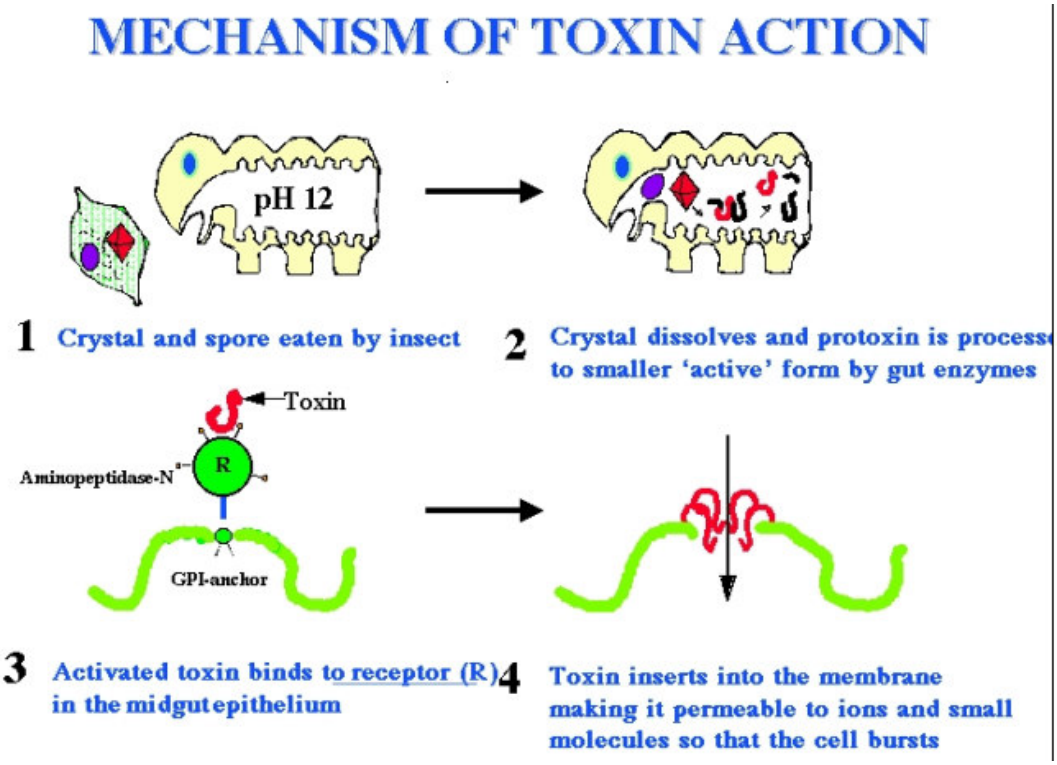


Figure 2: Mechanism of toxin action

3.9. DEVELOPMENT OF RESISTANCE TO *B. THURINGIENSIS*

The first evidence of resistance developing in the field against *B. thuringiensis* delta-endotoxins was published in 1985. Low levels of resistance were found in *Plodia interpunctella*, the Indianmeal moth, in storage bins of *B. thuringiensis*-treated grain. The *B. thuringiensis* resistance problem became greater when the first reports of high resistance to *B. thuringiensis* toxins in the field came in 1990 from Hawaii, Florida, and New York in the United States. The species found to be losing susceptibility to *B. thuringiensis* toxin was *Plutella xylostella*, and the diamondback moth. Resistance in *P. xylostella* was detected in several other countries, including Japan, China, the Philippines, and Thailand. Malaysia also reported *B. thuringiensis* resistance in the diamondback moth in 1990. Thus far *P. xylostella* is still the only insect species in

which very considerable resistance has been found to develop outside of the laboratory (Schnepf *et al.*, 1998).

Eleven species have developed resistance to various strains of *B. thuringiensis* toxin in the laboratory but not in the field: *Ostrinia nubilalis* (the European corn borer), *Heliothis virescens* (the tobacco budworm), *Pectinophora gossypiella* (the pink bollworm moth), *Culex quinquefasciatus* (the mosquito), *Caudra cautella* (the almond moth), *Chrysomela scripta* (the cottonwood leaf beetle), *Spodoptera exigua* (the beet armyworm), *Spodoptera littoralis* (the Egyptian cotton leaf worm), *Trichoplusia ni* (the tiger moth), *L. decemlineata* (the Colorado potato beetle), and *Aedes aegypti* (the yellow fever mosquito) (Schnepf *et al.*, 1998).

3.10. MECHANISM OF RESISTANCE

The mechanisms underlying *B. thuringiensis* resistance are found in the sequential steps in the proposed mode of action of *B. thuringiensis* toxins. Following the ingestion of *B. thuringiensis* crystals by susceptible insects, protoxins are solubilized and hydrolyzed by gut m-proteinases to an active toxin form. Activated *cry* proteins pass through the peritrophic membrane and bind to proteins in the brush border membrane of midgut epithelial cells. Toxin binding is followed by events that lead to cell lysis and disintegration of the brush border membrane, and eventually insect death. Any change in insect gut physiology that affects one or more steps in this process could prevent toxicity and lead to the development of resistant pest populations (Li *et al.*, 2004). Most studies have focused on two steps in the mode of action: proteolytic activation of protoxin and binding of active toxin to receptors. Reduced binding of Cry proteins to midgut receptors has been associated with resistance in several strains of *Plodia interpunctella, Plutella xylostella*, *Heliothis virescens*, *Spodoptera exigua*, and *Leptinotarsa decemlineata* (Li *et al.*, 2004)

However, a loss of toxin binding was not associated with resistance to *B. thuringiensis* in several insect. Therefore, other mechanisms of resistance may be operating in these

insects. Serine proteinases, such as trypsin, chymotrypsin, and elastase, are important in both the solubilization and activation of *B. thuringiensis* protoxins. In some insects, changes in these proteinases have been associated with resistance to *B. thuringiensis* toxin. A strain of *P. interpunctella* resistant to *B. thuringiensis* subsp. *Entomocidus* HD-198 processed *B. thuringiensis* protoxin at a slower rate than the parental susceptible strain. This resistant strain lacked a major gut proteinase involved in activation of *B. thuringiensis* protoxin, and the proteinase mechanism was responsible for about 90% of the total resistance to *cry*1Ab (Li *et al.*, 2004).

Enzymes from a strain of *H. virescens* resistant to *B. thuringiensis* subsp. *kurstaki* HD-73 were reported to activate the protoxin more slowly and degrade the toxin faster than enzymes from a susceptible strain. In *Spodoptera littoralis*, increases in the specific activity of gut proteinases were associated with the loss of sensitivity to Cry1C due to an increase in the degradation of active toxin. Aminopeptidase is an exopeptidase and a marker for membrane proteins. It is localized to the brush border membrane of midgut epithelial cells and is involved in the digestion of peptides and amino acid transport. A membrane-bound glycosyl-phosphatidylinositol anchored aminopeptidase N has been reported to bind Cry1Ac toxins in several different insects. Previously, a strain of *B. thuringiensis*-resistant *Ostrinia nubilalis*, selected for resistance to *B. thuringiensis* subsp. *kurstaki* (HD-1), was described with lower trypsin-like proteinase activity compared to the parental susceptible strain, but the reduced trypsin-like proteinase activity was in the soluble fraction of gut proteinases in the resistant strain. Soluble proteinases from the resistant strain incubated with Cry1Ab protoxin resulted in lower amounts of an active toxin fragment relative to incubations with proteinases from the susceptible strain. This reduction in toxin activation may account for lower susceptibility to *B. thuringiensis* toxins by resistant *O. nubilalis* larvae (Li *et al.*, 2004).

3.11. EFFECTS ON ANIMALS

Microbial pest control agents (MPCA) can, in principle, cause harmful effects via toxicity, inflammation, or a combination of these effects. The presence of bacteria in a

specimen derived from tissues does not necessarily mean infection. Colonization refers to the multiplication of MPCA either on the surface or within an animal/human organism without causing any tissue damage. Persistence refers to the ability to recover the inoculum of the MPCA over time. Persistence and transient disturbances of the normal microbial flora are to be expected after exposure of experimental animals to MPCA, since clearance of the inoculum is not instantaneous. Persistence may not be equated with infection (Siegel & Shadduck, 1990). Infection by a MPCA means that there is evidence of the establishment and proliferation of the MPCA in tissues, coupled with tissue damage. Evidence of multiplication includes a measurable increase in the total amount of MPCA, recovery of vegetative stages when spores were administered, and failure of the inoculum to clear. It cannot be determined solely on the basis of lesions since injection of foreign material can elicit an inflammatory process (Siegel *et al*., 1987). A classification of MPCA toxicity and infectivity has been proposed, in which MPCA is classified as toxic if an oral dose # 106 cfu per mouse causes mortality or clinical or pathological changes (Burges, 1980). However, any classification is very difficult because of the complexicity of the issue when dealing with living organisms (Ignoffo, 1973; Shadduck, 1983). Older reports do not discriminate between different strains of *B. t* but modern molecular techniques have proven that variability exists within strains with the same serotype (Helgason *et al*., 1998; Hansen and Hendriksen, 1997a,b). Mammalian toxicity studies on Bt-containing pesticides demonstrate that the tested isolates are not toxic or pathogenic (McClintock *et al*., 1995), as they occur in the products. Toxicity studies submitted to the US Environmental Protection Agency to support registration of *B. t* subspecies, and reviewed by McClintock *et al*. (1995), failed to show any significant adverse effects on body weight gain, clinical observations or upon necropsy.

3.12. EXPOSURE AND EFFECTS ON HUMANS

There are some case reports on the occurrence of *B.t* in patients with different infectious diseases. However, none of these studies demonstrate an actual risk to human health by the use of *B. thuringiensis*. They emphasize the need for further research on the

production of toxins, knowledge of factors causing the genes of the toxins to be expressed, and knowledge on the natural occurrence of *B. thuringiensis* and *B. cereus*. The medical practice does not discriminate between *B.t* and *B.c* as causative agents in infectious diseases. Therefore, the true proportion of *B.t* in *B.c*-induced disease is not known.

3.12.1. *Bacillus thuringiensis*

For aeons, humans have been exposed to Bt in their natural habitats, particularly from soil, water and the phylloplane. However in the recorded scientific literature, only few adverse effects to these environmental Bt levels have been documented. The manufacture and field application of Bt products can result in aerosol and dermal exposure of workers and the human population, especially by spraying programmes in populated areas. Agricultural and horticultural uses of Bt can also result in dietary exposure.

3.12.2. Experimental exposure of humans

Eight human volunteers ingested 1 gram of a Btk formulation (3 × 109 spores/g of powder) daily for 5 days. Of the eight volunteers, five also inhaled 100 mg of the Btk powder daily for five days. Comprehensive medical examinations immediately before, after, and 4 to 5 weeks later failed to demonstrate any adverse health effects, and all the blood chemistry and urinalysis tests were negative (Fisher and Rosner, 1959). Pivovarov *et al*. (1977) reported that ingestion of foods contaminated with Btg at concentrations of 105 to 109 cells/g caused nausea, vomiting, diarrhoea and tenesmus, colic-like pains in the abdomen, and fever in three of the four volunteers studied. The toxicity of the Btg strain may have been due to beta-exotoxin (Ray, 1990).

3.13. TECHNIQUES TO STUDY GENETIC POLYMORPHISM

Recent advances in molecular biology, principally in the development of the polymerase chain reaction (PCR) for DNA, DNA sequencing and data analysis, have

resulted in powerful techniques which amplifying can be used for the screening, characterization and evaluation of genetic diversity (Karp and Edward).

The polymorphic DNA markers that were shown to genetically link to a trait of interest could be used for individual and pedigree identification, pathogenic diagnostics, and trait improvement in genetics and breeding programs. Morphological criteria, biochemical data, isozyme electrophoresis, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNAs (RAPDs), and fluorescence *in situ* hybridization (FISH) have been so far used to analyze genetic similarity and diversity in genetics and breeding research of animal/plant/microbes (Yoon and Kim, 2001).

A whole range of different techniques can be used to detect polymorphisms at the DNA level, which falls into three broad categories with respect to the basic strategy (Karp and Edward).

a) Non-PCR based approaches,

b) PCR Arbitrary Priming, and

c) Targeted-PCR and sequencing.

3.13.1. Non-PCR based screening techniques

Restriction fragment length polymorphism (RFLP) analysis was the first technology developed which enabled the detection of polymorphisms at the sequence level. The approach involves digesting DNA with restriction enzymes, separating the resultant DNA fragments by gel electrophoresis, blotting the fragments to a filter and hybridizing probes to the separated fragments. RFLP analysis is used extensively in the construction of genetic maps and has been successfully applied to genetic diversity assessments, particularly in cultivated plants and in wild accessions. As a technique for diversity studies, there are three important advantages, which should be considered. The first is that RFLPs are highly reproducible between laboratories and the diversity profiles generated can be reliably transferred. The second is that RFLPs are co-dominant

markers, enabling heterozygotes to be distinguished from homozygotes. The third advantage is that no sequence-specific information is required and, provided suitable probes are available, the approach can be applied immediately for diversity screening in any system. (Karp and Edward).

3.13.2. PCR arbitrary priming techniques

With the advent of PCR, a number of techniques became available for the screening of genetic diversity. These require no prior sequence-specific information and can, therefore, be applied directly to any organism. The techniques are based on the use of a single 'arbitrary' primer, in a PCR reaction on genomic DNA and result in the amplification of several discrete DNA products. Each of these products will be derived from a region of the genome that contains two short segments with some homology to the primer, which is on opposite strands, and sufficiently close together for the amplification to work. A number of closely related techniques based on this principle were developed at the same time and are collectively referred to as multiple arbitrary amplicon profiling (MAAP) (Karp A and Edward K.J, http//www.ipgri.cjiar.org/publications).

The most commonly used is RAPDs (Random Amplified Polymorphic DNAs) analysis in which the primers are usually 10-mer or 20-mers and in which the amplification products are separated on agarose gels in the presence of ethidium bromide and visualized under ultraviolet light. AP-PCR (Arbitrary primed PCR) and DAF (DNA Amplification Fingerprinting) differ from RAPDs principally in primer length, the stringency conditions and the method of separation and detection of the fragments. In all cases, polymorphisms are detected as the presence or absence of bands and result from sequence differences in one or both primer binding sites (Karp and Edward).

The enormous attraction of RAPDs is that there is neither requirement for DNA probes, nor for any sequence information for the design of specific primers. The procedure involves no blotting or hybridizing steps. The technique is, therefore, quick, simple and efficient and only requires the purchase of a thermocycling machine and agarose gel

apparatus to set up in a laboratory for any new system under study. It requires small amounts of DNA (10ng per reaction) and sample throughput can be quite high (see annex). The procedure can also be made automatic with extremely high throughput. RAPDs have also been proved to detect higher levels of polymorphism compared with RFLPs in cases where the two techniques have been applied to the same material. They have been extensively used for screening diversity, particularly at intraspecific levels, including many population studies. Unfortunately, the approach has serious limitations (Karp and Edward).

The first concern is the nature of the data generated. RAPDs are dominant markers such that the homozygote conditions are the only genotypes discernible as presence or absence of the band. In addition, the presence of a band of apparently identical molecular weight in RAPD gels of different individuals cannot be taken as evidence that the two individuals have the same band, although this assumption is commonly made. Further complications are that single RAPD bands can be comprised of several co-migrating amplification products and, as in the case of DNA fingerprinting, there can be uncertainty in assigning markers to specific loci in the absence of preliminary pedigree analysis. Lynch and Milligan (1994) have recently discussed these limitations of RAPD for population genetic analysis and state 'provided there is only a single amplifiable allele per locus, this does not prevent the estimation of allele frequencies necessary for population-genetic-analysis, but it does reduce the accuracy of such estimation relative to analysis with co dominant markers (Karp and Edward).

The use of RAPDs for determining the distribution and extent of variation is challenged even further when the second general problem of RAPDs is considered concerning the robustness of the data generated. RAPDs are notoriously prone to user-error in that, unless the most consistent of conditions is strictly adhered to, the RAPD profiles obtained can vary considerably between different runs of the same sample. Even though careful practice quickly overcomes this problem, RAPD profiles are difficult to reproduce between laboratories, which may have different PCR machines or use different sources of polymerase and associated buffers. Even within a laboratory, the

time saved by the direct application of RAPDs is often lost in achieving consistency and in confirming the reproducibility of the results obtained. As PCR machines are being improved all the time and new thermostable polymerases continue to appear on the market, it is predictable that any particular data from RAPD profiles will have a transient life (Karp and Edward).

More recently, Keygene have developed a method which is equally applicable universally, which reveals very high levels of polymorphism and which is highly reproducible. This procedure, termed Amplified Fragment Length Polymorphism (AFLP) is essentially intermediate between RFLPs and RAPDs, in that the first step is restriction digestion of the genomic DNA but selective rounds of PCR amplification of the restricted fragments then follow this. The fragments are amplified by P^{33}- labelled primers designed to the sequence of the restriction site, plus one to three additional selected nucleotides. Only fragments containing the restriction site sequence plus the additional nucleotides will be amplified and the more selected nucleotides added on to the primer sequence (up to a maximum of three can be added at either site) the fewer the number of fragments amplified by PCR. This selection is necessary to achieve a total number of fragments within the range that can be resolved on a gel (approximately 150 to 200 fragments). The amplified products are normally separated on a sequencing gel and visualized after exposure to X-ray film. Recently, the technique has been automated, using fluorescent labelled primers and, therefore, high throughput can be achieved. Two different types of polymorphisms are detected: (1) point mutation in the restriction sites, or in the selective nucleotides of the primers which result in a signal in one case and absence of a band in the other; and (2) small insertions/deletions within the restriction fragment which results in different size bands. (Karp and Edward).

3.13.3. Targeted PCR and sequencing

The opposite approach to the arbitrary amplicon profiling procedures is to design primers to target specific regions of the genome. The targeted amplified product can be compared on an agarose gel to the corresponding product from another individual but

the resolution achievable will only detect differences in length of the fragment resulting from many base pair changes. In order to resolve all the possible sequence differences, it is necessary to sequence the entire fragment, either manually or using an automated DNA sequencer. Once this has been done, sequences from different individuals can be aligned, any differences detected and the data entered and analyzed in statistical packages. The fragment in which polymorphisms are studied is of known identity, therefore avoiding the ambiguities of analyzing RAPD and AFLP bands, or random RFLP probes. RFLPs, RAPDs and AFLPs provide indirect data that is only useful when converted into distance measures. This enables frequency data and distance measures to be determined for each genotype class but does not enable the classes to be ordered or grouped in any way. Data based on DNA sequences or restriction site mapping, on the other hand, provide the means of both classifying individuals into different classes and also of assessing relationships among the classes (Karp and Edward).

3.14.GENETICS OF *Bacillus thuringiensis*

3.14.1. The *B. thuringiensis* Genome

B. thuringiensis strains have a genome size of 2.4 to 5.7 million bp. Physical maps have been constructed for two *B. thuringiensis* strains (*B. thuringiensis* subsp, *Berliner*). Most *B. thuringiensis* isolates have several extrachromosomal elements, some of them circular and others linear. The proteins comprising the parasporal crystal are encoded by large plasmids. Sequences hybridizing to cry gene probes occur among *B. thuringiensis* chromosomes as well, although it is unclear to what degree these chromosomal homolog contribute to production of the crystal (Schnepf *et al*, 1998).

3.14.2. The Transposable Elements of *B. thuringiensis*

The *B. thuringiensis* species harbors a large variety of transposable elements, including insertion sequences and transposons (Schnepf *et al*., 1998).

There is structural association between *B. thuringiensis* transposable elements and the *cry* genes. The structural organization of the cry1A gene environment showed that genes of this type are flanked by two sets of inverted repeated sequences, designated as IS231 and IS232, revealed by nucleotide sequence analysis. IS231 belongs to the IS4 family of insertion sequences, and IS232 belongs to the IS21 family of insertion sequences. Because these elements can transpose it is likely that they provide mobility for the cry genes with which they form typical composite transposons (Schnepf *et al,* 1998).

Several IS231 variants have been isolated from various *B. thuringiensis* strains. In *B. thuringiensis* subsp. *israelensis*, an IS231 element (IS231W) is adjacent to the cry11Aa gene. Although IS231 elements are frequently associated with cry genes, IS231-related DNA sequences have also been found in strains of *B. cereus* and *Bacillus mycoides*. In contrast, IS232 has a much smaller range among the *B. thuringiensis* serovars, appearing in only 7 of 61. The cry4A gene of the *israelensis* subspecies is flanked by two repeated sequences in opposite orientations, designated IS240 and display features characteristic of insertion sequences. The IS240 transposase is homologous to those of the insertion sequences belonging to the IS6 family. IS240 is widely distributed in *B. thuringiensis* and is present in dipteran-active strains, upstream cry11B gene in the *B. thuringiensis* subsp. *jegathesan* and in the plasmid of the dipteran-active strain *B. thuringiensis* subsp. *fukuokaensis*. Insertion sequences have been found upstream of the cry1Ca gene and downstream of a cryptic cry2Ab gene. These elements encode putative transposase that have significant similarities with the transposase of the IS150 element from *Escherichia coli*. These potential transposable elements of *B. thuringiensis* consequently belong to the IS*3* family of insertion sequences (Schnepf *et al,* 1998).

The genetic and structural characteristics of this transposable element fulfilled the criteria of a Tn element, and it was designated Tn*4430*. Its transposase is homologous to those of the Tn*3* family. In contrast to Tn*3*, however, the site-specific recombinase that mediates Tn*4430* cointegrate resolution is not a resolvase but an integrase. Tn*4430* is

frequently found in the vicinity of genes of the *cry1A* type in various lepidopteran-active strains. A transposable element designated Tn*5401* was isolated from a coleopteran-active *B. thuringiensis* strain. Although nucleotide sequence analysis indicates that the structural organization of Tn*5401* is similar to that of Tn4430, they are not known to coexist in any *B. thuringiensis* strain. In *B. thuringiensis* subsp. *tenebrionis*, Tn5401 is located just downstream of the cry3Aa gene. Tn*5401* has been successfully used to construct a transposon insertion library in *B. thuringiensis* (Schnepf *et al*, 1998).

The transposable elements in *B. thuringiensis* are involved in the amplification of the *cry* genes in the bacterial cell and mediating the transfer of plasmids by a conduction process involving the formation of cointegrate structures between self conjugative plasmids and chromosomal DNA or non conjugative plasmids, although Tn*4430* mediates the transfer of non conjugative plasmids by a conduction process. Thus, a major adaptive function for these transposable elements may be the horizontal dissemination of genetic material, including *cry* genes (Schnepf *et al,* 1998).

3.14.3. Cry Gene Expression

A common characteristic of the *cry* genes is their expression during the stationary phase. Their products generally accumulate in the mother cell compartment to form a crystal inclusion that can account for 20 to 30% of the dry weight of the sporulated cells. The crystal protein synthesis in *B. thuringiensis* and its coordination with the stationary phase are controlled by a variety of mechanisms occurring at the transcriptional, posttranscriptional, and posttranslational levels (Schnepf *et al*, 1998).

3.14.3.1. Transcriptional Mechanisms

The *cry* genes are considered typical examples of sporulation-specific genes. However, it is necessary to distinguish, among the *cry* genes expressed during the stationary

phase, those that are dependent on sporulation from those that are not (Schnepf *et al,* 1998).

3.14.3.2. Sporulation-dependent *cry* Gene Expression

The *cry1Aa* gene is a typical example of a sporulation-dependent *cry* gene expressed only in the mother cell compartment of *B. thuringiensis* (Schnepf *et al*., 1998).

At the transcriptional level, the development of sporulation is controlled by the successive activation of σ factors, which bind the core RNA polymerase to direct the transcription from sporulation-specific promoters. These factors are the primary σ factor of vegetative cells, σA, and five factors called σH, σF, σE, σG, and σK, which appear in a temporally regulated fashion during development. The σA and σH factors are active in the predivisional cell, σE and σK are active in the mother cell, and σF and σG are active in the forespore (Schnepf *et al*, 1998).

Two-transcription start sites have been mapped (BtI and BtII), defining two overlapping, sequentially activated promoters. BtI is active between about *T*2 and *T*6 of sporulation and BtII is active from about *T*5 onwards (where *Tn* is *n* hours after the end of the exponential phase). Two other cry genes, cry1Ba and cry2Aa; also contain either BtI alone or BtI with BtII. The transcription of cry1Aa, cry1Ba, and cry2Aa, cry4Aa, cry4Ba, cry11Aa, cry15Aa etc, is likely to be σE- or σK-dependent. expression during sporulation and therefore considered to be sporulation dependent. However, low-level transcription of the cry4Aa, cry4Ba, and cry11Aa genes in *B. thuringiensis* has been detected during the transition phase, beginning at about T22 and lasting until the onset of sporulation. This expression may be due to the σH RNA polymerase, and it is suggested that Spo0A represses this weak expression, specific to the transition phase, when the cells enter the sporulation phase (Schnepf *et al,* 1998).

3.14.3.3. Sporulation-independent *cry* gene expression

The cry3Aa gene, isolated from the coleopteran-active *B. thuringiensis* var. *tenebrionis,* was found to be expressed during vegetative growth, but at a lesser extent than during the stationary phase. Analysis of lacZ transcriptional fusions and primer extension experiments indicates that the *cry3Aa* promoter is weakly but significantly expressed during vegetative growth, and is activated from the end of exponential growth until stage II of sporulation (about *T*3), and remains active until stage IV of sporulation (about *T*7). The cry3Aa promoter, although located unusually far upstream of the start codon (position 2558), resembles promoters recognized by the primary σ factor of vegetative cells, σA. A similar promoter was found 542 bp upstream of the start codon of the cry3Bb gene. The expression of cry3Aa is not dependent on sporulation-specific σ factors either in *B. subtilis* or in *B. thuringiensis*. Moreover, cry3Aa expression is increased and prolonged in mutant strains unable to initiate sporulation. The results indicate that cry3Aa expression is activated by a non-sporulation-dependent mechanism arising during the transition from exponential growth to the stationary phase (Schnepf *et al*, 1998).

3.14.3.4. Posttranscriptional Mechanisms

The stability of mRNA is an important contributor to the high level of toxin production in *B. thuringiensis*. The half-life of cry mRNA, about 10 min, is at least fivefold greater than the half-life of an average bacterial mRNA. Wong and Chang showed that the putative transcriptional terminator of the *cry1Aa* gene (a stem-loop structure) acts as a positive retro regulator. The fusion of a DNA fragment carrying this terminator with the 39 end of heterologous genes increases the half-life of their transcripts two- to threefold, which in turn increases the expression of their gene products. It has been demonstrated in other systems that the processive activities of 39-59 exoribonucleases are impeded by 39 stemloop structures. It is likely, then, that the *cry1Aa* transcriptional terminator increases the *cry* mRNA stability by protecting it from exonucleolytic

degradation from the 39 end. Similar terminator sequences, potentially able to form stable stem-loop structures, is found downstream from various *cry* genes and may contribute to their high-level expression by stabilizing the transcripts. Between the *cry3Aa* promoter, located from positions 2560 to 2600, and the translational start codon is a region involved at a posttranscriptional level with the accumulation of *cry3Aa* mRNA as a stable transcript with a 59 end corresponding to nucleotide position 2129. Deletion of 60 bp extending from nucleotide positions 2189 to 2129 has no detectable effect on the expression level or on the position of the 59 end of the transcript. It is likely, then, that the initial transcript, begun hundreds of bases upstream, is processed posttranscriptional. Insertion of the *cry3Aa* 59 untranslated region (extending from nucleotides 2129 to 212) between the *B. subtilis xylA* promoter and a *lacZ* reporter gene increases about 10-fold both the stability of the *lacZ* fusion mRNA and the production of β-galactosidase. Deletion and mutation analysis indicate that the sequence required for the stabilizing effect is a perfect Shine-Dalgarno sequence (GAAAGGAGG) mapping at a position between 2125 and 2117; this sequence has been designated STAB-SD. The stability of the *cry3Aa* mRNA could result from an interaction between the 39 end of 16S rRNA and STAB-SD. The binding of a 30S ribosomal subunit to this sequence may protect the mRNA against 59-39 ribonuclease activity, resulting in a stable transcript with a 59 end at nucleotide position 2129 (i.e., the limit of 30S subunit protection). Potential STAB-SD sequences are also present in similar positions upstream of the *cry3Ba*, *cry3Bb*, and *cry3Ca* genes (Schnepf *et al,* 1998).

3.14.3.5. Posttranslational Mechanisms

The Cry proteins generally form crystalline inclusions in the mother cell compartment. Depending on their protoxin composition, the crystals have various forms: bipyramidal (Cry1), cuboidal (Cry2), flat rectangular (Cry3A), irregular (Cry3B), spherical (Cry4A and Cry4B), and rhomboidal (Cry11A). This ability of the protoxins to crystallize may decrease their susceptibility to premature proteolytic degradation. However, the crystals

have to be solubilized rapidly and efficiently in the gut of insect larvae to become biologically active (Schnepf *et al,* 1998).

The structure and the solubility characteristics of a crystal presumably depend on such factors as the secondary structure of the protoxin, the energy of the disulfide bonds, and the presence of additional *B. thuringiensis*-specific components. Studies have shown that several cry1 genes cloned in *E. coli* or *B. subtilis* were able to direct the synthesis of biologically active inclusions, suggesting that the 130- to 140- kDa Cry1 protoxins can spontaneously form crystals. It is generally assumed that the cysteine-rich C-terminal half of the Cry1 protoxins contributes to crystal structure through the formation of disulfide bonds. A similar mechanism of protein self-assembly may be responsible for the crystal formation of other 130- to 140-kDa protoxins (e.g., Cry4, Cry5, and Cry7). The cysteine-rich C-terminal region is absent from the 73-kDa Cry3A protoxins. This protein forms a flat, rectangular crystal inclusion in which the polypeptides do not appear to be linked by disulfide bridges. Because this protein is able to form identical crystals in both *B. thuringiensis* and *B. subtilis*, it is possible that specific host factors are not required for the protein assembly. Analysis of the three-dimensional structure of the Cry3A toxin revealed the presence of four intermolecular salt bridges, which might participate in the formation of the crystal inclusion. It has been found that crystallization of Cry2A (71 kDa) and Cyt1A (27 kDa) requires the presence of accessory proteins. These proteins may act at a posttranslational level to stabilize the nascent protoxin molecule and to facilitate crystallization. However, the precise mechanism of their role in crystal formation has not been determined. Cry1Ia toxin can be found in the supernatant of *B. thuringiensis* cultures as a processed polypeptide of 60 kDa. Cry1Ia is an exported protein and therefore interacts with the cellular protein export machinery. Such a characteristic, together with the fact that this toxin is synthesized early in sporulation, may have implications for the significance of these toxins in the ecology of *B. thuringiensis* (Schnepf *et al*, 1998).

3.15. NUMERICAL INDEX OF THE DISCRIMINATORY ABILITY OF THE TYPING METHOD

Typing systems are used to define specific characteristics of the object under study. The procedures are specific for different phenotypic or genetic parameters and can be general (i.e, applicable to any microbial species) or species or genus specific (Belkum *et al*, 2001).

Epidemiological typing of bacterial strains can be carried by a variety of techniques, including serotyping, biotyping, bacteriophage and bacteriocin typing and genotyping. When microbial typing methods are compared, the three main characteristics that need to be considered are its typeability, reproducibility, and discriminatory power (Hunter and Gaston, 1988). The typeability of a method is the proportion of the population of distinct strains that can be assigned a type marker by that method (Hunter, 1990). The reproducibility of a typing method is the proportion of strains that are typed the same on repeat testing, preferably after a period of a few months (Hunter, 1990).

The discriminatory power of a typing method is its ability to distinguish between unrelated strains. It is determined by the number of types defined by the test method and the relative frequencies of these types (Hunter and Gaston, 1988). The discriminatory power of a method is an estimate of its ability to differentiate between two unrelated strains. Gaston and Hunter, 1988 have suggested that discriminatory power can be defined mathematically as the probability that the two strains chosen at random from a population of unrelated strains will be distinguished by that typing method. This probability can be calculated by Simpson's index of diversity, which was developed for the description of species diversity within an ecological habitat. This probability, numerical index of discriminatory power (D) is given by the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

Where s is the number of types, x_j is the number of population members falling into the j^{th} type, and N is the size of the population (Hunter and Gaston, 1988 and Hunter, 1990)

Thus, a D value of 1.0 would indicate that typing method was able to distinguish each member of a strain population from all other members of that population. Conversely, an index of 0.0 would indicate that all members of a strain population were of an identical type. An index of 0.50 would mean that if one strain was chosen at random from a strain population, there would be a 50% probability that the next strain chosen at random would be indistinguishable from the first (Hunter, 1990).

This equation can be applied both to a direct comparison of the discriminating power of typing methods and to analysis of the discriminating power of combined typing schemes (Hunter and Gaston, 1988). The acceptable level of discrimination will depend on a number of factors, but an index of greater than 0.90 would seem to be desirable if the typing results are to be interpreted with confidence (Hunter and Gaston, 1988).

3.16. DNA-FINGERPRINT SIMILARITY INDEX

DNA-fingerprint similarity is being used increasingly to make inferences about levels of genetic variation within and between natural populations. DNA-fingerprint similarity is generally defined as the fraction of shared bands. For individuals a and b, it is the number of common fragments in their fingerprint profiles (Nab) divided by the average number of fragments exhibited by both individuals,

$$Sab = 2\,Nab / Na+Nb$$

It would be useful if this index could be related to some standard population genetic parameter (Lynch, 1990).

CHAPTER – IV

4. MATERIALS AND METHODS

4.1 MATERIALS

The materials, different media and reagents used in this thesis work are given in Appendix-I, Appendix-II, Appendix-III and Appendix IV respectively.

4.2 METHODS

4.2.1 SAMPLE COLLECTION AND TRANSPORT

The soil samples were collected from the Biratnagar (Koshi, Nepal) and Tangboche region of Khumbu base Camp and were transported to Research Laboratory for Agricultural Biochemistry and Biotechnology (RLABB) where the study was carried out from May 2006 to May 2007. Soil samples were taken from 6-10 cm depth into sterile plastic bags sealed and then transported to the RLABB.

4.2.2 ISOLATION OF *BACILLUS THURINGIENSIS*

B. thuringiensis was isolated by Acetate selection method (Travers *et al.*, 1987). The soil samples (1gm) were taken in a sterile 100 ml conical flask containing one milliliter of 0.25M sodium acetate (pH 6.8) and 9 ml of Lauria Bertani (LB) broth (Appendix-II). The broth was incubated in the water bath shaker at 30^{o}C for 4 hours followed by heat treatment at 80^{o}C for 5 mins in hot water bath. One millilitre of the sample was dispensed in a sterile Petri plate in which 20 ml of molten Lauria agar media was poured, shake well and left till the media solidified and then incubated at 37^{o}C for 24 hours.

Selected colonies of *B. thuringiensis* were further isolated in pure form on the solidified nutrient agar (Appendix-II) plates by streak plate method.

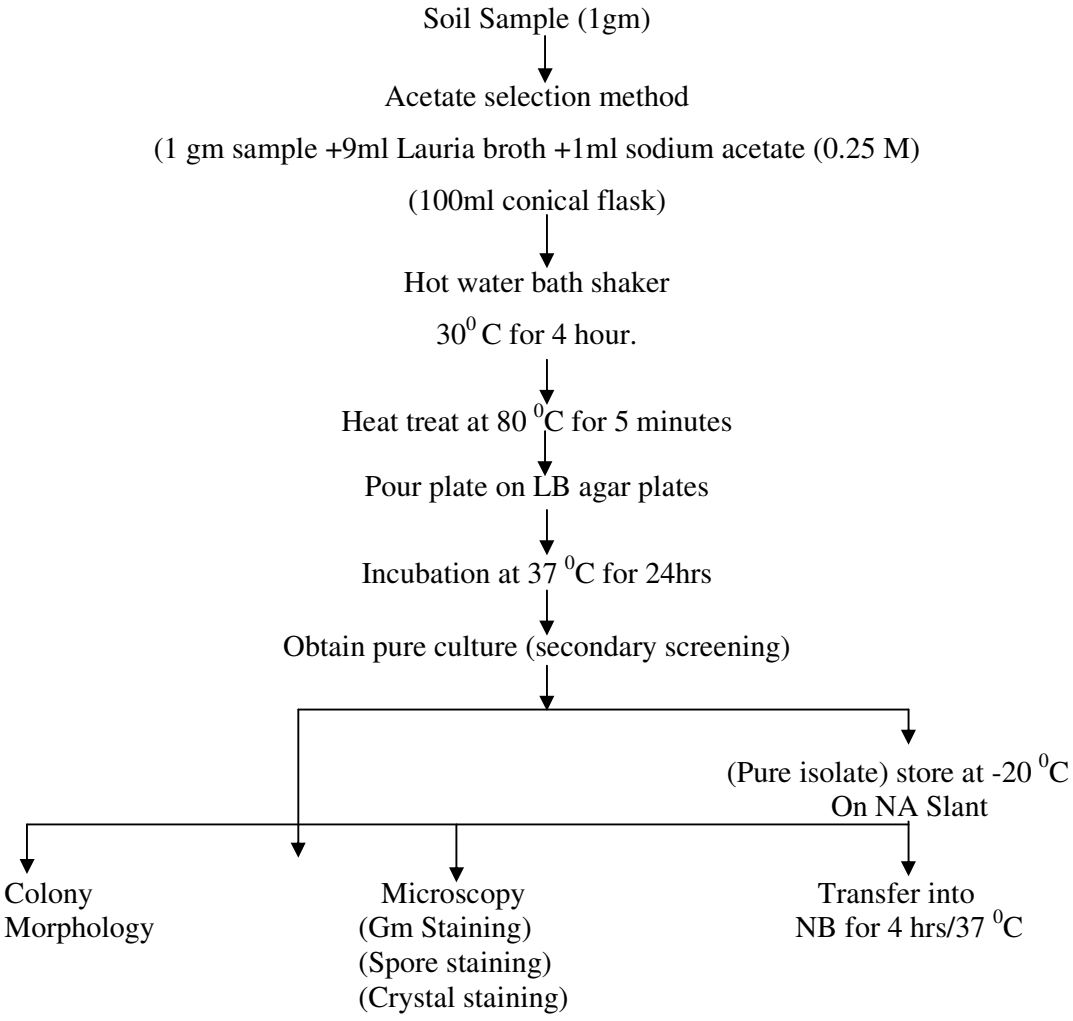
4.2.3 PRESERVATION OF THE ISOLATES

For long storage, purified *B. thuringiensis* were inoculated in Glycerol agar slants (Appendix II) and incubated at 30^{o}C for 24 hours to attain maximum growth with

sporulation. The tubes were then preserved in a refrigerator at -20^{o}C as a master culture and sub cultured at about three months interval. The sub culture was used for identification and RAPD-PCR.

4.2.4 IDENTIFICATION

The organisms were identified by standard microbiological techniques including colonial characteristics, morphological characteristics and biochemical characteristics (Bergey's Manual of Systematic Bacteriology, Volume 2, 1986) and biochemical types were also identified (Martin and Travers, 1989). The flowchart for the isolation of *Bacillus thuringiensis* is given below.



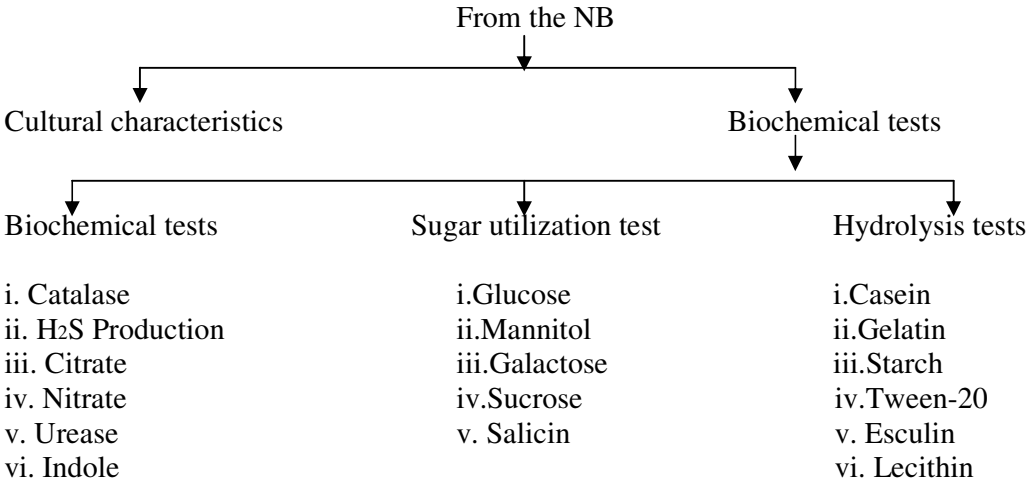


Figure 3. Flow chart for the isolation and identification of *B. thuringiensis* (Travers *et al*, 1987)

4.2.4.1 Microscopy

4.2.4.1.a Gram Staining

A thin smear of fresh culture was made in a clean, grease free glass slide and heat fixed. The heat fixed smear of bacterial culture was flooded with crystal violet (Apendix-III) for one minute and excess stain was washed out with distilled water. The slide was then flooded with Gram's iodine (Appendix-III) for 1 minute and again washed out with distilled water. Then it was flooded with decolorizer (95% ethyl alcohol) for 10-20 seconds. The smear was then again flooded with safranin (Appendix-III) for 1 minute and washed with distilled water. Excess water was removed by pressing the slide gently with blotting paper and finally air-dried slide was observed under the microscope.

4.2.4.1.b Spore Staining: (Schaeffer-Fulton spore stain method)

A thin film of culture was made on a clean, grease free glass slide. The smear was covered with malachite green (Appendix-III) and steamed over boiling water bath for

five minutes. Additional stain was added when stain boils off. After slide had cooled sufficiently, it was rinsed with water for 30 seconds followed by counter staining with safranin for about 20 seconds. Then it was rinsed with water and blot dried. The slide was examined under oil immersion.

4.2.4.1.c Crystals Staining

A thin film of five days old culture was made on a clean, grease free glass slide. The smear was air-dried and heat fixed by keeping at 110°C for 10 minutes. The smear was stained with 1.5% Amido black 10 B (Appendix-III) for 2 minutes and washed with distilled water. Then the slide was counter stained with Giemsa stain (Appendix-III) for one minute. It was again washed with water, blot dried and observed under microscope. The crystals were stained dark blue to black.

4.2.4.2 Biochemical Characteristics

For the study of biochemical characteristics of the organisms, the pure colonies were transferred into Nutrients broth (Appendix-II) and incubated at 37^0C for 4 hours. Then the isolates from Nutrient broth were used to inoculate respective biochemical media.

4.2.4.2.a Catalase test

A speck of the isolate was picked from the growth in agar plates with clean sterile glass rods and placed on a clean, flame sterilized glass slide. A drop of 3% H_2O_2 was added over the organism on a slide. Positive test was indicated by the production of gas bubbles.

4.2.4.2.b Oxidase test

Kovac's reagent coated stripe (Whatmann no. 1 filter paper impregnated with 1% tetra methyl –p phenylene diamine dihydrochloride) (Appendix-III) was taken in a clean dry

glass slide and moistened with distilled water. The colony to be tested was picked with a clean sterile glass rod and smeared over the moist area. Development of an intense deep purple blue indicated the positive reaction.

4.2.4.2.c Nitrate Reduction test

Each of the isolates was inoculated into test tubes dispensed with nitrate broth (Appendix-II) and incubated at 30°C for 24-48hours. Nitrate reduction was detected by adding few drops of Sulphanilic acid reagents and α-napthylamine reagents (Appendix-III) into the culture broth. A distinct red or pink color indicates the reduction of nitrate to nitrite.

4.2.4.2.d Urea Hydrolysis

Each test tube of sterile urea agar slant (Appendix-II) was inoculated with the isolates and incubated at 30°C for 24 hours. Observations were made to distinguish positive and negative results. Positive tests were confirmed by the change in color of the agar slant to pinkish red.

4.2.4.2.e Citrate utilization test

Organism was streaked on the slant of Simmon's citrate agar medium (Appendix-II) and incubated at 30°C for 24 hours. Positive tests were confirmed by growth of organism and development of intense blue color on the slant.

4.2.4.2.f Sugar utilization test

For this test, basal medium consisting of peptone, sodium chloride, and phenol red (Appendix-II) was incorporated with carbohydrate at the concentration of 1% (w/v). Each sugar was added to the separate broth, after the broth has been autoclaved. Then the mixture broth was dispensed into test tubes, which were inoculated with the isolates

and incubated at 30^{o}C for 24-48hours. Positive results were determined by the change in color of the broth from red to yellow. Sugars used were glucose, galactose, mannitol, sucrose and salicin.

4.2.4.2.g Hydrolysis tests

(i) Casein-hydrolysis

Solidified skim milk agar plates (Appendix-II) were divided into 6 sectors with each being streaked with the individual isolate. Observations were made after 24-48 hours of incubation at 30^{o}C, for the clear zone around the colony. To confirm, the plates were flooded with Mercuric chloride solution.

(ii) Gelatin hydrolysis

Each of the solidified gelatin agar plate (Appendix-II) was divided into 6-sectors and isolates were streaked on it. The plates were incubated at 30°C for 24-48 hours. Hydrolysis was confirmed by flooding the plates with mercuric chloride solution..

(iii) Starch-hydrolysis

Solidified starch agar plates (Appendix-II) were divided into 6 sectors and inoculated with the isolates and incubated for 24-48 hours at 30°C. The iodine solution was flooded onto the plates to ascertain the clear zone of hydrolysis around the colonies.

(iv) Tween-20 hydrolysis

The isolates were streaked on the solidified Tween 20 agar plates (Appendix-II) and incubated at 30^{o}C for 24-48 hours. Observations were made for white precipitation around the colony.

(v) Aesculin degradation

Aesculin degradation was determined after Kutzner (1976). Each of the isolates were inoculated into each Aesculin agar slants (Appendix-II) and incubated at 30^{o}C for 24-48

hours. Observations were made between the periods of incubation to check the blackening of the medium. Positive tests were confirmed comparing to the control, which was devoid, the substrate.

(vi) Lecithin degradation

The fresh culture was streaked on the egg-yolk agar medium (Appendix-II) and incubated at 37^{o}C for 24-48 hours. The presence of opalescence around the colony indicates the positive test.

4.2.5 DNA ISOLATION FROM *B. thuringiensis* ISOLATES

4.2.5.1 PREPARATION OF MASS CULTURE OF *B. thuringiensis*

The mass culture of *B. thuringiensis* was prepared in nutrient broth. The sterile broth in a conical flask was inoculated with pure cultures of *B. thuringiensis*. Flasks were then incubated at 30^{o}C in a water bath shaker for 20hrs.

4.2.5.2 ISOLATION OF GENOMIC DNA

Two methods of DNA extraction were used: Phenol-chloroform extraction and boiling cells method.

4.2.5.2.a. Boiling Cells Method

1. Bacteria were grown for 20 hours until log phase and were centrifuged (8,000rpm) for 5 minutes.
2. The pellet was washed with 0.85%NaCl solution and centrifuged again at 8,0000 rpm for 5 minutes.
3. The cell pellet was resuspended in 500µl of TE buffer (pH8.0) (Appendix-IV), freezed for 15 minutes at –20^{o}C and immediately boiled for 15 minutes in boiling water bath.

4. After centrifugation at 5,000 rpm for 3 minutes, the supernatant was transferred in clean tube and used for RAPD analysis.

4.2.5.2.b. Phenol-Chloroform Extraction Method

1. 1.5 milliliters of overnight culture was taken in a microfuge tube and centrifuged at 8000 rpm for 5 mins.
2. The cell pellet was resuspended in 500µl of 0.85% NaCl and centrifuged at 8000 rpm for 5 mins.
3. The pellet was again resuspended in 200µl of buffer A (0.2M Na-phosphate buffer in 20%sucrose, p^H-7.0) (Appendix-IV); 2.5 mg lysozyme was added and incubated at 37^0C for 1/2-1hr.
4. Then, 1200µl of lysis buffer (10mM Tris-Hcl, p^H-8.0, 1mM EDTA & 5%SDS) (Appendix-IV) was added and incubated at 37^oC for 1hour.
5. Equal volumes of Phenol: Chloroform (1:1) (Appendix-IV) was added and incubated at room temperature for 30 minutes with frequent shaking in between and centrifuged at 8000 rpm for 5 mins.
6. Upper clear supernatant was transferred into fresh, clean sterile microfuge tubes.
7. The DNA was precipitated by mixing with 0.1 volume of 3M sodium acetate solution (Appendix-IV) and two volumes of absolute (95%) ethanol and overnight incubation at -20^oC.
8. DNA was pelleted by means of centrifugation at 13,000 rpm for 15 minutes.
9. DNA was again precipitated with 1 ml absolute isopropanol and incubation at -20^oC for 30 minutes.
10. DNA was pelleted by centrifugation at 13,000 rpm for 15 minutes and supernatant discarded.
11. After completely drying the isopropanol, DNA was dissolved in 50 µl of TE buffer (10:0.1) (Appendix-IV).
12. DNA samples were stored at-20^oC until use.

4.2.6 DNA Quantization by spectrophotmetry

The DNA sample for quantization was removed from the cold storage, brought to room temperature and heated at 60^{o}C for 3 minutes (or 37^{o}C for 30 minutes). Bottom of the tube was flicked to ensure that the entire DNA has been suitably resuspended. Samples in microfuge tubes were given a quick spin and kept on ice prior to use. DNA concentrations were quantified photometrically at a wavelength of 260 nm as described by Sambrook et al. (1989).

1. The spectrophotometer was switched to on 30 minutes before the actual work or until the read-out had been stabilized.
2. Absorbance reading was adjusted to zero at wave length of 260nm using 500µl TE buffer as a blank.
3. 5 µl of buffer was carefully pipetted out of cuvette and 5 µl of sample DNA was added to the cuvette and mixed properly.
4. The absorbance reading of sample was taken at wave length of 260nm against the blank.
5. The concentration of DNA was calculated with assumption that an OD_{260} of 1.0 is equivalent to approximate 50µg/ml ds DNA.

$$[\mu g/ml = A_{260} \text{ X dilution X } 50].$$

4.2.7 RAPD-PCR Primer

A 10 bases long random sequence primer (obtained from University of British Columbia) was used in this study.

Table 1: Nucleotide sequence of RAPD-Primer

S. No.	Primer Code	Sequence of Primer (5'→3')	G+C mol% of Primer
1	284	CAG GCG CAC A	70

4.2.8 Random Amplified Polymorphic DNA (RAPD) PCR Amplification

The amplification reactions were carried out under a layer of mineral oil in 25 µl volumes containing 10mM Tris-HCl, 3mM $MgCl_2$, 40 pmol of a single RAPD primer,0.1% BSA, 200 µM dNTPs, 1U of Taq polymerase and 5µl of template DNA obtained from boiling cells method (Table 2).

Table 2: RAPD-PCR Reaction Mixture

Components		Volume (µl)	Final Concentrations
PCR Buffer pH 8.3	100mM Tris-HCl	2.5	10mM Tris-HCl
	30 mM $MgCl_2$		3mM$MgCl_2$
dNTP (2.5 mM)		2.0	200µM
Taq Polymerase (1U)		1.0	1U
Template DNA		5.0*	
Primer (10 µM)		4	40pmol
Bovine Serum Albumin (1%)		2.5	0.1%
Double distilled water		8	
Final Volume		25	

- 5-µl template obtained from boiling cells method.

The PCR program was run as follows:

Table 3: Thermal-cycling Profile for *Bacillus thuringiensis* isolates

Parameters	**Conditions**		**Number of Cycles**
	Temperature (^oC)	Time (min)	
Initial Denaturation	95	2	
Denaturation	94	2	4
Annealing	34	5	
Extension	72	5	
Denaturation	94	1	35
Annealing	38	2	
Extension	72	3	
Final Extension	72	10	

4.2.9 Gel Electrophoresis of DNA

Agarose gel electrophoresis was done essentially as described by Sambrook *et al.* (1989).

1. Approximately 1/10 volume of loading buffer (Appendix-IV) was added to the DNA solutions prior to loading samples into wells.
2. DNA molecules were separated on 1.5% Agarose gels (Appendix-IV) containing 0.5 µg/ml ethidium bromide.
3. Electrophoresis was carried out at 5-10 V/cm. in 1X TAE (Appendix-IV) buffer as a running buffer.
4. DNA was visualized by illumination with long wavelength UV-light (320nm).
5. Fragment sizes were determined by comparison to λDNA/*HindIII* cut as a marker.

4.2.10 RAPD Data Analysis

The molecular size of the RAPD bands obtained after electrophoresis was calculated by using standard curve (Appendix VIII). The index of similarity among strains was calculated using the formula:

$$Sab = 2\ Nab / Na+Nb,$$

Where Nab is the number of common fragments observed in individuals a and b and Na and Nb are the total number of fragments scored in a and b respectively (Lynch, 1990).

CHAPTER - V

5. RESULTS

5.1 COLONY CHARACTERISTICS OF THE ISOLATES

After acetate selection, bacterial colonies were morphologically examined. Presumptive *B. thuringiensis* were selected for further studies. A total of 40 colonies were picked from 19 soil samples from Tangboche, and 75 colonies were picked from 24 soil samples from Biratnagar. The colony characteristics of these isolates were observed (Appendix V).

5.2 MORPHOLOGICAL CHARACTERISTICS

All the isolates were found to be Gram-positive spore forming rods. Out of total 115 isolates, 74 were found to have crystal proteins (Table 4).

Table 4: Crystal staining of the isolates from Tangboche and Biratnagar

Sampling site	Crystal staining results	Isolates
Tangboche (40)	Positive (28)	T1, T3b, T4, T5c, T6a, T6b, T7a, T7b, T7d, T8a, T8b, T9a, T9b, T10a, T10c, T10d, T11, T12a, T12b, T13a, T13b, T13c, T15a, T15b, T15c, T15d, T16a, T17
	Negative (12)	T2a, T2b, T3a, T5b, T7c, T10b, T13d, T16b, T18, T19a, T19b, T20
Biratnagar (75)	Positive (46)	B1a, B4a, B4b, B5b, B6a, B7a, B8a, B8c, B8d, B9b, B10a, B10d, B11a, B11b, B11c, B11d, B12b, B13b, B14c, B14d, B15a, B15d, B16a, B16b, B16c, B17a, B17b, B17c, B17d, B18a, B19a, B19b, B19c, B19d, B20a, B20b, B20c, B20d, B20e, B20f, B23a, B23b, B23c, B23d, B23e, B24a
	Negative (29)	B1b, B2a, B3a, B3b, B3c, B4c, B4d, B5a, B5c, B7b, B7c, B8b, B9a, B9c, B10b, B10c, B12a, B12c, B13a, B14a, B14b, B15b, B15c, B16d, B17e, B18b, B21a, B22a, B24a

5.3. DISTRIBUTION OF *B.thuringiensis* IN SOIL SAMPLES

Of the 43 samples, 35 (81.4%) contained at least one crystal-forming *B. thuringiensis*. The numbers of *B. thuringiensis* isolates were expressed as a decimal fraction or index of the number of colonies examined (Table 5). This index represents the fraction of *B. thuringiensis* (crystal formers) in the total acetate-selected bacterial population examined. The other bacteria that made up the total acetate selected population were other members of the genus *Bacillus* (not identified in this study).

Table 5: Distribution of crystalliferous *B. thuringiensis* in soil samples, by region.

Location	**No. of soil samples Examined**	**No. of colonies examined**	**No. of *Bt** isolate**	**No. of soil samples with at least one *Bt** isolate**	**% of soil samples with at least one *Bt** isolate**	***Bt** index[a]**
Tangboche	19	40	28	15	78.9 (15/19)	0.7 (28/40)
Biratnagar	24	75	46	20	83.4 (20/24)	0.61 (46/75)
Total	**43**	**115**	**74**	**35**	**81.4 (35/43)**	**0.64 (74/115)**

* *B. thuringiensis*

[a] The *B .thuringiensis* index was calculated as a number of *B .tnuringiensis* isolates recovered divided by the number of colonies of all bacteria examined.

5.4 BIOCHEMICAL IDENTIFICATION

All crystal positive *B. thuringiensis,* screened by light microscopy, were processed for biochemical tests (Appendix-VI). Subdivisions within the *B. thuringiensis* by biochemical types were made as described by Martin and Travers (1989) (Appendix-VII).

B. thuringiensis were divided into 16 biochemical types, based on the following four biochemical tests: Esculin utilization, acid formation from salicin and sucrose, and lecithinase production, which are more variable among *B. thuringiensis* isolates (Martin and Travers, 1989).

There were three subspecies isolated from geographical areas, *thuringiensis*, *indiana*, and type 16. Four subspecies (*darmstadiensis*, type 11, *ostriniae* and type 15) were isolated from Khumbu region only and three subspecies (*kurstaki*, *sotto*, *morrisoni*) from Terai region only.

Table 6: Distribution of biochemical types of *B. thuringiensis*

Biochemical types	***B. thuringiensis* Subspecies**	**Location**		**Total**	**%**
		Tangboche	**Biratnagar**		
1	*thuringiensis*	6	4	10	13.52
2	*kurstaki*	0	1	1	1.35
3	*indiana*	10	19	29	39.19
4	*galleriae*	0	0	0	0
5	*sotto*	0	1	1	1.35
6	*dendrolimus*	0	0	0	0
7	*morrisoni*	0	19	19	25.68
8	*darmastadiensis*	2	0	2	2.70
9		0	0	0	0
10		0	0	0	0
11		2	0	2	2.70
12	*ostriniae*	2	0	2	2.70
13		0	0	0	0
14	*israelensis*	0	0	0	0
15		4	0	4	5.4
16		2	2	4	5.40
Total		**28**	**46**	**74**	**100**

5.5 STRAINS FOR RAPD-PCR ANALYSIS

The strains from Tangboche and Biratnagar were selected randomly for RAPD-PCR analysis (Table 7).

Table 7: Randomly selected *B. thuringiensis* strains for RAPD-PCR

Bio-chemical types	Subspecies	Location			
		BT from Tangboche		BT from Biratnagar	
		No.	Isolates	No.	Isolates
1	*thuringiensis*	1	T10a	-	-
2	*kurstaki*	-	-	1	B4a
3	*indiana*	6	T3b, T7b, T9b, T13a, T15a, T16a,	5	B8a, B8c, B11a, B16b, B16c
5	*sotto*	-	-	1	B6a
7	*morrisoni*	-	-	1	B10a,
8	*darmastadiensis*	1	T7a	-	-
11	Not known	1	T8b	-	-
12	*ostriniae*	1	T8a	-	-
15	Not known	1	T6b	-	-
16	Not known	-	-	1	B7a

5.6 RAPD PATTERN OF THE *B. thuringiensis* SUBSPECIES

Ten isolates (representative of each subspecies isolated in this study) were subjected to RAPD analysis. The total genomic DNA isolated from *B. thuringiensis* subspecies were analyzed using RAPD-PCR with 284 random decamer oligonucleotide primers (Table 1). All amplification products were found to be reproducible when reactions were repeated using the same reaction conditions. Negative controls contained all the ingredients except DNA template.

Under the amplification condition used in this study, different informative arrays of amplified fragments (Photograph 6) ranging in size from 1838 bp to 237 bp (Appendix IX) were observed. Almost all comparisons showed highest polymorphism (100%) in 42 among 45 comparisons (Table 8).

Table 8: Estimated genetic similarities among *B. thuringiensis* subspecies

Comparison (aXb)	Na	Nb	Nab	% Similarity
indiana X *thuringiensis*	2	3	1	40
indiana X *sotto*	2	2	0	0
indiana X *morrisoni*	2	1	0	0
indiana X *darmstadiensis*	2	2	0	0
indiana X 11	2	3	0	0
indiana X *ostriniae*	2	4	0	0
indiana X 15	2	6	0	0
indiana X 16	2	5	0	0
indiana X *kurstak*i	2	0	0	0
thuringiensis X *sotto*	2	2	0	0
thuringiensis X *morrisoni*	2	1	0	0
thuringiensis X *darmstadiensis*	2	2	0	0
thuringiensis X 11	2	3	0	0
thuringiensis X *ostriniae*	2	4	0	0
thuringiensis X 15	2	6	0	0
thuringiensis X 16	2	5	0	0
thuringiensis X *kurstaki*	2	0	0	0
sotto X *morrisoni*	2	1	0	0
sotto X *darmstadiensis*	2	2	0	0
sotto X 11	2	3	0	0
sotto X *ostriniae*	2	4	0	0
sotto X 15	2	6	0	0
sotto X 16	2	5	0	0
sotto X *kurstaki*	2	0	0	0
morrisoni X *darmstadiensis*	1	2	0	0
morrisoni X 11	1	3	0	0
morrisoni X *ostriniae*	1	4	0	0
morrisoni X 15	1	6	0	0
morrisoni X 16	1	5	0	0
morrisoni X *kurstaki*	1	0	0	0
darmstadiensis X 11	2	3	1	40
darmstadiensis X *ostriniae*	2	4	0	0
darmstadiensis X 15	2	6	0	0
darmstadiensis X 16	2	5	0	0
darmstadiensis X *kurstaki*	2	0	0	0
11 X *ostriniae*	3	4	0	0
11 X 15	3	6	0	0
11 X 16	3	5	0	0
11 X *kurstaki*	3	0	0	0
ostriniae X 15	4	6	0	0
ostriniae X 16	4	5	0	0
ostriniae X *kurstaki*	4	0	0	0
15 X 16	6	5	5	90.90
15 X *kurstaki*	6	0	0	0
16 X kurstaki	5	0	0	0

Na: Number of fragments in a

Nb: Number of fragments in b

Nab: Number of common fragments in both a and b

5.6.1 RAPD profile of the *B. thuringiensis* subspecies *indiana*

Out of total 29 *B. thuringiensis* subspecies *indiana* (10 isolates from Tangboche and 19 isolates from Biratnagar), 10 isolates (5 from each region) were randomly selected for RAPD analysis.

5.6.1.1 RAPD pattern of *B. thuringiensis* subspecies *indiana* isolated from Tangboche

A particular RAPD pattern was observed (Photograph 6) with the used primer, amplified fragments ranging in size from 505 bp to 197 bp. Under the conditions used in this study, most of the isolates gave the common bands, *viz.,* 386 bp. 315 bp and 197 bp. Genetic similarities among the isolates were estimated as band sharing between the two isolates (Table 9).

Table 9: Estimated genetic similarities among *B. thuringiensis* subspecies *indiana* isolates from Tangboche

Comparisons (aXb)	Na	Nb	Nab	% Similarity
T3b X T7b	3	4	3	85.71
T3b X T9b	3	3	2	66.67
T3b X T15a	3	3	2	66.67
T3b X T16a	3	3	2	66.67
T7b X T9b	4	3	3	85.71
T7b X T15a	4	3	3	85.71
T7b X T16a	4	3	3	85.71
T9b X T15a	3	3	3	100
T9b X T16a	3	3	3	100
T15a X T16a	3	3	3	100

Under the amplification conditions**,** highest similarities (100%) were observed in three comparisons followed by 85.71% and 66.67% similarities in four and three comparisons respectively.

5.6.1.2: RAPD pattern of *B. thuringiensis* subspecies *indiana* isolated from Biratnagar

Arrays of amplified fragments ranging in size from 386bp to 197bp were observed (Photograph 7). Particular RAPD pattern was observed which was able to distinguish the isolates (Table 10).

Table 10: Estimated genetic similarities among *B. thuringiensis* subspecies *indiana* isolates from Biratnagar

Comparisons (aXb)	Na	Nb	Nab	% Similarity
B8a X B8c	3	3	2	66.67
B8a X B11a	3	2	2	80
B8a X B16b	3	2	2	80
B8a X B16c	3	2	0	0
B8c X B11a	3	2	2	80
B8c X B16b	3	2	1	40
B8c X B16c	3	2	0	0
B11a X B16b	2	2	1	50
B11a X B16c	2	2	0	0
B16b X B16c	2	2	0	0

Only three comparisons were more than 70% similar. On the other hand, no similarities (100% polymorphism w.r.t primer 284) were observed in four comparisons indicating more diversity among the strains (Table 10).

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

Bacillus thuringiensis is Gram-positive, spore forming bacteria that synthesize a large diversity of crystal proteins (Cry and Cyt) during sporulation. Some of these are toxic for a wide range of insects belonging to the orders Lepidoptera, Coleoptera, Diptera, Hymenoptera and Homoptera as well as being active against nematodes, mites and protozoa (Jhonson *et al*., 1998; Schnepf *et al*., 1998). This characteristics has resulted in *B. thuringiensis* being the most widely used bacterium over the last 50 years for biological control of pests and vectors of disease and its safety to non target insects, birds and mammals has been well demonstrated (Seigel 2001; Jensen *et al*, 2002).

Several collections of *B. thuringiensis* strains from different regions of the world have been recently characterized. These strains have been isolated from diverse microhabitats such as soil, plant leaves, dead insects and stored grains, as well as aquatic environments such as marine sediments mangroves and fresh water. Characterization of these collections has revealed the great variability and diversity of *B. thuringiensis* in nature (Ben-Dov *et al*., 1997; Bravo *et al*., 1998; Ben-Dov *et al*. 1999; Ichimatsu *et al*., 2000; Iriarte *et al*., 2000; Kim 2000; Maeda *et al*., 2001; Mizuki *et al*., 2001, Arango *et al*., 2002; Maduell *et al*., 2002; Uribe *et al*., 2003).

Nepal has a heterogeneous climate that gives unique geographical features and abundant biological resources to study ecological distribution of the organism in soil. The objective of this work was to study the composition, ecological distribution and genetic similarities in *B. thuringiensis* isolates from two ecological niches of Nepal having different climates using RAPD-PCR and different biochemical tests.

B. thuringiensis isolates were obtained from soil samples from different geographical areas of Nepal. Soil samples were collected from both non-cultivated (Tangboche) and cultivated (Biratnagar) fields in order to have correlative study in varied altitude and temperature. To our knowledge, none of the areas had been previously treated with *B. thuringiensis* based bioinsecticides but in case of samples from cultivated land of Biratnagar, there might be chemical pesticides.

After acetate selection (Travers *et al*., 1987), out of 115 isolates from both areas, 74 were classified as *B. thuringiensis* because of their content of crystal proteins as shown after crystal staining and visualize by light microscopy. Frederiksen *et al*., 2006, observed cry genes in some strains of *B. thuringiensis* but they had no visible protein crystals. In this study, only 74 isolates having crystal proteins were included for biochemical and molecular characterization.

We have found variable percentage of samples with *B. thuringiensis* depending on their origin, 78.9% and 83.4% in the Tangboche and Biratnagar, respectively, which are in agreement with the general percentages obtained from samples of Asia and Central and South Africa (94%), Europe (84%), USA(60%) and New Zealand (56%) (Meadows, 1993). Nevertheless, these percentages were obtained not only from soil samples but also from samples from other sources as insects, silos and mills, which may be more successful sources of *B. thuringiensis* than soil samples (Chaufax *et al*., 1997; Zhang *et al.*, 2000). In fact, the higher *B. thuringiensis* index was found in Tangboche, 0.7 and the lower one 0.61 in the Biratnagar.

The characterization of the 74 *B. thurin*giensis isolates obtained from two different geographical areas of Nepal by means of biochemical tests and molecular techniques (RAPD-PCR) supplements the work of others (Subedi, 1999; Bhattarai, 2000; Shrestha *et al.*, 2006 and Sahukhal, 2006) and contributes to a better knowledge of *B. thuringiensis* in Nepal and in the world.

Biochemical characterization of the isolates was done as described by Martin and Travers, 1989 (Appendix). These biochemical types were based on esculin utilization, acid production from sucrose and salicin, and lecithinase production, which were the most variable among *B. thuringiensis* isolates. While some of these types differed only by a single biochemical test, these differences were significant.

Biochemical type was not indicative of location. Most biochemical types could be found everywhere. However, most locations had a distinct distribution of types. *B. thuringiensis* subsp. *israelensis*, the most common type overall, was the most prevalent type only in the United States (18.8%) and Europe (29.7%). In Asia, *B. thuringiensis* subsp. *kurstaki* (Es^+, Sa^+, Le^+, Su^+) was the most common type. In Africa and South and Central America, biochemical type 15 (Es^-, Sa^-, Le-, Su^+), which was similar to the biochemical type *B. thuringiensis* subsp. *israelensis* was the most common biochemical type. Biochemical type 10 (Es^-, Sa^+, Le^+, Su^-), which was similar to *B. thuringiensis* subsp. *kurstaki*, was the most common biochemical type found in New Zealand (Martin and Travers, 1989). Surprisingly, in our study, biochemical type *B. thuringiensis* subsp. *indiana* was the most prevalent representing 39.19% (29 isolates) of the total *B. thuringiensis* isolates and was isolated from both places (Tangboche and Biratnagar). Another biochemical type *B. thuringiensis* subsp *morrisoni* made up 25.68% (19 isolates) and isolated only from Biratnagar. This may be due to the environmental adaptation of this subspecies or may be due to the presence of suitable host for that biochemical type. Biochemical type *B. thuringiensis* subsp. *thuringiensis* and type 16 were also found in both places. But, biochemical type *B. thuringiensis* subsp. *kurstaki* and subsp. *sotto* were found only in Biratnagar. Similarly, biochemical type *B. thuringiensis* subsp. *darmastadiensis*, subsp. *ostriniae*, type 11 and type 15 were found only in Tangboche. These results indicate that most locations had a distinct distribution of biochemical types. Of particular interest is that we have predominantly isolated subspecies *indiana* indicating soils of Nepal were very rich in subspecies *indiana* even by the fact that it has been obtained from the two geographical areas of our study.

RAPD-PCR is a simple and rapid mean for establishing the polymorphisms between biology without DNA sequencing (Chen *et al.* 2003). Moreover, RAPD-PCR has proved to be an informative method suitable for the study of a large number of strains in a short time. RAPD is a well established easy method used to classify closely related strains (Williams *et al*., 1990; Ghelardi *et al*., 2002; Pinchuk *et al*., 2002). The applied RAPD technology was very useful, fast and informative in differentiating BT strains similar to related studies of Bt (Hansen *et al*., 1998; Gaviria and Priest, 2003a). The arbitrary primer recognizes differences in the prevalence and positions of annealing sites in the genome producing sets of fragments that are considered to reflect the genomic composition of the strains (Gaviria and Priest, 2003a). Furthermore, no information is needed in advance of any sequence (Hansen *et al*., 1998), therefore it gives a good opportunity to detect biodiversity of a group of isolates and also makes it a tool of great power and general applicability. In the present work, RAPD method was used to evaluate genetic similarity among *B. thuringiensis* isolates from two different regions, *viz.* Tangboche of Khumbu region and Biratnagar of Terai region, of Nepal having differences in altitude, temperature, vegetation etc. In this study, the isolates used in RAPD-PCR were selected randomly in such a way that 5 isolates of the same biochemical type *B. thuringiensis* subsp *indiana* from each two region for one set of RAPD-PCR and 10 isolates which were representative of each biochemical type for another set of RAPD-PCR.

In this study, the template DNA for RAPD-PCR was prepared by boiling cell method, which offers many advantages over other conventional DNA extraction method (Araujo *et al*., 2004). They showed no significant differences among the RAPD profile of the PCR reactions derived from the boiling and phenol extraction methods, suggesting the utilization of this method for genetic population analysis. To ensure that the amplified DNA bands originated from genomic DNA and not primer artifacts, negative control was carried out for each preparation of PCR reaction mixture. No amplification was detected in control reactions. Salem *et al*., 2006, used primers with GC% ranging from 50 to 100% for characterization of *B. thuringiensis* by RAPD-PCR and the results

showed that GC content is not essential requirements for generation of polymorphic bands. This result is in agreement with the findings of other researchers (Gomes *et al*., 2005) On the contrary, it has been observed that random primers with high GC content (>60%) resulted in a greater and better reproducible number of strain specific bands in enterotoxigenic *E. coli* (Pacheco *et al*., 1996; Makino *et al*., 1994; Akopyanz *et al*., 1992). In this study, primer having GC content 70% was used. The RAPD analysis of ten isolates (representative of each subspecies isolated in this study) showed that these Bts have, generally, a heterogeneous genetic background. The amplified fragment size ranges from 1838 bp to 237 bp, which is expected because the strains included were of different biochemical types and isolated from two different locations. Genetic similarities among the isolates showed highest polymorphism (100%) in 42 among 45 comparisons. The highest similarity was recorded only in between biochemical type 15 and 16 in this study. The large number of RAPD types within *B. thuringiensis* suggests a genetically heterogeneous background. Plasmid DNA exchange in nature is well documented in *B. thuringiensis* strains (Vilas-Boas *et al*., 1998).

RAPD profile of *B. thuringiensis* subspecies *indiana* from Tangboche showed a particular RAPD pattern (Photograph 6). The amplified fragments ranging in size from 505 bp to 197 bp. Producing similar band patterns suggest that the strains are closely related to each other within each group, however, the data must be interpreted with caution since PCR bands of similar size do not necessarily mean that the molecules are identical in sequence (Brousseau *et al*., 1993). Most of the isolates gave the common bands *viz*., 386bp, 315bp and 197 bp. can be used as RAPD markers for the identification of i*ndiana* subspecies, under the amplification conditions used in this study. However, to our knowledge, such study for *indiana* subspecies were not found yet and not compared to any reference. Under the amplification conditions, highest similarities (100%) were observed in three comparisons followed by 87.71% and 66.67% similarities in four and three comparisons respectively. Genomically similar or highly related strains of *B. thuringiensis* from geographically distinct locations show that clones of this species have successfully colonized the biosphere. Strains of these

clones apparently undergo limited chromosomal DNA exchange (Gaviria Rivera and Priest, 2003a), as found here. On the other hand, the highest polymorphism was observed in four comparisons of subspecies *indiana* isolated from Biratnagar indicating highly divergent strains. This difference may be due to ecological environment which was not highly selective as of high altitude region and also may be due to various agricultural and industrial practices, which could not be explained from the present study.

6.2 CONCLUSION

This study shows that the soil is a very important source of *B. thuringiensis* strains providing large genetic resources for its use in the development of bioinsecticides to control insect pests. *B. thuringiensis* presents a great physiological and genetic diversity even in isolates from the same geographical area. It can be concluded that RAPD-PCR is powerful tool to study genetic similarity and/or polymorphisms among bacterial strains of same or different subspecies. The ecological aspects of the distribution of subspecies and RAPD profiles of the isolates from soils of two different climatic zones could contribute to a better understanding of the role of *B. thuringiensis* in the environment.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

7.1 SUMMARY

1. After acetate selection, a total of 40 and 75 presumptive *B. thuringiensis* were isolated from 19 and 24 soil samples from Tangboche and Biratnagar respectively.

2. Out of total 115 isolates, 74 were found to have crystal proteins.

3. The *B. thuringiensis* index was variable depending on the territory where the samples were taken, the higher value being found in the samples from the Khumbu region (an index of 0.7), while the lower ones in the Terai region (an index of 0.61).

4. In order to study the biochemical type of our isolates, crystalliferous spore formers were identified by four biochemical tests: esculin utilization, acid production from salicin and sucrose and lecithinase production and 74 isolates appeared to belong to 10 subspecies.

5. There were three subspecies isolated from both geographical areas, *thruingiensis*, *indiana*, and type 16. Four subspecies (*darmstadiensis*, type 11, *ostriniae* and type 15) were isolated from Khumbu region only and three subspecies (*kurstaki*, *sotto*, *morrisoni*) from Terai region only.

6. Among 10 subspecies, *B. thuringiensis* subspecies *morrisoni* was predominantly isolated from Terai region only.

7. Among the 10 randomly selected *B. thuringiensis* isolates (representative of each subspecies isolated in this study) for RAPD-PCR, different amplified fragments ranging in size from 1838 bp to 237 bp were obtained. Each isolate was compared with each other for the estimation of genetic similarities. Almost all comparisons showed highest polymorphism (100%) in 42 among 45 comparisons.

8. Ten randomly selected isolates of *B. thuringiensis* subspecies *indiana* (5 from each region) were subjected to RAPD-PCR.

9. RAPD analysis of isolates from Tangboche showed highest genetic similarities (100%) in three comparisons followed by 85.71% and 66.67% similarities in four and three comparisons respectively. Under the amplification conditions used in this study, most of the isolates gave the common bands, viz., 386 bp, 315 bp and 197 bp.

10. *B. thuringiensis* subspecies *indiana* from Biratnagar showed no similarities (100% polymorphism with respect to primer 284) in four comparisons more than 70% similarities in three and the 40-70% similarities in three comparisons indicating more diversity among the strains.

7.2 RECOMMENDATIONS

- As this study was aimed to study genetic similarities among *B.thuringiensis* isolates from two different geographical locations (Khumbu region and Terai region), more samples from other natural regions and different habitats of Nepal need to study to explore the ecological distribution of *B. thuringiensis*.

- Other RAPD primers should also be used to obtain reliable RAPD marker for the identification of *B. thuringiensis*.

- Using specific primers, multiplex- PCR should be done so as to study the distribution of *cry* genes throughout the natural regions of Nepal.

CHAPTER-VIII

8. REFERENCES

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APPENDIX-I

MATERIALS

Equipment

Autoclave	Life, Portable steam stericlave-800
Cryocentrifuge	Fischer Scientific Microcentrifuge, 235C
Distillation Unit	
DNA Thermal Cycler	Perkin Erlmer Cetus
Electric balance	Mettler Instrument corp. Switzerland
Electric Heater	Jindal
Elecrophoretic tank	Fotodyne
Glasswares	Pyrex and Borosil
Hot air oven	Ambassadors, Laboratory Electronics Oven
Hot water bath	NSW, India
Incubator	Ambassadors, Laboratory Electronics Oven
Laminar Flow	Yorco Sales
Microcentrifuge	Fischer Scientific
Micropipettes	Hi-media
Microscope	Olympus
Microwave Oven	White Westinghouse
Millipore filter	
pH meter	Deluxe, Model-151-R
Refrigerator	Sanyo
Spectrophotometer	Milton Roy company
UV illuminator	Fotodyne
Vortex	Lab.Line Instrument, Inc
Water bath shaker	NSW
Micropipettes tips	
Ependorff tubes	

Media

i. Culture media

Agar powder	Oxoid, Unipath Ltd., England
Beef extract	Qualigens Fine Chemicals
Nutrient agar	Hi-media Laboratory
Nutrient broth	Hi-media Laboratory
Peptone	Qualigens Fine Chemicals, India.
Tryptone	Qualigens Fine Chemicals, India.
Yeast extract	Qualigens Fine Chemicals, India.

ii. Biochemical media

Aesculin Sequihydrate	Hi-media chemical, Lab. India
Casein	Sd-fine chemicals
D (+) Galactose	Hi-media chemical, Lab. India
Gelatin	Hi-media chemical, Lab. India
D-Glucose	Hi-media chemical, Lab. India
Mannitol	Hi-media chemical, Lab. India
Salicin	Loba Chemical, India
Simmon's citrate agar	Hi-media chemical, Lab. India
Starch powder	S.D. Fine chemicals Ltd. Boisar
Sucrose	Qualigens Fine Chemicals, India
Urease broth base	Hi-media chemical, Lab. India
Tween 20	Qualigens Fine Chemicals, India.

Chemicals/reagents

i. Staining reagents

Acetic acid	Ranbaxy Laboratories, India
Acetone	Qualigens Fine Chemicals, India
Alcohol	Bengal Chemical & Pharmaceutical, India
Amido black	S.D. Fine chemicals Ltd. Boisar

Bromophenol Blue	Qualigens Fine Chemicals, India
Calcium chloride	S.D. Fine chemicals Ltd. Boisar
Crystal violet	Loba Chemical, Indoaustrailian, India
Disodium hydrogen phosphate	S.D. Fine chemicals Ltd. Boisar
Glycerol	Qualigens Fine Chemicals, India
Iodine	Loba Chemical, Indoaustrailian, India
Malachite green	S.D. Fine chemicals Ltd. Boisar
Mono sodium hydrogen phosphate	S.D. Fine chemicals Ltd. Boisar
Safranine	Loba Chemical, Indoaustrailian, India

ii. Tests regents

α-naphthylamine	Central Drug House Pvt. Ltd, India
3% hydrogen peroxide	Qualigens Fine Chemicals, India
Ethyl alcohol	Qualigens Fine Chemicals, India
Isoamyl alcohol	Qualigens Fine Chemicals, India
Hydrogen peroxide	Qualigens Fine Chemicals, India
Mercuric chloride	E. Merck, India
Methyl red	Qualigens Fine Chemicals, India
Phenol red	Qualigens Fine Chemicals, India
p-dimethyl aminobenzaldehyde	Qualigens Fine Chemicals, India
Potassium hydroxide	Central Drug House Pvt. Ltd, India
Sodium carbonate	S.D. Fine chemicals Ltd. Boisar
Sodium chloride	S.D. Fine chemicals Ltd. Boisar
Sulphanillic acid	S.D. Fine chemicals Ltd. Boisar
Tetramethylene paraphenylene-Diamine dihydrochloride	S.D. Fine chemicals Ltd. Boisar
Zinc Powder	S.D. Fine chemicals Ltd. Boisar

iii. Reagents for DNA isolation and PCR

Agarose	MD Bio, Inc.
Bovine Serum Albumin	
Chloroform	Qualigens fine chemicals
DMSO	Hi media
Ethidium bromide	Sigma chemicals
Ethylene Diamine tetramethyl acetic acid	Qualigens
Isoamyl alcohol	Merck
Iso propanol	Merck
Lysozyme	Hi Media
Paraffin oil	Yogi pharmaceutical works
Phenol Crystals	Qualigens fine chemicals
Pronase E	Sigma chemicals
Sodium dodecyl sulphate	Sd-fine chemicals
Tris (hydroxymethyl) methylamine	Qualigens fine chemicals
Triton X 100	Hi media

Others:

Sodium Acetate	Qualigens fine chemicals
Hydrochloric acid	Qualigens Fine Chemicals, India
Methanol	Qualigens fine chemicals
Sodium hydroxide pellets	Lab. Rasayen S.D.v fine
Ferric Citrate	Loba chemicals
Sodium acetate	S.D. Fine chemicals Ltd. Boisar
Urea Crystals	S.D. Fine chemicals Ltd. Boisar

APPENDIX-II

NAME AND COMPOSITION OF MEDIA

I. CULTURE MEDIA

1. Lauria Bertani Broth

Ingredients	(Grams/Litre)
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	5.00
Final pH (at 25°C)	6.8 ±0.2

2. Lauria Bertani Agar

Ingredients	(Grams/Litre)
Tryptone	10.00
Yeast extract	5.00
Sodium chloride	5.00
Agar	15.00
Final pH (at 25°C)	6.8 ±0.2

3. Nutrient Broth

Ingredients	(Grams/Litre)
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Final pH (at 25°C)	7.4 ±0.2

4. Nutrient Agar

Ingredients	(Grams/Litre)
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00
Final pH (at 25°C)	7.4 ±0.2

II. BIOCHEMICAL MEDIA

1. Nitrate Broth

Ingredients	(Grams/Litre)
Beef extract	3.0
Peptone	5.0
Potassium Nitrate	1.0
Sodium thiosulphate	0.025
Final pH at (25°C)	7.3±0.2

2. Peptone Water

Ingredients	(Grams/Litre)
Peptone	10.0
Sodium chloride	5.0
pH	7.0-7.4

3. Urea agar base

Ingredients	(Grams/Litre)
Peptone	1.0
Dextrose	1.0
Sodium chloride	5.0
Monopottasium phosphate	0.8
Phenol red	0.012
Agar	2.0
Final pH at 25°C	6.8±0.2

21 grams urea agar base was suspended in 1000 of distilled water .it was boiled to dissolve completely and sterilized by autoclaving .it was cooled down to 55°C and aseptically introduced urea extrapure crystal solution and mixed well. It was distributed in sterile test tubes and allowed to solidify in slanted position.

4. Carbohydrate fermentation medium

Ingredients	(Grams/Litre)
Peptone	10.0
Sodium chloride	5.0
Phenol red	0.018
Carbohydrates	
Glucose-1%	
Sucrose-1%	
Mannitol-1%	
Galactose-1%	
Salicin-1%	
Final pH at (25°C)	7.2±0.2

Except sugars all the ingredients were weighed separately and dissolved in 1000 ml distilled water. It was dispensed in 100ml amounts and was sterilized in the autoclave for 15 minutes at 15lbs pressure at 121°C.to the 100ml of sterile medium aseptically sterile sugars were added. Each flask was shacked to allow mixing thoroughly and 15 ml amounts were dispensed into sterile test tubes aseptically.

5. Hydrolysis Agar Media

i. Casein Agar Medium

Ingredients	(Grams/Litre)
Nutrient Agar	2.8
Casein	1.0
Final pH at (25°C)	7.2±0.2

ii. Esculin Agar medium

Ingredients	(Grams/Litre)
Beef extract	3
Peptone	5
Aesculin Sequihydrate	1
Ferric Citrate	0.5
Agar	15
Final pH	7

iii. Egg yolk agar

Ingredients	(Grams/Litre)
Nutrient Agar	200 ml
Egg-yolk emulsion	8.0 ml

Egg was sterilized with 70% w/v ethanol for 10 minutes. Egg yolk was separated from the egg white aseptically. 4 ml of egg yolk was mixed with 4 ml sterile physiological saline.

Nutrient agar was sterilized by autoclaving. The sterile egg yolk emulsion was added aseptically to the sterile molten nutrient agar cooled to 50-55^{o}C and mixed quickly by swirling the flask. The media was then immediately dispensed to the sterile Petri plates.

iv. Gelatin Agar Medium

Ingredients	(Grams/Litre)
Nutrient Agar	2.8
Gelatin	1.0
Final pH at (25°C)	7.2±0.2

v. Starch Agar Medium

Ingredients	(Grams/Litre)
Nutrient Agar	2.8
Starch	1.0
Final pH at (25°C)	7.2±0.2

vi. Tween-20 Agar Medium

Ingredients	(Grams/Litre)
Nutrient Agar	2.8
Tween-20	1
$CaCl_2$	0.05
Final pH at (25°C)	6.8±0.2

APPENDIX-III

COMPOSTION OF TEST AND STAINING REAGENTS

1. Amido Black

Amido black	1.5gm
98% methyl alcohol	50 ml
Glacial acetic acid	10 ml
Distilled water	40 ml

Amido black was dissolved in 50 ml methyl alcohol and 10 ml glacial acetic acid solution. The volume was then adjusted to 100 ml by adding distilled water. It was stored at 3 days before using.

2. Crystal Violet

Solution A:

Crystal violet	2 gm
95% ethanol	20 ml

Solution B:

Ammonium oxalate	0.8 gm
Distilled water	30.0 ml

Crystal violet was dissolved in ethyl alcohol and ammonium oxalate in distilled water. Then solution A and B were mixed.

3. 95% Ethyl Alcohol

5 ml of distilled water was added to 95 ml of absolute alcohol to make 95% ethyl alcohol solution.

4. Gram's Iodine

Iodine crystal	1.0 gm
Potassium iodine	2.0 gm
Distilled water	300 ml

Iodine and potassium iodine were dissolved in distilled water.

5. Giemsa Stain

Giemsa Powder	3.8 gm
Glycerol (Glycerine)	250 ml
Methanol	250 ml

6. Kovac's Reagent

Isoamyl or Amyl alcohol	150 ml
P-dimethylaminobenzaldehyde	10.0 gm
Conc. Sulphuric acid	50.0 ml

P-dimethylaminobenzaldehyde was dissolved in amyl alcohol and acid was slowly added. It was stored at 4°C.

7. Mercuric Chloride Solution

Mercuric Chloride	15.0 gm
Conc. HCI	20.0 ml
Distilled water	100.0 ml

8. Nitrate reduction Test Reagent

Solution A:

Sulphanilic acid	8 gm
Acetic acid (5N)	1000 ml

Solution B:

α -naphylamine	5 gm
Acetic acid (5N)	1000 ml

9. Oxidase Strips

First of all oxidase reagent was prepared by dissolving 1 gm of tetra methyl paraphenylene diaminedihydrochloride in 100 ml of distilled water.

Whatman No.1 filter paper was cut into stripes and placed them in pertridish. Oxidase reagent was poured into the pertridish. After 10 seconds the stripes were dried in an incubator by covering them with cover, and stored in cold and dark place in amber colored bottle.

10. Safranin

Safranin (2.5% solution in 95% ethyl alcohol)	10 ml
Distilled water	100 ml

APPENDIX-IV

COMPOSITION OF REAGENTS AND SOLUTIONS FOR DNA EXTRACTION, GEL ELECRTOPHORESIS AND DNA QUANITZATION

1. Agarose gel (0.8%):

Agarose	0.28gm
TAE (1X)	35ml

2. Blue juice (6X):

Glycerol	350µl
EDTA (0.25M)	40µ l
SDS (20%)	5 µl
Bromophenol blue (10%)	30µ l

Store at 4^{o}C.

3. Phenol:Chloroform:Isoamyl alcohol:

Melted Phenol crystals	25ml
Chloroform	24ml
Isoamyl alcohol	1ml

Store at room temperature.

4. Sodium acetate (3M), pH 4.8:s

Sodium acetate	40.82gm
Distilled water	80ml
Glacial acetic acid	to make pH4.8
Final volume	100 ml with distilled water

Store at 4^{o}C.

5. Sodium chloride (5M):

Sodium chloride	5.844g
Distilled water	100ml

Store at 4^{o}C.

6. Sodium dodecyl sulphate (10%):

SDS	10gm
Distilled water	100ml

7. Tris acetate buffer (50X), pH 8.0:

Tris	24.2gm
Acetic acid	5.71ml
EDTA (0.5M)	11.1ml
Final volume	100ml with distilled water

Dilute to 1X before use and store at 4^{o}C until use.

8. Tris Buffer (pH 7.6):

a. TE buffer, 10.0:1.0:

Tris HCl	10mM
EDTA	1mM
Final volume	100 ml with distilled water

b. TE buffer, 10.0:0.1:

Tris HCl	10mM
EDTA	0.1mM

APPENDIX-V

Table I: Isolation of *Bacillus thuringiensis* from the soil sample of Khumbu region and Biratnagar, Nepal.

I. Tangboche

Sample	No. of isolates	Code of isolates
T1	1	T1a
T2	2	T2a,T2b
T3	2	T3a,T3b
T4	1	T4
T5	2	T5b,T5c
T6	2	T6a,T6b
T7	4	T7a,T7,T7c,T7d
T8	2	T8a,T8b
T9	2	T9a,T9b
T10	4	T10a.T10b,T10c,T10d
T11	1	T11
T12	2	T12a,T12b
T13	4	T13a,T13b,T13c,T13d
T15	4	T15a,T15b,T15c,T15d
T16	2	T16a,T16b
T17	1	T17
T18	1	T18
T19	2	T19a,T19b
T20	1	T20

II. Biratnagar

Sample	No. of isolates	Code of isolates
B1	2	B1a,B1b
B2	1	B2a
B3	3	B3a, B3b,B3c
B4	4	B4a,B4b,B4c,B4d
B5	3	B5a,B5b,B5c
B6	1	B6a
B7	3	B7a,B7b,B7c
B8	4	B8a,B8b,B8c,B8d
B9	3	B9a,B9b,B9c
B10	4	B10a,B10b,B10c,B10d
B11	4	B11a,B11b,B11c,B11d
B12	3	B12a,B12b,B12c
B13	2	B13a,B13b
B14	4	B14a,B14b,B14c,B14d
B15	4	B15a,B15b,B15c,B15d
B16	4	B16a,B16b,B16c,B16d
B17	5	B17a,B17b,B17c,B17d,B17e
B18	2	B18a,B18b
B19	4	B19a,B19b,B19c,B19d
B20	6	B20a,B20b,B20c,B20d,B20e,B20f
B21	1	B21a
B22	1	B22a
B23	5	B23a,B23b,B23c,B23d,B23e
B24	2	B24a,B24b

Table II: Colony morphology of the isolates.

I. Tangboche

Isolates	Colour	Configuration	Margin	Consistency	Opacity	Elevation
T1	Dull white	Round	Smooth		Opaque	Convex
T2a	Creamy	Round	Smooth		Opaque	Convex
T2b	White	Round	Smooth		opaque	Convex
T3a	White	Round	Smooth		opaque	Convex
T3b	Dull white	Irregular	Branching	Dry	Opaque	Flat
T4			crenated	Non mucoid	Opaque	Flat
T5b	Creamy	Irregular		Non mucoid	Opaque	Flat
T5c	White	Round	Entire	Non mucoid	Opaque	Convex
T6a	White		Smooth		Translucent	Flat
T6b	White	Round	Smooth		Translucent	Flat
T7a	Dull white	Round	Smooth		Transparent	Flat
T7b	White	Irregular	Branching	Dry	Opaque	Raised
T7c	Dull white		Irregular	Dry	Opaque	Flat
T7d	Dull white	Round	Smooth	Dry	opaque	Flat
T8a						
T8b						
T9a	Dull white	Irregular	Branching	Dry	Opaque	Flat
T9b	Dull white	Irregular	Branching	Dry	Opaque	Flat
T10a	White	Round			Opaque	Low convex
T10b		Round	Smooth		Opaque	Low convex
T10c		Round	Smooth		Translucent	Low convex
T10d	White					Low convex
T11		Round	Smooth		Translucent	Low convex
T12a	Dull white	Round	Smooth	Matty	Opaque	Flat
T12b		Round	Smooth		Translucent	Low convex
T13a	White	Round			Opaque	Low convex
T13b	White	Round	Irregular		Opaque	Low convex
T13c	White	Round	Irregular		Opaque	Low convex
T13d		Round	Smooth		Translucent	Low convex
T15a	White	Round	Irregular	Dry	Opaque	Flat
T15b		Round	Smooth		Translucent	Low convex
T15c	White	Round	Irregular	Matty	Opaque	Low convex
T15d	Creamy	Round	Irregular	Matty	opaque	Low convex
T16a	Creamy	Round	Smooth	Mucoid	Opaque	Flat
T16b	White	Round	Irregular	Mucoid	Opaque	Raised
T17						
T18	Dull white	Round	Smooth		Opaque	Convex
T19a		Round	Smooth		Translucent	Convex
T19b		Round	Smooth		Translucent	Convex
T20	Dull white	Round	Smooth		Opaque	Convex

II. Biratnagar

Isolate	Colour	Configuration	Margin	Consistency	Opacity	Elevation
B1a	White	Irregular	Irregular	Non mucoid	Opaque	Flat
B1b	White	Irregular	Irregular	Non mucoid	Opaque	Flat
B2a	White	Irregular	Irregular	Dry	Opaque	Flat
B3a	White	Oval	Irregular	Dry	Opaque	Flat
B3b	Creamy white	Irregular	Irregular	Non mucoid	Translucent	Flat
B3c	Creamy white	Round	Smooth	Non mucoid	Opaque	Flat
B4a	White	Round	Smooth	Non mucoid	Opaque	Flat
B4b	White	Oval	Irregular	Non mucoid	Opaque	Flat
B4c	Off white	Oval	Smooth	Non mucoid	Opaque	Flat
B4d	Creamy	Round	Irregular	Non mucoid	Opaque	Flat
B5a	Creamy	Round	Irregular	Non mucoid	Opaque	Flat
B5b	White	Oval	Smooth	Non mucoid	Opaque	Flat
B5c	Creamy	Oval	Irregular	Non mucoid	Opaque	Flat
B6a	Creamy	Oval	Irregular	Non mucoid	Opaque	Flat
B7a	Creamy	Round	Smooth	Non mucoid	Opaque	Flat
B7b	Off white	Round	Irregular	Non mucoid	Opaque	Flat
B7c						
B8a	White	Round	Smooth	Non mucoid	Opaque	Convex
B8b						
B8c	White	Round	Smooth	Non mucoid	Opaque	Convex
B8d	Off white	Round	Smooth	Non mucoid	Opaque	Low convex
B9a	Yellow	Round	Irregular	Non mucoid	Translucent	Flat
B9b	Creamy	Round	Smooth	Non mucoid	Opaque	Flat
B9c						
B10a	Off white	Round	Smooth	Non mucoid	Opaque	Convex
B10b	White	Round	Smooth	Non mucoid	Translucent	Flat
B10c	White	Oval	Irregular	Non mucoid	Opaque	Flat
B10d	White	Round	Smooth	Mucoid	Opaque	Raised
B11a	White	Round	Irregular	Mucoid	Opaque	Raised
B11b	White	Round	Irregular	Mucoid	Opaque	Raised
B11c	White	Round	Irregular	Non mucoid	Opaque	Flat
B11d	White	Irregular	Branching	Non mucoid	Transparent	Flat
B12a	White	Round	Irregular	Non mucoid	Translucent	Flat
B12b	White	Round	Smooth	Mucoid	Opaque	Raised
B12c	White	Round	Smooth	Non mucoid	Opaque	Low convex
B13a	White	Round	Smooth	Mucoid	Opaque	Raised
B13b	White	Round	Smooth	Mucoid	Opaque	Raised

B14a	Off white	Round	Irregular	Non mucoid	Opaque	Flat
B14b	White	Round	Smooth	Non mucoid	Opaque	Flat
B14c	White	Round	Smooth	Mucoid	Opaque	Raised
B14d	White	Round	Smooth	Mucoid	Opaque	Raised
B15a	Creamy	Round	Smooth	Non mucoid	Opaque	Convex
B15b	Yeloowish	Oval	Irregular	Non mucoid	Opaque	Low convex
B15c	Creamy white	Round	Smooth	Non mucoid	Opaque	Low convex
B15d	White	Irregular	Irregular	Non mucoid	Opaque	Flat
B16a	White	Round	Irregular	Mucoid	Opaque	Raised
B16b	White		Smooth	Mucoid	Opaque	Raised
B16c	White	Round	Irregular	Mucoid	Opaque	Raised
B16d	Creamy white	Round	Smooth	Non mucoid	Translucent	Low convex
B17a	White	Round	Irregular	Mucoid	Opaque	Raised
B17b	White	Round	Irregular	Mucoid	Opaque	Raised
B17c	White	Round	Irregular	Mucoid	Opaque	Raised
B17d	White	Round	Irregular	Mucoid	Opaque	Raised
B17e	Creamy white	Round	Smooth	Non mucoid	Translucent	Low convex
B18a	White	Round	Irregular	Mucoid	Opaque	Raised
B18b	Creamy	Oval	Smooth	Non mucoid	Translucent	Flat
B19a	White	Round	Irregular	Mucoid	Opaque	Raised
B19b	White	Oval	Irregular	Non mucoid	Opaque	Flat
B19c	White	Round	Irregular	Mucoid	Opaque	Low convex
B19d	Creamy	Round	Smooth	Non mucoid	Opaque	Flat
B20a	White	Round	Irregular	Mucoid	Opaque	Raised
B20b	White	Round	Irregular	Mucoid	Translucent	Convex
B20c	Creamy	Round	Irregular	Mucoid	Translucent	Cnvex
B20d	Creamy	Round	Smooth	Mucoid	Opaque	Convex
B20e	White	Round	Irregular	Mucoid	Opaque	Raised
B20f	Creamy	Round	Smooth	Mucoid	Opaque	Low convex
B21a	White	Round	Irregular	Mucoid	Opaque	Raised
B22a	Yellow	Round	Smooth	Non mucoid	Opaque	Convex
B23a	White	Oval	Irregular	Non mucoid	Opaque	Flat
B23b	White			Mucoid	Translucent	Convex
B23c	White	Round	Irregular	Mucoid	Opaque	Raised
B23d	Creamy	Round	Smooth	Non muoid	Opaque	Low convex
B23e	White	Oval	Irregular	Non mucoid	Opaque	Flat
B24a	White	Round	Irregular	Non mucoid	Opaque	Flat
B24b	Creamy white	Round	Smooth	Non mucoid	Translucent	flat

APPENDIX-VI

Table III: Biochemical tests of crystal positive *Bacillus thuringiensis*

Isolates	Biochemical tests					Sugar utilization tests					Hydrolysis tests					
	Catalase	Oxidase	Citrate	Nitrate	Urease	Glucose	Mannitol	Galactose	Sucrose	Salicin	Casein	Gelatin	Starch	Tween 20	Esculin	Lecithin
T1	+	+	+	-	+	+	+	-	+	+	-	+	-	+	+	-
T3b	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-
T4	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-
T5c	+	+	+	-	+	+	+	-	+	-	-	-	-	+	-	-
T6a	+	+	-	-	+	+	+	-	-	-	+	+	-	+	-	-
T6b	+	+	+	-	+	-	+	-	+	-	+	+	-	+	-	-
T7a	+	+	-	-	-	+	+	+	-	-	+	+	-	+	+	-
T7b	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	-
T7d	+	+	-	-	+	-	+	-	-	-	+	+	-	+	-	-
T8a	+	+	-	-	+	-	+	-	-	+	+	+	-	+	-	-
T8b	+	+	-	+	-	+	+	-	+	+	+	+	+	+	-	-
T9a	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-
T9b	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-
T10a	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	-
T10c	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+
T10d	+	+	+	+	-	+	+	-	-	-	+	+	+	+	+	+
T11	+	+	-	-	+	+	+	-	+	+	+	+	-	+	-	-
T12a	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-
T12b	+	+	-	-	+	-	+	-	-	+	+	+	-	+	-	+
T13a	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-

	Biochemical tests					Sugar utilization tests					Hydrolysis tests					
	Catalse	Oxidase	Citrate	Nitrate	Urease	Glucose	Mannitol	Galactose	Sucrose	Salicin	Casein	Gelatin	Starch	Tween 20	Esculin	Lecithin
T13b	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	-
T13c	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+
T15a	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+
T15b	+	+	+	-	+	+	+	-	+	-	+	+	-	+	-	-
T15c	+	+	-	+	-	+	+	-	+	+	+	+	-	+	+	+
T15d	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-
T16a	+	+	-	+	-	+	+	-	+	+	+	+	+	+	+	-
T17	+	+	-	+	+	-	+	-	+	-	+	+	-	+	-	-
B1a	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B4a	+	+	-	-	-	+	-	-	-	+	+	+	+	+	+	+
B4b	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
B5b	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-
B6a	+	+	-	-	-	+	-	+	+	-	+	+	+	+	+	+
B7a	+	+	+	+	+	+	-	-	+	-	+	+	-	+	-	-
B8a	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-
B8c	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B8d	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B9b	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-
B10a	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
B10d	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
B11a	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B11b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B11c	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B11d	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B12b	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-

Isolates	Biochemical tests					Sugar utilization tests					Hydrolysis tests					
	Catalase	Oxidase	Citrate	Nitrate	Urease	Glucose	Mannitol	Galactose	Sucrose	Salicin	Casein	Gelatin	Starch	Tween 20	Esculin	Lecithin
B13b	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-
B14c	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-
B14d	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
B15a	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+
B15d	+	+	-	+	-	+	-	-	+	+	+	+	+	+	+	-
B16a	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+
B16b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B16c	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
B17a	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-
B17b	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-
B17c	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
B17d	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
B18a	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	-
B19a	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
B19b	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+
B19c	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
B19d	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B20a	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-
B20b	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
B20c	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
B20d	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-
B20e	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-
B20f	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B23a	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+
B23b	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-

Isolates	Biochemical tests					Sugar utilization tests					Hydrolysis tests					
	Catalase	Oxidase	Citrate	Nitrate	Urease	Glucose	Mannitol	Galactose	Sucrose	Salicin	Casein	Gelatin	Starch	Tween 20	Esculin	Lecithin
B23c	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
B23d	+	+	+	-	+	-	-	+	-	-	+	+	+	+	-	-
B23e	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+
B24b	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-

APPENDIX-VII

TableIV: Biochemical types of *B. thuringiensis*

Biochemical type (described subspecies)	**Biochemical test result[a]**			
	Esculin	**Salicin**	**Lecithinase**	**Sucrose**
1 (*thuringiensis*)	+	+	+	+
2 (*kurstaki*)	+	+	+	-
3 (*indiana*)	+	+	-	+
4 (*galleriae*)	+	+	-	-
5 (*sotto*)	+	-	+	+
6 (*dendrolimus*)	+	-	+	-
7 (*morrisoni*)	+	-	-	+
8 (*darmstadiensis*)	+	-	-	-
9	-	+	+	+
10	-	+	+	-
11	-	+	-	+
12 (*ostriniae*)	-	+	-	-
13	-	-	+	+
14 (*israliensis*)	-	-	+	-
15	-	-	-	+
16	-	-	-	-

[a.] Positive reaction, i.e., utilization of esculin, acid production from salicin and sucrose, and production of lecithinase.

APPENDIX-VIII

Table V: Standard Curve for molecular weight determination of DNA

S.No.	Rf value	Log(molecular size)
1	0.2643	4.3641
2	0.2988	3.9738
3	0.3563	3.8167
4	0.4137	3.6395
5	0.5287	3.3658
6	0.5632	3.3068

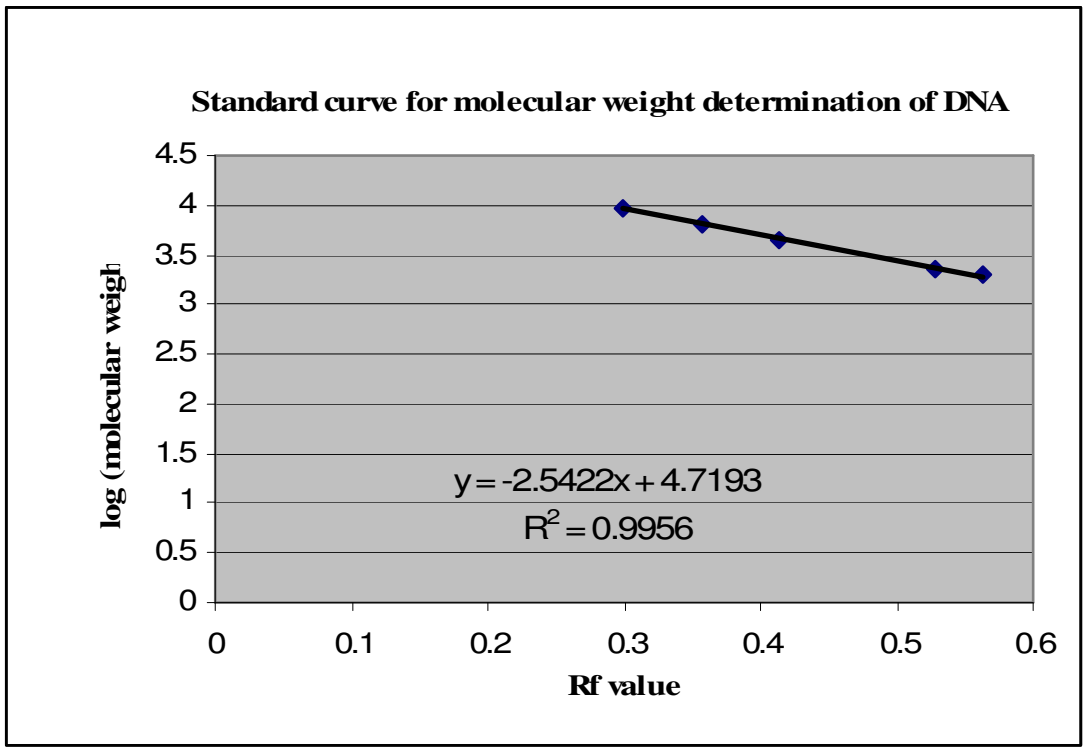

Table VI: Determination of molecular weight (Gel1)

Lane No.	Isolates	Distance travelled by each band (cm)	Rf value	Log size of band	Size of band (Kbp)
1	λDNA/Hind IIIcut	2.3	0.2643	4.3641	23.130
		2.6	0.2988	3.9738	9.416
		3.1	0.3563	3.8167	6.557
		3.6	0.4137	3.6395	4.361
		4.6	0.5287	3.3658	2.322
		4.9	0.5632	3.3068	2.027
2	T3b	6.9	0.7931	2.7029	0.505
		7.3	0.8390	2.586	0.386
		7.6	0.8735	2.4984	0.315
3	T7b	6.9	0.7931	2.7029	0.505
		7.3	0.8390	2.5860	0.386
		7.6	0.8735	2.4984	0.315
		8.3	0.9540	2.2938	0.197
4	T9b	7.3	0.8390	2.5860	0.386
		7.6	0.8735	2.4984	0.315
		8.3	0.9540	2.2938	0.197
5	T15a	7.3	0.8390	2.5860	0.386
		7.6	0.8735	2.4984	0.315
		8.3	0.9540	2.2938	0.197
6	T16a	7.3	0.8390	2.5860	0.386
		7.6	0.8735	2.4984	0.315
		8.3	0.9540	2.2938	0.197
7	B8a	7.3	0.8390	2.5860	0.386
		7.6	0.8735	2.4984	0.315
		8.3	0.9540	2.2938	0.197
8	B8c	7.3	0.8390	2.5860	0.386
		7.7	o.8850	2.4692	0.295
		8.3	0.9540	2.2938	0.197
9	B11a	7.3	0.8390	2.5860	0.386
		8.3	0.9540	2.2938	0.197
10	B16b	7.6	0.8735	2.4984	0.315
		8.3	0.9540	2.2938	0.197
11	B16c	7.4	0.8505	2.5568	0.360
		7.9	0.9080	2.4107	0.257
		8.3	0.9540	2.2938	0.197
12	Negative control	No amplification			

*Distance travelled by blue juice: 8.7cm

$Y = -2.5422x+4.7193$

$R^2 = 0.9956$

Table VII: Determination of molecular weight (Gel 2)

Lane No.	Isolates	Distance travelled by each band (cm)	Rf value	Log size of bands	Size of bands (kbp)
13	λDNA/*Hind IIIcut*	2.5	0.2551	4.36417	23.130
		3.0	0.3061	3.9738	9.416
		3.4	0.3469	3.8167	6.557
		4.0	0.4081	3.6395	4.361
		5.3	0.5408	3.3658	2.322
		5.6	0.5714	3.3068	2.027
14	T13a	8.3	0.8469	2.6213	0.418
		8.8	0.8979	2.4976	0.315
15	T10a	6.5	0.6632	3.0665	1.165
		6.9	0.7040	2.9675	0.928
		8.8	0.8979	2.4976	0.315
16	B6a	8.1	0.8265	2.6707	0.469
		8.9	0.9081	2.4729	0.297
17	B10a	8.0	0.8163	2.6955	0.496
18	T7a	7.8	0.7959	2.7449	0.556
		8.4	0.8571	2.5965	0.395
19	T8b	7.8	0.7959	2.7449	0.556
		8.7	0.8877	2.5223	0.333
		9.2	0.9387	2.3987	0.250
20	T8a	7.0	0.7142	2.9428	0.877
		7.7	0.7857	2.7697	0.588
		8.6	0.8775	2.5471	0.352
		9.3	0.9489	2.3739	0.237
21	T6b	5.7	0.5816	3.2643	1.838
		6.4	0.6530	3.0912	1.234
		7.4	0.7551	2.8439	0.698
		7.6	0.7755	2.7944	0.623
		8.2	0.8367	2.6460	0.443
		9.1	0.9285	2.4234	0.265
22	B7a	6.4	0.6530	3.0912	1.234
		7.4	0.7551	2.8439	0.698
		7.6	0.7755	2.7944	0.623
		8.2	0.8367	2.6460	.443
		9.1	0.9285	2.4234	0.265
23	B4a	No amplification			
24	Negative control	No amplification			

APPENDIX-IX

Table VIII: Molecular size of RAPD-fragments of the isolates

Lane no.	*B. thuringiensis* (subspecies)	Size of amplified fragments (bp)
2	T3b	505, 386, 315
3	T7b	505, 386, 315, 197
4	T9b	386, 315, 197
5	T15a	386, 315, 197
6	T16a	386, 315, 197
7	B8a	386, 315, 197
8	B8c	386, 295, 197
9	B11a	386,197
10	B16b	315, 197
11	B16c	360, 257
12	Negative control	No amplification
14	T13a	418, 315
15	T10a	1165, 928, 315
16	B6a	469, 297
17	B10a	496
18	T7a	556, 395
19	T8b	556,333, 250
20	T8a	877, 588, 352, 237
21	T6b	1838, 1234, 698, 623, 443, 265
22	B7a	1234, 698, 623, 443, 265
23	B4a	No amplification
24	Negative control	No amplification

APPENDIX-X

Table IX: Known *cry* and *cyt* gene sequences with revised nomenclature assignments (Crickmore et al., 1998)

Revised gene name	Original gene or protein name	Accession no.	Coding region[a]	Reference
cry1Aa1	*cryIA(a)*	M11250	527–4054	92
cry1Aa2	*cryIA(a)*	M10917	153–>2955	98
cry1Aa3	*cryIA(a)*	D00348	73–3600	99
cry1Aa4	*cryIA(a)*	X13535	1–3528	62
cry1Aa5	*cryIA(a)*	D17518	81–3608	113
cry1Aa6	*cryIA(a)*	U43605	1–>1860	63
cry1Ab1	*cryIA(b)*	M13898	142–3606	119
cry1Ab2	*cryIA(b)*	M12661	155–3622	111
cry1Ab3	*cryIA(b)*	M15271	156–3620	31
cry1Ab4	*cryIA(b)*	D00117	163–3627	50
cry1Ab5	*cryIA(b)*	X04698	141–3605	40
cry1Ab6	*cryIA(b)*	M37263	73–3537	37
cry1Ab7	*cryIA(b)*	X13233	1–3465	36
cry1Ab8	*cryIA(b)*	M16463	157–3621	69
cry1Ab9	*cryIA(b)*	X54939	73–3537	13
cry1Ab10	*cryIA(b)*	A29125	—[b]	28
cry1Ac1	*cryIA(c)*	M11068	388–3921	3
cry1Ac2	*cryIA(c)*	M35524	239–3769	117
cry1Ac3	*cryIA(c)*	X54159	339–>2192	18
cry1Ac4	*cryIA(c)*	M73249	1–3534	84
cry1Ac5	*cryIA(c)*	M73248	1–3531	83
cry1Ac6	*cryIA(c)*	U43606	1–>1821	63
cry1Ac7	*cryIA(c)*	U87793	976–4509	38
cry1Ac8	*cryIA(c)*	U87397	153–3686	71
cry1Ac9	*cryIA(c)*	U89872	388–3921	33
cry1Ac10		AJ002514	388–3921	107
cry1Ad1	*cryIA(c)*	M73250	1–3537	79
cry1Ae1	*cryIA(e)*	M65252	81–3623	60
cry1Af1	*icp*	U82003	172–>2905	49
cry1Ba1	*cryIB*	X06711	1–3684	10
cry1Ba2		X95704	186–3869	105
cry1Bb1	ET5	L32020	67–3753	25
cry1Bc1	*cryIB(c)*	Z46442	141–3839	6
cry1Bd1	*cryE1*	U70726		12
cry1Ca1	*cryIC*	X07518	47–3613	45
cry1Ca2	*cryIC*	X13620	241–>2711	88
cry1Ca3	*cryIC*	M73251	1–3570	79
cry1Ca4	*cryIC*	A27642	234–3800	114
cry1Ca5	*cryIC*	X96682	1–>2268	106
cry1Ca6	*cryIC*	X96683	1–>2268	106
cry1Ca7	*cryIC*	X96684	1–>2268	106
cry1Cb1	*cryIC(b)*	M97880	296–3823	48
cry1Da1	*cryID*	X54160	264–3758	42
cry1Db1	*prtB*	Z22511	241–3720	56
cry1Ea1	*cryIE*	X53985	130–3642	115
cry1Ea2	*cryIE*	X56144	1–3513	7
cry1Ea3	*cryIE*	M73252	1–3513	82
cry1Ea4		U94323	388–3900	47
cry1Eb1	*cryIE(b)*	M73253	1–3522	81
cry1Fa1	*cryIF*	M63897	478–3999	14
cry1Fa2	*cryIF*	M73254	1–3525	80
cry1Fb1	*prtD*	Z22512	483–4004	56
cry1Ga1	*prtA*	Z22510	67–3564	56
cry1Ga2	*cryIM*	Y09326	692–4210	96
cry1Gb1	*cryH2*	U70725		12
cry1Ha1	*prtC*	Z22513	530–4045	56
cry1Hb1		U35780	728–4195	53
cry1Ia1	*cryV*	X62821	355–2511	108
cry1Ia2	*cryV*	M98544	1–2157	34
cry1Ia3	*cryV*	L36338	279–2435	100
cry1Ia4	*cryV*	L49391	61–2217	54
cry1Ia5	*cryV159*	Y08920	524–2680	94
cry1Ib1	*cryV465*	U07642	237–2393	100
cry1Ja1	ET4	L32019	99–3519	25
cry1Jb1	ET1	U31527	177–3686	116
cry1Ka1		U28801	451–4098	52
cry2Aa1	*cryIIA*	M31738	156–2054	20
cry2Aa2	*cryIIA*	M23723	1840–3738	123
cry2Aa3		D86064	2007–3911	89
cry2Ab1	*cryIIB*	M23724	1–1899	123
cry2Ab2	*cryIIB*	X55416	874–2775	17
cry2Ac1	*cryIIC*	X57252	2125–3990	124
cry3Aa1	*cryIIIA*	M22472	25–1956	39
cry3Aa2	*cryIIIA*	J02978	241–2172	93
cry3Aa3	*cryIIIA*	Y00420	566–2497	41
cry3Aa4	*cryIIIA*	M30503	201–2132	65
cry3Aa5	*cryIIIA*	M37207	569–2500	22
cry3Aa6	*cryIIIA*	U10985	569–2500	1
cry3Ba1	*cryIIIB2*	X17123	25–>1977	101
cry3Ba2	*cryIIIB*	A07234	342–2297	85
cry3Bb1	*cryIIIB5*	M89794	202–2157	24
cry3Bb2	*cryIIIC(b)*	U31633	144–2099	23
cry3Ca1	*cryIIID*	X59797	232–2178	59
cry4Aa1	*cryIVA*	Y00423	1–3540	121
cry4Aa2	*cryIVA*	D00248	393–3935	95
cry4Ba1	*cryIVB*	X07423	157–3564	16
cry4Ba2	*cryIVB*	X07082	151–3558	112
cry4Ba3	*cryIVB*	M20242	526–3930	125
cry4Ba4	*cryIVB*	D00247	461–3865	95
cry5Aa1	*cryVA(a)*	L07025	1–>4155	102
cry5Ab1	*cryVA(b)*	L07026	1–>3867	67
cry5Ac1		I34543	1–>3660	76
cry5Ba1	PS86Q3	U19725	1–>3735	76
cry6Aa1	*cryVIA*	L07022	1–>1425	68
cry6Ba1	*cryVIB*	L07024	1–>1185	67
cry7Aa1	*cryIIIC*	M64478	184–3597	58
cry7Ab1	*cryIIIC(b)*	U04367	1–>3414	75
cry7Ab2	*cryIIIC(c)*	U04368	1–>3414	75
cry8Aa1	*cryIIIE*	U04364	1–>3471	29
cry8Ba1	*cryIIIG*	U04365	1–>3507	66
cry8Ca1	*cryIIIF*	U04366	1–3447	70
cry9Aa1	*cryIG*	X58120	5807–9274	104
cry9Aa2	*cryIG*	X58534	385–>3837	32
cry9Ba1	*cryX*	X75019	26–3488	97
cry9Ca1	*cryIH*	Z37527	2096–5569	57
cry9Da1	N141	D85560	47–3553	4
cry9Da2		AF042733	<1–>1937	122
cry10Aa1	*cryIVC*	M12662	941–2965	111
cry11Aa1	*cryIVD*	M31737	41–1969	21
cry11Aa2	*cryIVD*	M22860	<1–235	2
cry11Ba1	Jeg80	X86902	64–2238	19
cry11Bb1	94 kDa	AF017416		72
cry12Aa1	*cryVB*	L07027	1–>3771	67
cry13Aa1	*cryVC*	L07023	1–2409	90
cry14Aa1	*cryVD*	U13955	1–3558	77
cry15Aa1	34kDa	M76442	1036–2055	11
cry16Aa1	*cbm71*	X94146	158–1996	5
cry17Aa1	*cbm72*	X99478	12–1865	5
cry18Aa1	*cryBP1*	X99049	743–2860	126
cry19Aa1	Jeg65	Y07603	719–2662	86
cry19Ba1		D88381		87
cry20Aa1	86kDa	U82518	60–2318	61
cry21Aa1		I32932	1–3501	74
cry22Aa1		I34547	1–2169	76
cyt1Aa1	*cytA*	X03182	140–886	118
cyt1Aa2	*cytA*	X04338	509–1255	120
cyt1Aa3	*cytA*	Y00135	36–782	26
cyt1Aa4	*cytA*	M35968	67–813	30
cyt1Ab1	*cytM*	X98793	28–777	109
cyt1Ba1		U37196	1–795	78
cyt2Aa1	*cytB*	Z14147	270–1046	51
cyt2Ba1	*"cytB"*	U52043	287–655	35
cyt2Bb1		U82519	416–1204	15

[a] The symbols < and > indicate that the coding region extends up- or downstream, respectively, from the known sequence data.
[b] Only the polypeptide sequence has been reported.

Table X: Phylogram demonstrating amino acid sequence identity among cry and cyt proteins (Crickmore et al., 1998)

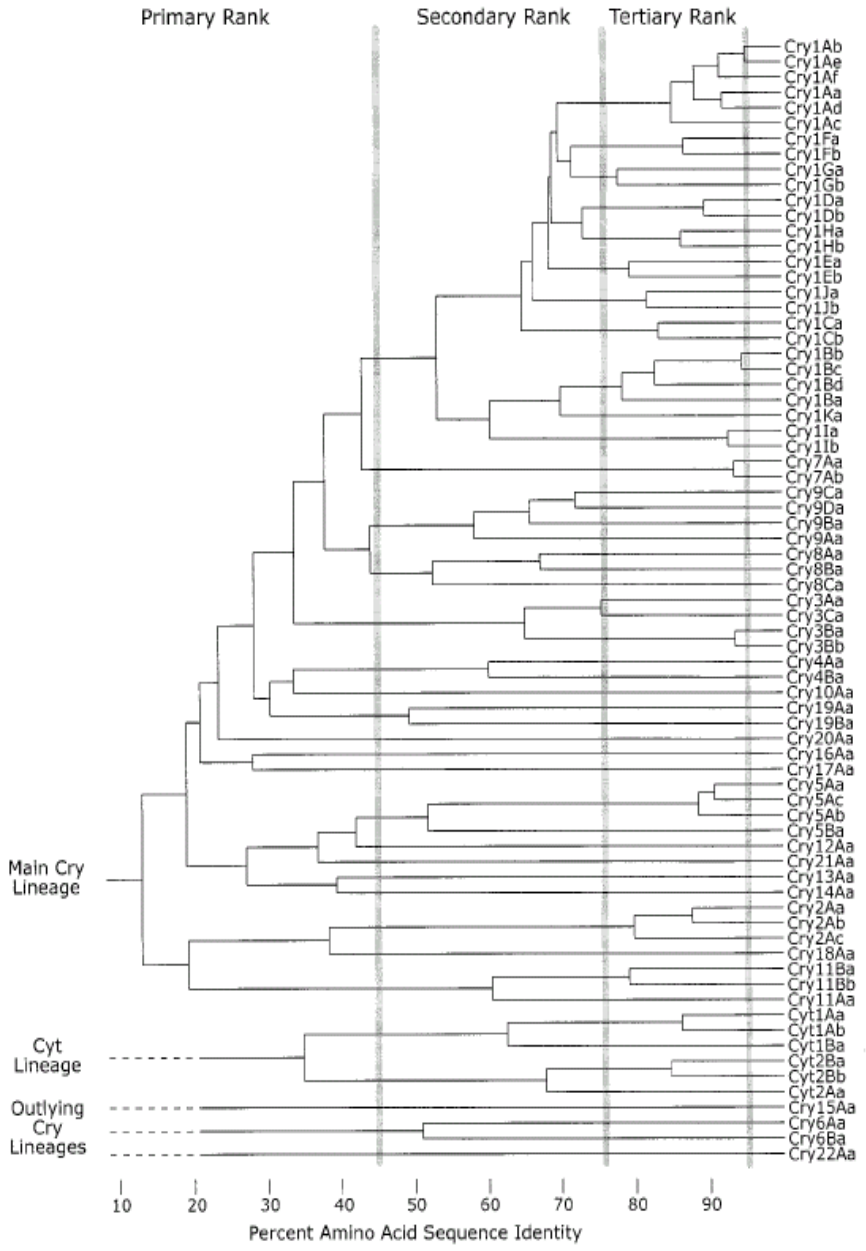

FIG. 1. Phylogram demonstrating amino acid sequence identity among Cry and Cyt proteins. This phylogenetic tree is modified from a TREEVIEW visualization of NEIGHBOR treatment of a CLUSTAL W multiple alignment and distance matrix of the full-length toxin sequences, as described in the text. The gray vertical bars demarcate the four levels of nomenclature ranks. Based on the low percentage of identical residues and the absence of any conserved sequence blocks in multiple-sequence alignments, the lower four lineages are not treated as part of the main toxin family, and their nodes have been replaced with dashed horizontal lines in this figure.