QATAR UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

DEVELOPMENT OF BIO-INSECTICIDES BASED ON LOCAL QATARI BACILLUS THURINGIENSIS STRAINS FOR THE BIOLOGICAL CONTROL OF HARMFUL DIPTERAN DISEASE VECTORS

BY

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ABSTRACT

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Title: Development of Bio-insecticides Based on Local Qatari Bacillus Thuringiensis Strains for the Biological Control of Harmful Dipteran Disease Vectors

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The very friendly bacterium *Bacillus thuringiensis* (*Bt*) is the main source of environment-friendly and safe bioinsecticides used in the control of plant pests and animal and human disease vectors. A collection of 441 Qatari *Bt* strains were characterized and classified based on crystal morphology, plasmid patterns, crystal protein patterns and *cry* and *cyt* genes. In summary, the *Bti* strain *QBT*220 was recognized as the most efficient against Dipteran insects *Aedes Aegypti*. Two of its clones obtained by plasmid curing showed an increase of 115% in the δ-endotoxins. As example of novel *Bt* strains, *QBT*674 is a spherical crystal producing strain having *cry2* gene but no cuboidal crystals. *QBT*555 is a Non-*Bti* strain with molecular profile very different from *Bti* strains, but expresses proteins like Cry11, Cry10 and Cyt1A. *QBT*229 showed high cytolytic activity due to five amino acid replacements in its β sheet that enhanced its anti-cancer activity.

DEDICATION

This thesis is dedicated to all my teachers who guided me through the years, academically and personally.

Thanks to them for not only imparting knowledge but also help me be the person I am today.

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CHAPTER 1: INTRODUCTION

Bacillus thuringiensis (*Bt*) is a rod shaped, Gram positive, spore forming and entomopathogenic bacterium (Soberon *et al*., 2013; Palma *et al*., 2014). It is a ubiquitous microorganism found in many different habitats like soil, water, insect guts, plant leaves, etc. (Mashtoly *et al*., 2010; Despres *et al*., 2011; Koppenhofer *et al*., 2012). *Bt* produces many toxins like chitinases, exotoxins, δ-endotoxins, bacteriocins, parasporins, etc (Luara-Salarazar *et al*., 2016; Periyasamy *et al*., 2016; Honda *et al*., 2017). *Bt* has been largely explored for their insecticidal parasporal crystals containing one or more δ-endotoxins. The latter produced by *Bt* depend on the strain and are specifically toxic to insect families, depending on the nature of *cry* genes expressed (Jouzani *et al*., 2008; Periera *et* al., 2013). The *Bt* crystals and spores are ingested by the insect larvae and reach the midgut. The crystals are solubilized into different proteins due to the high pH of the insect midgut. The solubilized δ-endotoxins are also called protoxins, which are cleaved by the proteases of the midgut of the larvae to form activated toxins. Once cleaved, the toxins bind specifically to midgut epithelial cells membrane receptors on the and bind. The binding of these toxins to the receptors trigger the lysis of the cells and forming leaks in the midgut. This causes starvation in the larvae and the consequent death (Lopez *et al*., 2013).

Most widely studied strains of *Bt* are *Bacillus thuringiensis kurstaki* (*Btk*) and *Bacillus thuringiensis israelensis* (*Bti*). *Btk* strains produce bipyramidal and cuboidal shaped parasporal crystals. The δ-endotoxins forming the bipyramidal crystals are toxic to Lepidopteran insects and that of cuboidal crystals are toxic to Dipteran insects. On the other hand, *Bti* produces spherical crystals that contain δ-endotoxin proteins that are toxic to Dipteran insects. *Bt* provides an economic, environment-friendly and very safe alternative of insect control (Thomas and Read, 2007). The δ-endotoxins proteins are

encoded by genes carried on one or more extra chromosomal plasmids and their expression is under the control of the sporulation dependent sigma factors. Some of the δ-endotoxins proteins are sporulation-independent and are expressed during the vegetative phase of life cycle. Each type of δ-endotoxin is specifically toxic to insect families (Abdelmalek *et al*., 2015).

The Dipteran specific δ-endotoxins belong to two groups: Cry (crystalizing protein) and Cyt (cytolytic protein). For the complete insecticidal activity, there is a need for some accessory proteins like p19 and p20. Cry proteins that are toxic to Dipteran insects include Cry4, Cry2, Cry11 and Cry10 and the Cyt proteins including Cyt1A, Cyt2A, Cyt1C. *Bti* strain carry the genes encoding all these Cry, Cyt and accessory proteins.

The objective of this PhD research work was to characterize, classify and screen the *Bt* strains isolated locally to identify strains that carry Dipteran specific δ- endotoxins. Finding a sustainable solution to control Dipteran insects like mosquitoes is the need of the hour. World health organization (WHO) considered Malaria and Dengue the two most dangerous vector borne diseases prevalent in the world today that need an urgent and effective control system (WHO, 2019). More than 50% of the earth's population face the threat of contracting these diseases as they are the cause of the highest morbidity and mortality among infectious diseases (WHO, 2012). Their control depends on the control of the vector as the treatment of these diseases is neither a feasible solution for eradication, nor affordable by everyone (Monath and Vasconcelos, 2015). Dengue virus is transmitted mostly by *Aedes aegypti* and preferentially transferred among humans (Carrington and Simmons, 2014). Malaria is caused by the parasite *Plasmodium* that is transmitted by Dipteran insect called *Anopheles* (Mernard *et al*., 2013).

The human disease-causing vectors like mosquitoes are controlled mostly by methods

like chemical insecticides, traps, environmental management strategies, social awareness, etc. But, these methods are not sufficient to control the vectors to a level where the diseases can be completely eradicated (Horatick *et al*., 2010; Achee *et al*., 2019). Although existing chemical insecticides occupy the major share of the market, they are being rendered ineffective every day due to three main reasons: behavioral changes in the vector to avoid toxicity, the development of resistance to different chemical moieties (Kongmee *et al*., 2004; Miller *et al*., 2009), the harmful effects on humans, other non-target organisms and environment (Scholte *et al*., 2004; Ffrench-Constant, 2005). A more sustainable method of controlling mosquitoes in the form of *Bti* was found about four decades back (Dammak *et al*., 2010; Lacey *et al*., 2015). Unlike chemical insecticides, *Bti* δ-endotoxins are effective at much lower concentrations, do not affect non-target organisms, have very low risk of developing resistance and can be produced at a large scale at a much smaller cost (Walker 2002; Lacey *et al*., 2015; Jain *et al*., 2016). The safety of *Bti* as a bio-control agent of mosquitoes have been shown by many studies (Vasquez *et al*., 2009; Loke *et al*., 2010; Wirth, 2010; Boyer *et al*., 2011; Tetraeu *et al*., 2013; Mossa *et al*., 2018).

Qatar, like many countries, also rely mainly on chemical insecticides to control these vectors of human diseases. Qatar does not produce these insecticides but imports them. According to United Nations Commodity Trade Statistics Database, Qatar's annual insecticide imports increased from 800 tons in 2005 to 11000 tons in 2010. Just considering the mosquito population of Qatar, it harbors a high diversity of mosquitos. A study done in different regions like Doha (highly populated) and Al Khor (less populated), found the presence of vectors of both Dengue and Malaria (Mikhail *et al*., 2009). Preliminary exploration studies showed the presence of high diversity of *Bt* strains in Qatar. This gave impetus to this project of exploring Qatari environment for

Bt strains producing Dipteran specific δ- endotoxins. Extreme climatic conditions and lack of use of *Bt* based commercial insecticides in the unexplored ecologies are the advantages in exploring the Qatari ecology for novel and interesting *Bt* strains.

Apart from its insecticidal property, *Bti* is also known for its cytolytic property against cancer cells. The cytolytic activity of *Bt* is contributed to two factors: the anticancer parasporin proteins and Cyt proteins (Ohba *et al*., 2009; Correa *et al*., 2012). This property of the *Bt* strains were also explored in this study as there is a great need to find solution to the increasing mortality rates of cancer (Banner *et al*., 2009).

The objectives of the PhD project were:

(1) The characterization of *Bt* strains producing spherical crystals based on crystal morphology, plasmid content, δ-endotoxins protein patterns

(2) The classification of *Bt* strains into classes and choose true representatives to be analyzed on molecular levels

(3) The screening of *Bt* strains and identification of the most effective insecticidal strain (s) against Dipteran insects

(4) The investigation of the *Bt* strains for the presence of δ-endotoxins proteins and coding *cry* genes

(5) The evaluation of the cytolytic activity among the *Bt* strains and identification of the most cytolytic strain(s)

(6) The comparison and identification of the *Bti* strains that have a higher δ-endotoxin yield than the commercially available *Bti* strains H14

(7) The exploration of the structural instability among the *Bti* strains and chose of the most promising strains with highly stable plasmids carrying δ-endotoxins

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Dipteran insects like mosquitoes are disease-transmitting vectors that are present worldwide in all types of ecologies. They transmit diseases like yellow fever, dengue, malaria, filariasis, etc among humans and animals. More than 3500 species of mosquitoes studied and reported, belong to the Dipteran family of insects. These disease-carrying vectors are responsible for about 17% deaths due to infections caused by parasites, viruses and bacteria (WHO, 2017). According to World Health Organization (WHO, 2015), if we consider just one of these diseases, malaria, half the world population is at risk. The death tolls of these diseases are too high in developing countries and their control using only medications is not a plausible solution (WHO, 2008; Tolle, 2009). Malaria, transmitted by *Anopheles* mosquitoes, has caused the death of more than 400,000 individuals in 2015 alone. The cases of Dengue, transmitted by *Aedes aegypti* mosquitoes, have increased 30-fold in the last three decades and more and more countries are reporting new outbreaks of this disease (WHO, 2018). The lack of availability and exorbitant costs of the prophylactic medications and vaccines against these diseases make it inaccessible to most of the disease prone developing countries. It has been recognized that the control of the vectors of the diseases is a more practical solution. From the beginning, all the countries rely heavily on synthetic chemical insecticides to eradicate these vectors. These insecticides give quick and prolonged results and are hence still occupying almost the entire market for insecticides, despite the availability of other alternatives (Federici *et al.*, 2003). Also, today we recognize the disadvantages of these chemical compounds and their harmful effects on humans, animals, vegetation and environment. The vectors are continuously acquiring resistance to these compounds and industries are developing new varieties every day. These

compounds give prolonged effect because they are retained in the ecologies and their harmful effects on non-target entities are seen for a long time. They develop into persistent pollutants of nature that are difficult to remediate.

Qatar was declared a malaria free country by WHO in 1990 (WHO, 1990). But, many cases of malaria have been reported in the last few years among the expatriates immigrating to the country (Al-Kuwari, 2009; Khan *et al.,* 2009). As the incidences of these cases become more frequent, the country could become vulnerable to this disease, especially because now it is evident that the malaria-carrying vector is present in the country (Baldacchino *et al.*, 2015). Mikhail *et al.,* (2009) found that Qatar has a huge diversity among mosquito species in different areas, rural and urban. According to this study, areas like Al Rayyan, Doha, Al Daayan, Al Khor, Al Shamal and Al Zahakira have many sub-species of *Anopheles*, *Culex pipiens* and *Aedes groups*. From 2009 to 2015, the study showed the presence of 5 new species of mosquitoes in Al Khor area alone (Kardousha, 2015). The vulnerability is further increased due to the incidences of endemic cases reported in the neighbouring country of Saudi Arabia (Fakeeh, 2001; Madani *et al.,* 2003; Alsheikh *et al.,* 2013; Memish *et al.,* 2014;). Other vectors like *Culex tritaeniorhynchus* carrying parasite for Rift Valley fever (Balkhy & Memish, 2003), *Culex univittatus* carrying virus for dengue like fever, *Anopheles stephensi* carrying parasite for malaria (Ahmed *et al.,* 2011; Alsheikh *et al.,* 2013) are considered the main disease-causing vectors in Saudi Arabia. All these vectors are now present in Qatar (Mikhal *et al.*, 2009; Kardousha, 2015).

Biocontrol of mosquitoes is the best method as the products used as insecticides can easily decompose in nature into products that are not toxic to other organisms or environment (Sanjay and Tiku, 2009). Biological insecticides that are being researched include plant-based insecticides (Bilal *et al*., 2012), bacteria-based insecticide, essential

oil-based insecticides (Muturi *et al*., 2019), bio-diesel by products (Pant *et al*., 2016), etc. *Bacillus thuringiensis* proteins are preferred among these alternatives because of their specificity and lack of harmful effects on humans or other organisms (Regis *et al.*, 2001, Federici *et al.*, 2005; Bravo *et al.*, 2007; Benfarhat-Touzri *et al.*, 2013). There are accounts of using the insecticidal activities of the *Bt* even before the bacteria itself was discovered. It is believed that in ancient Egypt, *Bt* spores were used as biocontrol agents (Sanahuja *et al.*, 2011). In the modern days, the bacterium was discovered when there was a sudden outbreak of sotto (sudden) disease that killed the *Bombyx mori* (silkworm) larvae at a very high rate in Japan. In 1901, a Japanese bacteriologist, Ishiwata Shigetane, isolated and identified the cause of the disease as a soil bacterium residing in the larval midgut causing the death (Bravo *et al*., 2012). He also recognized that the presence and growth of this bacterium was not enough to kill the larvae. The death was caused only when the bacterium entered the sporulation stage. A German scientist named Ernst Berliner found a similar bacterium in dead flour moth larvae didn't characterize the bacterium itself until 1915. He named the bacterium as thuringiensis as he was working in the region called Thuringia (Berliner, 1915). He also named the crystal proteins as parasporal bodies but did not associate them with the insecticidal properties. Subsequently many different scientists isolated different *Bt* species from different insect larvae. Their combined studies revealed the relationship between the parasporal crystals and the insecticidal properties (Angus, 1954). In 1955, Philip Fitz-James and Hannay characterized these crystals and found that they were proteins expressed by the bacteria during the sporulation stage only (Hannay & Fitz-James, 1955). The first successful field application was done in 1938 and since then improvements in the production technology is being continuously done. By 1950s, cheaper fermentation technologies were available for the commercial large-scale

production of *Bt* (Qaim and Zilberman, 2003; Kleter *et al*., 2007). Ever since, spore formulations are being used for field applications in countries like United States of America (USA) and France (Andrews *et al.*, 1987). As the chemical pesticides were already available in the market that gave very efficient results, *Bt* formulations could not compete with them in the market as an insect control agent. In spite of their many advantages, they still had some limitations in the commercial productions when compared to the synthetic chemical compounds. Research in this field has been focusing on these limitations – persistence, stability, resistance, high yield / production, toxicity, etc. There is always a need to screen for more strains to identify novel genes that could be more efficient against these disease vectors. Depending on the ecology from which these strains are isolated, they might have better combinations of δ-endotoxins, which can be used for more than one disease vector at the same time (Ammouneh *et al.,* 2011). Also, these novel strains could overcome some of the limitations of these bio-insecticides that are discussed below. In nature, bacteria and insects have developed many symbiotic relationships and hence exist together (Feldhaar, 2011). But, in rare cases like this, the bacteria behave like an insect pathogen. *Bt* has achieved this by developing strategies to enter the insects, avoid the immune system, and kill it. It is believed that the efficiency of these strategies is due to the long process of co-evolution (Vilcinskas, 2010; Baxter *et al.*, 2011).

Among the different strategies, strain improvement has enhanced the *Bt* commercial production. Some of the strains that were developed gave 10-50-fold more protein production than the ones that were initially used. Initially, most of the pesticides were produced using *Bt kurstaki* that produced δ- endotoxins toxic to the lepidopteran insects. Later, other strains of *Bt israelensis* and *Bt tenebrionis* were used for the toxin production against *Dipteran* and *Coleopteran* species, respectively (Kaur, 2000). While

the research for improving this technology was ongoing, their demand fell sharply in 1970s as more effective chemical pesticides were introduced in the markets. But, the advancements in the field of Biotechnology gave an impetus to this field of research (Schnepf & Whiteley, 1981). The cloning technologies changed the face of this research completely by introducing the genes from *Bt* into vectors that are more suitable for large-scale production, like *E coli*.

Among the large-scale screenings for *Bt* with insecticidal activities, it was found that in nature, there is always a very high percentage of non-insecticidal strains compared to the insecticidal ones (Ohba, 1996; Mizuki *et al.*, 1999; Ohba *et al.*, 2000; Ohba *et al.*, 2002; Yasutake *et al.*, 2006; Yasutake *et al.*, 2007). This negated the earlier assumption that these bacteria co-evolved with the insects that they are toxic to (Maeda *et al.*, 2000; Lee *et al.*, 2003). These non-insecticidal strains were screened for other activities and it was found that some of them are toxic to nematode parasites and some even have cytolytic properties against vertebrate cells including human cancer cell lines. In 1999, Mizuki *et al.* found for the first time parasporal proteins that had cytolytic activities against human leukemia T cells. He also tested these against the normal T cells. The non-cancerous cells were not sensitive to these parasporal proteins, which were later named as parasporins.

2.2 *Bacillus thuringiensis*

2.2.1 Introduction

Bt is a rod shaped, Gram positive, spore-forming bacteria that are present in a variety of ecologies including soil, water, insect midgut, plants, etc. (Margalith & Ben-Dov, 2000, Fillinger & Lindsey, 2006). They have high adaptability to their individual ecologies owing to their 60,000 genes pan-genome (Bazinet, 2017). They are commonly used as bio-pesticides or bio-insecticides. The life cycle of *Bt* consists of

two phases: the vegetative division phase and spore development phase (Bulla *et al.*, 1980). The rod-shaped bacteria that divides into two daughter cells characterizes the vegetative phase. On the other hand, sporulation stage constitutes seven stages: axial filament formation, forespore septum formation, engulfment and appearance of crystals, formation of exosporium along with primordial cell wall and spore coats, spore maturation and sporangial lysis (Ibrahim *et al.*, 2010). During this stage, they produce proteinaceous inclusion crystals called δ-endotoxins that have insecticidal properties. This has identified them as biocontrol agents and tools for genetic engineering of crops. There are also many *Bt* strains that produce crystal proteins but do not have any insecticidal properties (Abulreesh *et al.*, 2012). During both phases, they also produce other types of proteins that have insecticidal, cytotoxic or bactericidal properties. The production of the crystal proteins is seen only simultaneously with the spore formation and their appearance is regulated by the same system and hence occurs together. These crystals have different physical structures depending on the *Bt* species and each form is specific to certain insect groups. For example, the bipyramidal crystals have insecticidal properties against *Lepidopteran* species while the spherical crystals are toxic to *Dipteran* species. These bacteria and their protein crystals have been used commercially as biocontrol agents in field applications for almost four decades. Most of the products are based on the spore-crystals formulations from *Bt kurstaki* HD1 or HD73. Also, *Bt aizawai* HD137 that produces slightly different Cry proteins are also used commercially. These two groups are used against *Lepidopteran* species (Soberon *et al.*, 2009). *Bt* var *israelensis* has proved to be a successful bio-insecticide against *Dipteran* insects like mosquitoes, black flies, etc.

2.2.2 Ecology of *Bacillus thuringiensis*

Bt is indigenous to many environments including soil, insect cadavers (Carozzi *et al.*, 1991), plant leaves (Smith & Couche, 1991), aquatic bodies (Iriarte *et al.*, 2000), sediments in the sea (Maeda *et al.*, 2000), etc. As is evident, the bacterium can be found in a wide variety of ecologies but is most frequently found in soil ecologies. They are naturally saprophytes that feed on dead and decaying matter. In the absence of these conditions, they remain in the form of spores, until nutrients are available again. Depending on their presence in different ecologies, their functions in these environments are still not clear as some of these bacteria are also found in these ecologies without any insecticidal properties (Iriarte *et al.*, 2000). Because the *Bt* formulations have been used for field applications for many decades now, their ecologies are broadly classified as native or artificial. The native ecologies are ones where the *Bt* formulations have never been applied. The strains found in these ecologies are strictly local or naturally occurring. Artificial ecologies, on the contrary are the ecologies where commercially available *Bt* formulations have been introduced and even the local strains have been eventually affected by these applications (Stahly *et al.*, 1991).

2.2.3 Life cycle of *Bacillus thuringiensis*

The life cycle of *Bt* is divided into two phases: the symmetrically dividing vegetative phase and the asymmetrically dividing sporulation phase (Bulla *et al.*, 1980). The vegetative phase is the normal phase that is continued until the bacteria encounters a stressful condition. Once it encounters a stress environment or conditions, the sporulation stage is self-induced. Interestingly, during sporulation, the cell also forms some crystal proteins that are lethal to many of its susceptible insect targets. It is believed that these proteins are a survival strategy of the bacterium. By killing the host

larvae, *Bt* ensures the regeneration of a suitable environment for it to enter the vegetative phase again and continue the divisions (Ibrahim *et al.*, 2010).

The vegetative phase is characterized by 2-5 µm long and 1µm wide, rod shaped bacteria. During this phase, each bacterium grows and doubles in size and forms a septum exactly in the middle and the new large bacterium divides into two equal daughter cells. This continues till the bacteria have favourable conditions. The sporulation phase is characterized by the formation of spores and other associated proteins and their subsequent release after lysis of the rod-shaped bacterium. This phase is broadly divided into seven stages (Bechtel & Bulla, 1982).

During the first stage, the axial filament is formed followed by septum formation in stage II. Stage III marks the beginning of the appearance of parasporal crystals and endospores. During the next three stages, the cell transforms the nucleoid of the spore and forms the spore-coat, cell wall and cortex. The fidelity in these stages are important to ensure that the spores can regenerate and enter the vegetative phase when favourable conditions are available. The final stage is marked by spore maturation, lysis of the mother cell and release of spores and parasporal crystals (figure 1).

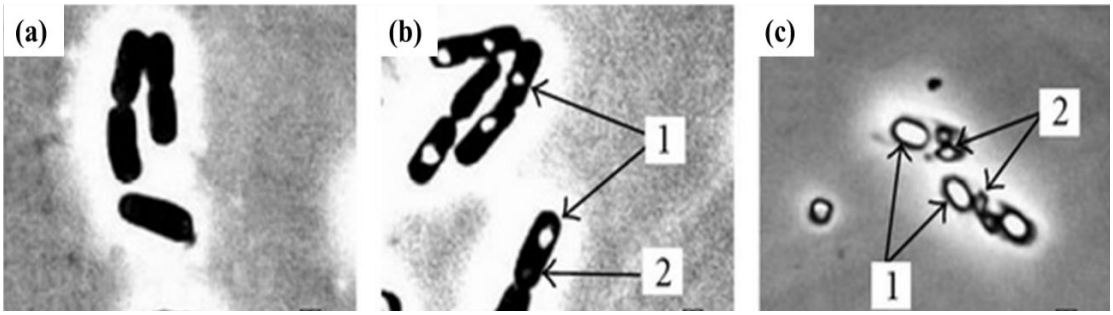

Figure 1. Stages of life cycle of *Bt* (Li *et al*., 2012)

[1: spore; 2: crystal; (a): vegetative stage; (b): mid-sporulation stage (c): complete sporulation and spores and crystal released]

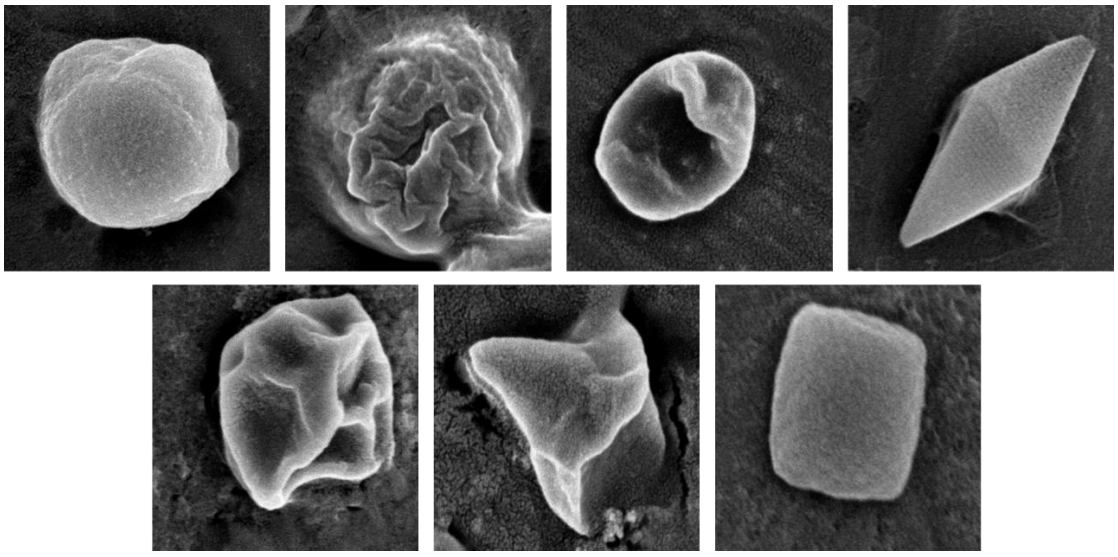

Figure 2. Types of parasporal crystals observed among the *Bt* strains (Nair *et al*., 2018b)

2.3 *Bt* toxins

Bt produces many proteins that are toxic to a wide range of organisms. Depending on the subspecies and strains, the proteins produced are toxic to different insects, nematodes, molluscs, and human cancer cells, other bacteria, etc (Kondo *et al.*,

2002; Van Frankenhuyzen, 2009; Ohba *et al.*, 2009; Pena *et al.*, 2013; Wang *et al.*, 2013; Van Frankenhuyzen, 2013;). The evolution of such diversified protein families produced by different subspecies of single bacteria is not very well understood. Because most of the research involving *Bt* has focused on its insecticidal activities, the mode of action of these insecticidal δ- endotoxins are fairly understood. By laboratory and field selection pressure experiments, it is now known that the pH of the larval gut, the types of proteases in the gut and the receptors present on the epithelial cells of the gut could induce the evolution of the types of δ- endotoxins produced by the *Bt* strains (De Maagd *et al.*, 2003; Chougule & Bonning, 2012).

Nine different types of toxins have been identified in *Bt* so far – α-exotoxin, β-exotoxin, γ-exotoxin, δ-endotoxin, louse factor exotoxin, water soluble toxin, vegetative insecticidal protein (Vip), mouse factor toxin and enterotoxin (Rowe & Margartis, 1987; WHO, 1999). The δ-endotoxins and Vip are given more importance due to their diverse insecticidal activities and commercial applications. Among these δ-endotoxins, two major groups are Cry (crystal) and Cyt (cytolytic) proteins. These two families of proteins combine and form the parasporal crystals during the sporulation stage. The other proteins expressed by *Bt* include vegetative insecticidal proteins, secretory insecticidal proteins, chitinases, bacteriocins, parasporins, helper proteins like p19 and p20, proteases, phospholipases, etc.

Bacteriocins are antimicrobial proteins produced by *Bt* against a wide range of microorganisms (Delves-Broughton, 2005; Fguira *et al.*, 2014). Many study groups have isolated strains that produce these proteins. They have also proposed possible industrial application of these bacteriocins: prevention of food-spoilage, prevention of plant diseases, prevention of honeybee bacterial diseases, etc (Cherif *et al.*, 2003; Chehimi *et al.*, 2007; Cherif *et al.*, 2008; Lee *et al.*, 2009; Rea *et al.*, 2010; Kamoun *et*

al., 2011). These bacteriocins have both bacteriolytic as well as bacteriostatic effects (de la Fuentes-Salcido *et al.*, 2012).

The vegetative insecticidal proteins (Vip), as the name suggests, are produced during the vegetative phase of the *Bt* and are secreted out (Bi *et al.*, 2014). They also have insecticidal activities and are usually found in *Bt* strains that are toxic to *Lepidopteran, Hemipteran* and/or *Coleopteran* insects (Sattar & Maiti, 2011). These strains that express Vip proteins have broader range of target organisms (Donova *et al.*, 2001; Milne *et al.*, 2008). These Vip proteins are widely classified into two groups: Class I with Vip1, Vip4 and Vip2 and Class II with Vip3 (Sun *et al.*, 2013; Cickmore *et al.*, http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/*Bt*/). The first class consists of binary proteins that are cleaved and activated, and the other single chain proteins are included in the second class. So far, more than 80 different types of Vip proteins have been identified

(http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/*Bt*/vip.html).

Secretory insecticidal proteins (Sip), like Vip, are expressed by *Bt* during vegetative phase and are secreted outside into the medium. It shows insecticidal activities against *Coleopteran* insects. Although it is proposed that their mode of action is through pore formation, the exact mechanisms is not understood well (Donovan *et al.*, 2006). Also, they have been shown to have additive effect with the δ- endotoxins, but the mechanism of synergism has not been reported yet.

Chitinases are enzymes that convert the complex, insoluble chitin into its monomers. The gene for chitinase has been isolated from many different organisms including gram positive and negative bacteria, fungi, animals, plants, etc. Chitinase is produced by *Bt* and is secreted outside which makes *Bt* a very attractive tool for

chitinase production on industrial level. Apart from this, the chitinase production enhances the insecticidal activity of the *Bt* δ- endotoxins (Wiwat *et al.*, 2000; Liu *et al.*, 2002; Sneh *et al.*, 2009). Many different *chitniase* genes have been isolated from different *Bt* strains and cloned with the δ- endotoxins in order to develop or just enhance the insecticidal activity against different insect groups (Barboza-Corona *et al.*, 2003; Thamthiankul *et al.*, 2001; Arora *et al.*, 2003).

Like Cry proteins, some parasporins also have a three-domain structure and have low to medium homology with some of the Cry proteins. Hence, all the six known parasporins are included under the Cry nomenclature

(http://parasporin.fitc.pref.fukuoka.jp/). But, they do not show any insecticidal activities (Mizuki *et al.*, 1999). On the contrary they specifically act against human cancer cell lines (Brasseur *et al.*, 2015). It has been recognized that these different parasporins have toxicity to a range of cell types, but the pathways triggered by these proteins are not completely understood. Different studies have given explanations like, induction of apoptotic pathways (Lee *et al.*, 2000; Ohba *et al.*, 2009), increasing plasma membrane permeability (Abe *et al.*, 2008), selective pore-formation (Zhuang *et al.*, 2002; Ohba *et al.*, 2009), or a combination of these modes (Phonnok *et al.*, 2010). They also form spherical crystals and need solubilisation at a higher pH and activation by proteases to be converted to a toxin (Mizuki *et al.*, 1999; Mizuki *et al.*, 2000; Ohba *et al.*, 2009).

Cry proteins usually have their own domain that will help them crystallize, but some Cry proteins require the helper or accessory proteins like p19 and p20 for this conformational change which are part of the operon encoding the *cry11A* gene (Shao *et al.*, 2001; Berry *et al.*, 2002; Ibrahim *et al.*, 2010). These helper proteins also have functional roles like enhancing the insecticidal activities of certain Cry proteins like

Cry11A (Xu *et al.*, 2001). The accessory protein p20 especially has been shown to be involved in stabilization of certain δ- endotoxins also, like in case of Cyt1Aa during heterologous expression (Manasherob *et al.*, 2006; Nisnevitch *et al.*, 2006)

2.4 *Bt* δ-endotoxins

2.4.1 Introduction

During sporulation, *Bt* produces one or more δ-endotoxins and usually form a parasporal crystalline structure. Cry (crystal) and Cyt (cytolytic) proteins are the two major families of proteins that constitute δ-endotoxins (Mahler & Halvorson, 1980). Both these groups of proteins are usually encoded by genes on large mega-plasmid in *Bt*. This mega-plasmid is called p*Bt*oxis in *Bti* and are about 128 kb long (Ben-Dov *et al.*, 1999; Berry *et al.*, 2005). *Bti* also contains many large and small plasmids depending on the species. The number and size of plasmids differ with one species to another (Aptosoglou *et al.*, 1997; Gonzalez *et al.*, 1980). Gel electrophoresis of genome extraction samples from each strain shows a specific plasmid patterns (Vilas-Boas *et al.*, 2004). This is one way of characterizing newly isolated strains.

Cry proteins are the δ- endotoxins with a structural domain that is involved in crystallization of the parasporal inclusions. They usually have strong insecticidal activity against some insect groups or at least have amino acid sequence similarity to the existing Cry proteins. There are currently about 800 *cry* genes that have been reported (Crickmore *et al.*, 2011) and grouped into 74 families of genes. The translated polypeptide lengths vary from 369 amino acids to 1344 amino acids. Among these Cry toxins, other groups are also included and designated with a Cry name due to their sequence similarities – ETX/MTX like toxins (Bokori-Brown *et al.*, 2011), Binary like toxins (Berry, 2012), parasporins (Mizuki *et al.*, 1999; Akiba *et al.*, 2009), etc.

Cyt proteins on the other hand form a part of the crystal structure, but none of

them can form crystals on its own. They are more specific to certain subspecies like *Bt israelensis*. Their target insects are also specific. They are more toxic to *Dipteran* insects like mosquitoes and black flies (de Maag *et al.*, 2003). It also has cytolytic activity against mammalian and insect cells. It has been divided into three gene families and 11 halotypes (Crickmore *et al.*, 2014). It is characterized by its haemolytic and cytotoxic properties. Cyt proteins on its own are not highly insecticidal, but greatly enhance the toxicity of Cry proteins by synergistic actions (Bravo *et al.*, 2007).

2.4.2 Taxonomy and Nomenclature of *Bt* δ endotoxins

In early 1960s, *Bt* was classified into the *Bacillus* genera. Their insecticidal activities, serological analysis and biochemical tests allowed the scientists to group them into different subspecies (De-Barjac *et al.*, 1980). H serotyping (immunological response to the flagellar antigens) divided the *Bt* into 69 serotypes and 82 serovars (Lecadet *et al.*, 1999). However, this system has its limitations as recognized by Soufiane & Cote (2009). As the economic demand for *Bt* strains increased, there was a need to have a better system of categorizing the isolates. In 1973, *Bt* was defined as a separate species in the *Bacillus* genus. Differentiation techniques like 16s rRNA and 23s rRNA sequences, amplified fragment length polymorphism (AFLP), multi-locus sequence typing, and multi-locus enzyme electrophoresis have been used, but at the same time supported the idea of putting *Bt* under the species *Bc* (Helgason *et al.*, 2000; Ticknor *et al.*, 2001; Helgason *et al.*, 2004; Zhong *et al.*, 2007). The gene *gyrB* has been the only technique so far that has help resolve this issue. It showed immense differences between these three groups and hence *Bt* is now a separate species of genus *Bacillus* (Park *et al.*, 2007).

The first *Bt* crystal (*cry*) gene was identified in 1981 (Schnepf & Whiteley, 1981). Since then, more than 700 δ- endotoxin genes have been identified. This huge

database demanded a systematic nomenclature. The very first system developed for the nomenclature included the name of the δ- endotoxin followed by a roman number. This system depended on the insecticidal specificity of each δ- endotoxin. For example, all the δ- endotoxins that are toxic to *Lepidopteran* strains, *Lepidopteran* and *Dipteran* insects, *Coleopteran* insects and *Dipteran* alone were grouped as CryI, CryII, CryIII & CryIV, respectively (Hofte & Whiteley, 1989). The complications and limitations of this system included: lack of system to include the non-toxic strains, the delay in naming the toxin till all the bioassays against each group of insects analysed, lack of information about the structural or sequence similarities between the δ- endotoxins, etc (Crickmore *et al.*, 1998). In order to overcome these limitations, a committee was appointed to develop a new Toxin Nomenclature system (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/*Bt*/ (accessed on 30 November, 2018); Crickmore *et al.*, 1998). In this new system, the type of protein is followed by four parts according to its similarity to an existing protein sequence. For the four parts, Arabic number is assigned for the first and the last part and the second and third part is assigned with capital and small alphabets. The primary ranking depicts less than 45% identity, secondary ranking depicts less than 78% identity, tertiary ranking depicts less than or equal to 95% identity and the quaternary ranking depicts more than 95% sequence identity.

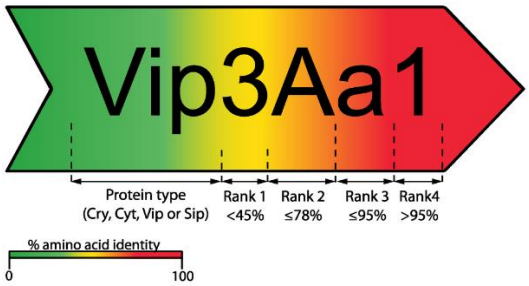

Figure 3. Overview of the current nomenclature system (Palma *et al.*, 2014)

This nomenclature is used for the Cry, Cyt, Vip and Sip proteins (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/*Bt*/ (accessed on 30 November, 2015); Crickmore *et al.*, 1998). The main advantage of this system is the possibility to include even the δ- endotoxins that have amino acid sequence similarity to the existing δ- endotoxins without any insecticidal activity. Also, it can include the similar proteins that do not form a part of the crystal structures (Barloy *et al.*, 1996) and some that are even expressed by entirely different bacteria (Varani *et al.*, 2013).

2.4.3 Structure of *Bt* δ endotoxins

Almost all Cry proteins share a three-domain structure. The domain I share structural similarities with the pore-forming domain of another protein named α-PFTs colicin A. This indicates that the first domain is involved in the pore formation in the insect gut epithelial cell membranes. Seven amphipathic helices have been recognized for this domain: α1, α2, α3, α4, α5, α6, α7. The length of these helices was found to be enough to span the insect cells's lipid bilayer membrane. All this gave evidence that this domain is involved in penetration of the lipid bilayer plasma membrane and consequent pore formation (Florez *et al.*, 2012). Domain II consists of three anti-parallel β sheet structures that form a β prism. They are involved in receptor binding,

oligomerization and membrane insertion (Pigott & Ellar, 2007; Fujii *et al.*, 2013). Domain III has a more complex structure as well as function in Cry.

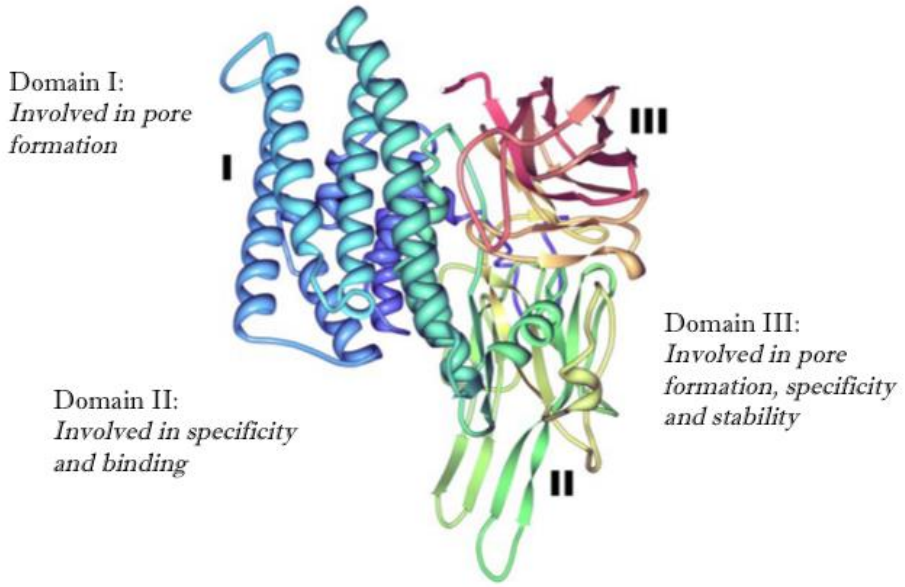

Figure 4. Structure of Cry proteins (Palma *et al.*, 2014)

Structurally, it is a β sandwich of two anti-parallel β sheets that is folded into a jellyroll topology. Each β sheet consists of two loops at the Domain I - Domain III interface and five strands. Considering the structural similarity with other known proteins and the 3D structure itself, it is deduced that this domain is involved in receptor binding (Jurat-Fuentes *et al.*, 2004; Sarkar *et al.*, 2009; Sengupta *et al.*, 2013) and maintaining protein stability (Masson *et al.*, 2002).

Structures of all Cyt proteins are known and they have high similarities between each other. Cyt protein has a single α-β domain. The core is formed by a β sheet, which is surrounded by two α helix hairpins (Cohen *et al.*, 2008; Cohen *et al.*, 2011). The β

sheet consists of six anti-parallel β strands. These strands are bordered by α_1 and α_2 on one side, and α_3 and α_4 on the other (Li *et al.*, 1996; Cohen *et al.*, 2011; Cohen *et al.*, 2008;). Analysis of the 3D structure and the sequence similarities with other known proteins, suggest that the structure of Cyt protein has the ability of non-specific pore-forming ability (Lin *et al.*, 2004; Weng *et al.*, 2004) and can trigger the oligomerization of other protein domains especially the Cry proteins (Weng *et al.*, 2004).

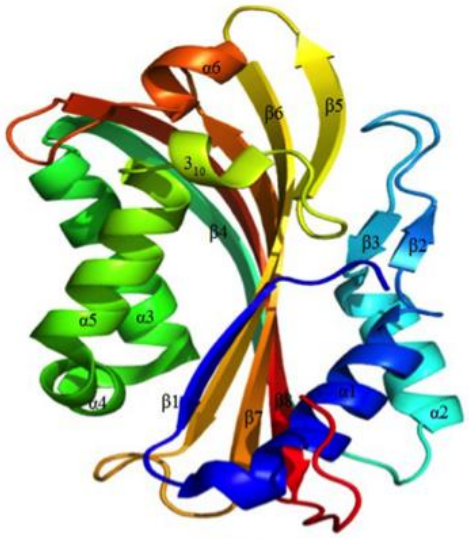

Figure 5. Structure of a Cyt protein (Xu *et al.*, 2014)

2.4.4 Mode of action of *Bt* δ endotoxins

Once the insecticidal larvae ingest the Cry toxins, it is solubilized due to the alkaline conditions of the gut. The disulphide bonds are broken to form a protoxin. This step is affected by factors like the type of Cry protein, physiological conditions of the midgut, presence of helper proteins, etc (Aronson *et al.*, 1991; Naimov *et al.*, 2011).

Once solubilized, these protoxins are activated by specific midgut proteases to form the active toxin molecule. This cleavage occurs on both N and C terminal of the protoxin (Bravo *et al.*, 2004; Yamaguchi *et al.*, 2010; Ai *et al.*, 2013; Guo *et al.*, 2012). Once activated these toxin molecules cross the peritrophic matrix, which may reduce the availability of Cry toxins that reach the membrane receptors. The next crucial step is the binding of these activated toxin molecules to specific receptors on the membrane. This decides the host range of the *Bt* strains. The receptors that Cry proteins bind to, are aminopeptidase, cadherin, alkaline phosphatase, ABC transporter and other Cry binding proteins and molecules (Pigott & Ellar, 2007; Bravo *et al.*, 2011; Jurat-Fuentes & Jackson, 2012; Stalinski *et al.*, 2015). Once the binding is complete, the mode of action has been explained by many study groups but is still a controversial topic. Two models have been proposed: Pore formation model and Signal pathway model. According to the pore formations pathway, the activated toxin binds to multiple primary receptors. Once localized, they bind to the secondary receptors, which are usually cadherin molecules (Arenas *et al.*, 2010; Bravo *et al.*, 2011). This interaction leads to cleavage of N terminal, which induces the oligomer formation (Gomez *et al.*, 2002) and insertion into the membrane. The pores thus formed will cause the osmotic lysis of the cell (Pacheco *et al.*, 2009; Soberon *et al.*, 2009; Bravo *et al.*, 2011; Zhang *et al.*, 2017). The second model is signal pathway. As per this model, specific binding between the Cry toxins and the cadherin proteins will induce the Mg^{2+} dependent signalling pathway that ultimately causes cell death (Zhang *et al.*, 2005).

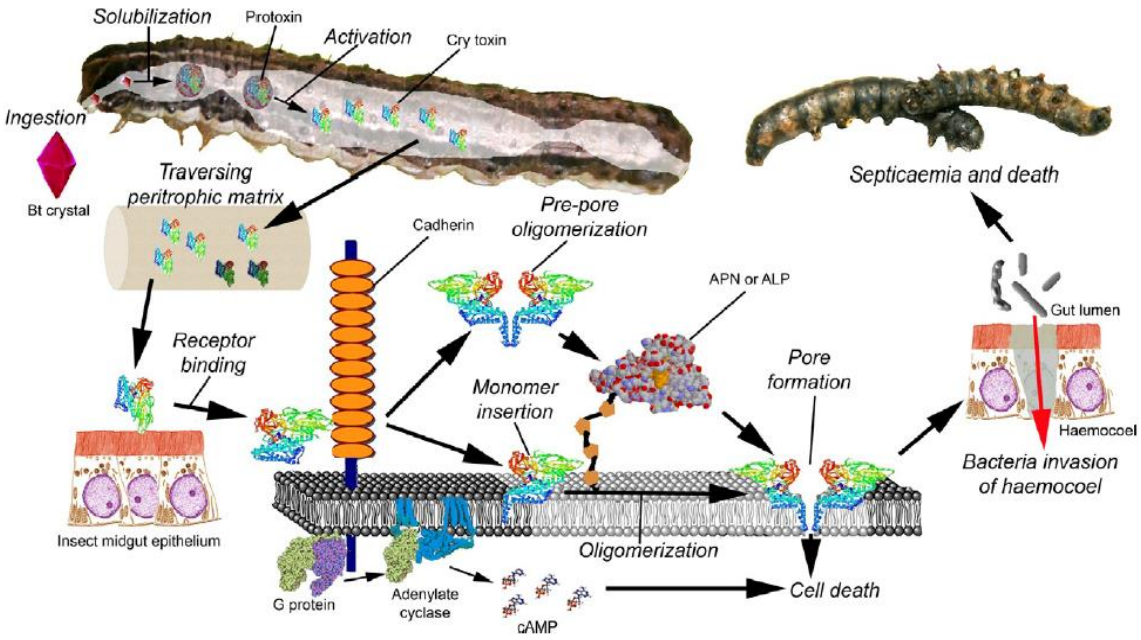

Figure 6. Mode of action of δ- endotoxins (Adang *et al.*, 2014)

Cyt proteins do not require specific receptors on the insect epithelial cell membrane. Instead, they bind directly to saturated membrane lipids such as sphingomyelin, phosphatidylcholine and phosphatidylethanolamine (Rodriguez-Almazan *et al.*, 2011). The proteases of the insect midgut activate the Cyt protoxin by cleaving at the N terminal. The C terminal α1 and α2 bind to the membrane lipids, which triggers the self-oligomerization. Then the C terminal β5, β6 and β7 penetrate the membrane and 6 such domains come together to form the β barrel across the membrane. This creates channels and makes the membrane permeable. The cytosol components start leaking and cause cell death subsequently (Rodriguez-Almazan *et al.*, 2011). Another model says that the binding of the C terminal α1 and α2 to the membrane and insertion of β5, β6 and β7 domains in the membrane act as a detergent and disturb the integrity of the membrane and dissolve causing cell death (Butko, 2003). Both the models are considered true and is thought to be toxin concentration dependent.

At the low concentrations, the permeability model may be true, while the high Cyt concentration might trigger a detergent like model.

Another important function of Cyt protein is its synergistic action with Cry proteins. Cyt has been found to be able to bind to the Cry proteins (Perez *et al.*, 2005; Canton *et al.*, 2011). This enables the Cry proteins to bind to the membrane at more sites than their own receptors alone. This in turn enhances the insecticidal properties of the Cry proteins. It not only binds, but also triggers the oligomerization of the Cry toxins like Cry11A (Carmona *et al.*, 2011; Perez *et al.*, 2007; Hua *et al.*, 2008). This is also the reason why the insects do not develop resistance against the Cry proteins of the strains that also express the Cyt proteins.

The δ- endotoxins once bound to the insect midgut epithelial cells trigger a cascade of histopathological events in the larval midgut, which eventually leads to its death (Abdelkefi-Mesrati *et al.*, 2011). An insect larval midgut typically consists of a chitin rich peritropic membrane and midgut epithelial cells. The epithelial cells are of two types: columnar and goblet cells. The columnar cells with the microvilli give the brush border appearance along with the closely intercalated goblet cells (Jamoussi *et al.*, 2013). The cells affected by the δ- endotoxins show some common characteristics: loss of tight associations among the epithelial cells, degenerated columnar and goblet cells, loss of microvilli, vacuolated organelles, clustered chromatin, shrunk nucleus, appearance of degenerated cells in the lumen, etc (Rouis *et al.*, 2007, El-Ghany *et al.*, 2015). These events are then followed by starvation and larval death. The sequence of these events or the pathways of signalling molecules that trigger these events are not very clear (Canton *et al.*, 2015).

2.5 Resistance of insects to *Bt*

It was believed that the insects would never develop resistance to the bio-pesticides based on *Bt* δ- endotoxins as these two organisms have co-evolved. But, considering the multi-step toxicity of the δ- endotoxins, resistance should have been expected. The resistant varieties of insects developed in laboratory as well as in the field after applications (Ferre & Van Rie, 2002). It was seen that the development of the resistance was much easier in the field conditions with high genetic diversity compared to the laboratory selections. However, laboratory selection experiments were carried out to identify the possible mechanism of resistance development. Most insect species developed resistance to the subspecies *Bt kurstaki*. Substituting this subspecies with *Bt aizawai* could resolve the issue. But, resistance to this new subspecies also started developing rapidly (Lambert *et al.*, 1992). The mechanisms used by the insects to develop resistance include: alteration of the activation of δ- endotoxin step (Oppert *et al.*, 1997), enhanced immune response (Hernandez-Martinez *et al.*, 2010), alteration of the membrane receptors that were specific to the δ- endotoxins (Griffith & Aroian, 2005), sequestering the toxin molecule (Gunning *et al.*, 2005; Ma *et al.*, 2005), etc. Most cases reporting the resistance are due to the alterations in receptor molecules.

So, to control the *Lepidopteran* and *Coleopteran* insects, new and toxic subspecies or strains are constantly being isolated or engineered for field applications. Resistance management was also included in this field of research. The possible strategies for this purpose included using high dosages, spatial or temporal refugia, using combinations of δ- endotoxins or crop rotations (Alstad & Andow, 1995; Gould, 1998).

Among the *Bti* strains, the resistant strains were isolated from the field as well as selected in the laboratory among the *Culex* species (Wirth, 2010). But this resistance

was unstable in both cases. These resistances were developed against individual Cry proteins. But when two or more Cry proteins were expressed together, the resistance was greatly reduced. When Cyt protein was included with the Cry protein (s), the resistance developed was negligible or non-existent (Wirth & Georghiou, 1997; Georghou & Wirth, 1997; Wirth *et al.*, 1997). This is due to the ability of Cyt protein to act as a receptor for the Cry proteins to bind to more sites other than their specific receptors (Wirth *et al.*, 2012). Many studies have shown that the combinations of Cry proteins definitely delayed the development of resistance but including Cyt protein with these combinations avoided the development of resistance all together, under both field and laboratory selection pressures (Wirth, 2010; Wirth *et al.*, 2005). This strategy can overcome the resistance developed due to alteration in the genes encoding the membrane receptors. This makes *Bti* a very strong candidate as a biocontrol agent for a prolonged period.

2.6 Non-target effects of *Bt*

δ- endotoxins from *Bt* have been used extensively for many applications including transgenic plants, bio-pesticides, sources of industrially important proteins, etc. As with any technology, environmental safety of *Bt* has been questioned. The effects of transgenic plants have been the most studied as the field applications of this technology have been going on for more than half a century. Studies to test the effect on arthropods could not find any adverse effects of the transgenic plants despite many crop rotations (Naranjo, 2005; Marvier *et al*., 2007; Duan *et al*., 2010; Li *et al*., 2011; Xu *et al*., 2012). Studies on the effects of transgenic crops on soil nematodes showed that a two year long study could not detect any difference between the community structure of the fields with transgenic plants and the fields with non-transgenic plants. The same studies on the laboratory level showed that the number of nematodes

increased after application of transgenic plants because they were becoming resistant to these *Bt* toxins (Li & Liu, 2013). Studies conducted to test the safety of these toxins towards mammalian cells have also been conducted and could not find any adverse effects (Brodirick *et al*., 2006; Gill & Ellar, 2002). Even the microbiota of the fields growing the transgenic plants do not show significant effects (Farias *et al*., 2014). In case of applications including direct use of δ- endotoxins as bio-pesticides on the field as well as laboratory levels, no non-target effects have been found by the many studies that have been conducted worldwide (Saxena & Stotzky, 2000; Sears *et al*., 2001). In conclusion, although no toxic effects have been detected towards non-target organisms, the presence of transgenic plants with *Bt* toxins has been found to develop resistance among the non-target insects against the *Bt* δ- endotoxins, but only due to the transgenic plants harbouring *Bt* δ- endotoxins. Direct applications of the proteins do not allow them to persist in nature for long and hence do not have an effect of toxicity or resistance development.

2.7 Cytolytic activity / Anti-cancer property of *Bt*

As mentioned before, majority of *Bt* isolates in all ecologies are non-insecticidal. On testing, it was found that some of these strains have anti-cancer activities. This activity is attributed to two groups of proteins: Parasporins (also called as non-insecticidal Cry proteins as they are included in the nomenclature) and the cytolytic proteins Cyt. Cyt1Aa and Cyt2Ba have shown anticancer activities (Nair *et al*., 2018b). At the same time, dosages that showed toxicity in cancer cell lines, did not affect the normal cells (Celandroni *et al.*, 2014). This activity could also be directed specifically to certain cell types by conjugating the Cyt proteins with a signal peptide. This construct specifically lysed the murine hybridoma cells (Cohen *et al.*, 2007) and did not affect the normal cells. Other properties of cells can also be utilized for cytolytic

activity (Correa *et al.*, 2012; Aldeewan *et al.*, 2014). For example, conjugating the insulin to the Cyt protein directed the cytolytic activity towards only the cells that had high number of insulin receptors (Al-yahyee & Ellar, 1996).

Bt also expresses parasporal proteins that have no known effects on invertebrates but show cytotoxic effects on cancer cell lines. These are commonly known as parasporins and they have no structural homology and very low sequence homology with the known Cry proteins. These parasporins also have no insecticidal activities (Knowles *et al.*, 1992). But they are included in the Cry protein groups for nomenclature. Cry31A (Parasporin 1), Cry41A (Parasporin 3), Cry45A (Parasporin 4), Cry46A (Parasporin 2), Cry63A (Parasporin 6) and Cry 64A (Parasporin 5) (Ohba *et al.*, 2009; http://parasporin.fitc.pref.fukuoka.jp/). They preferentially kill the cancer cells of human leukemic T cell lines and HeLa cell lines, without affecting the normal cells like erythrocytes or T-cells (Mizuki *et al.*, 1999; Knowles *et al.*, 1992). Interestingly, like Cry proteins and their spectrum of target organisms, parasporins also have a spectrum of cancer cell lines that are sensitive (Ito *et al.*, 2004; Yamashita *et al.*, 2005; Katayama *et al.*, 2005; Okumura *et al.*, 2005; Kitada *et al.*, 2006). But, unlike Cry proteins, some parasporins' mechanism of action does not involve the membrane pore-forming strategy. Instead, the parasporin induces controlled apoptosis in human cancer cells lines (Katayama *et al.*, 2007). Other parasporins increase the membrane permeability by oligomerization at the membrane after binding to specific receptors, like the Cry toxin strategy.

Overall parasporins are considered as possible anti-cancer drugs that need a few improvements. It is expected that these proteins could induce some immunological complications. The solution would be to find the specific receptors on the cancer cells that are recognized by the parasporins and work towards a targeted therapy (Ohba *et*

al., 2009; Periyasamy *et al*., 2016).

2.8 *Bacillus thuringiensis israelensis* (*Bti*)

2.8.1 Introduction

Bti is the first subspecies that was found to be toxic to the *Dipteran* species. It is the most efficient biological alternative for controlling the disease vectors (Fillinger *et al.*, 2006; Margalith & Ben-Dov, 2000). *Bti* forms spherical crystals composed of δ-endotoxins. The most attractive feature of this biocontrol agent is the absence of resistance among the insect species even after applications for many decades (Kamgang *et al.*, 2011; Loke *et al.*, 2010; Tetreau *et al.*, 2013; Vasquez *et al.*, 2009). The lack of resistance development is due to the different modes of actions among the δ- endotoxins and their synergistic activities. There are four main toxins that are present: Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa (Crickmore *et al.*, 1995). *Bti* reproduces and persists in different ecologies under natural conditions. Applied formulation of spores and crystals usually settles to the bottom of the water bodies within 24 to 48 hours (Boisvert & Boisvert, 1999: Tilquin *et al.*, 2008; Tetreau *et al.*, 2012a; 2012b; 2012c). If ingested, the larvae are killed, and the carcass is used by this sporophyte to multiply and make the carcasses toxic to other larvae (Khawaled *et al*., 1990).

2.8.2 Extra chromosomal genome (plasmids) of *Bti*

Bti that was originally isolated from dead *Culex pipiens* larvae had eight circular plasmids having size ranges between 5 kb and 210 kb, and a linear plasmid of about 16 kb (Carlton & Gonzalez, 1985; Sekar, 1990). The identified and studied plasmids are pTX14-1, pTX14-2, pTX14-3, pGIL01, p*Bt*oxis, p*Bti*c100, p*Bti*c235, pXO16 and p*Bti*c360. These nine plasmids constitute about 13% of the whole genome of *Bti*. These plasmids provide the bacteria with high adaptability to changing environment and other complications arising with their host (Gillis *et al*., 2018). To facilitate gene transfer and

genome dynamics, the plasmids carry several mobile elements like insertion sequences, introns, prophages, transposons, integrons, conjugative elements, mobile insertion cassettes, etc (Fiedoruk *et al*., 2017). Among the nine plasmids, two of the plasmids carry the insecticidal δ- endotoxin genes: p*Bt*oxis and p*Bti*c100. The 128 kb p*Bt*oxis plasmid has been widely studied and was considered the only plasmid involved in insecticidal properties until recently. A new study reported the presence of two new *cry* genes (*cry60Aa* and *cry60Ba*) on the 100 kb plasmid designated as pBtic100 (Bolotin *et al*., 2017). Apart from this, not much is known about this 100 kb plasmid.

pBtoxis plasmid, on the other hand, is very well studied and completely sequenced (Berry *et al*., 2002). Apart from the genes encoding insecticidal proteins, pBtoxis carries the genes for sporulation, transcription regulation, antimicrobial peptides, plasmid replication, germination, etc. A representation of pBtoxis plasmid is shown in the figure 7. The insecticidal protein genes are closely associated with transposons, insertion sequences and other mobile elements. These elements enable exchange of plasmids among the strains as well as many recombinant events. Many studies have reported that the presence of mobile elements lead to DNA rearrangements in the plasmid and consequent structural instability (Zghal & Jaoua, 2006; Driss *et al*., 2011). This property makes the plasmids of *Bti* unstable in changing environmental conditions. Research groups have taken advantage of this instability by artificially pushing the *Bt* strains to lose parts of their whole genome by plasmid curing technique. The idea is to make the bacteria selectively lose certain unwanted metabolic activity or randomly lose parts of genome to enhance the remaining activities. Some of the examples are regulation of gene expression (Park *et al*., 2007), synthesis of antimicrobial bacteriocins (Kamoun *et al*., 2009), production of δ- endotoxins (Gonzalez *et al*., 1981), etc.

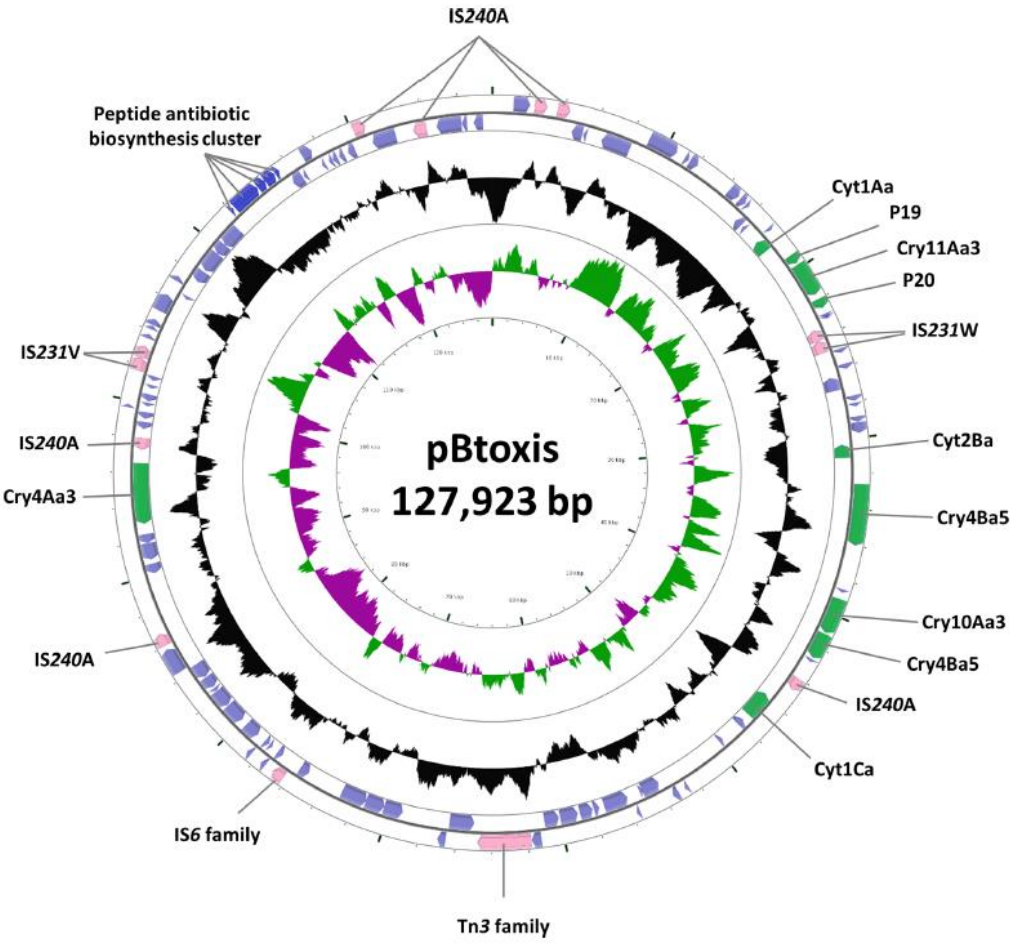

Figure 7. Representation of the pBtoxis mega-plasmid encoding the δ- endotoxins (Gillis *et al*., 2018)

2.8.3 δ- endotoxin genes of *Bti*

The insecticidal activity of *Bti* depends on four main genes *cry4Aa*, *cry4Ba*, *cry11Aa* and *cyt1Aa* that gave protein products of 134 kD, 128 kD, 72 kD and 27 kD, respectively. These genes are encoded by the mega-plasmids called as pBtoxis (Berry *et al.*, 2002). Apart from these, the mega-plasmid also contained other δ- endotoxins like *cry10Aa*, *cyt2Ba* and *cyt1Ca* (Lee *et al*., 1985; Thorne *et al*., 1986). Although the

function of Cyt1Ca protein is not clear, Cry10Aa and Cyt2Ba were found in small quantities and yet contributing to the insecticidal activity. The largest protoxins have C terminal domain that is involved in the crystallization of the entire parasporal crystal (Bietlot *et al.*, 1999). The other smaller δ- endotoxins lack this property and hence need assistance forming the disulphide bonds of crystallization (Xu *et al.*, 2001). The *cry11Aa* is encoded by an operon that also codes for two other proteins, p19 & p20. These helper proteins help stabilize the heterologous expression of δ- endotoxin genes, especially Cry11Aa and Cyt1Aa.

2.8.4 Toxicity of *Bti* towards Dipteran insects

Cry4Aa is highly toxic to the larvae of *Culex*, and less toxic to *Anopheles* and *Aedes*. Cry4Ba, on the contrary is highly toxic to *Anopheles* and *Aedes* but low toxicity against *Culex* (Otieno-Ayayo *et al.*, 2008; Beltrao & Silva-Filha, 2007; Abdulla *et al.*, 2003). These two proteins are cleaved into active forms of about 65 kD. But, unlike the other Cry proteins, these two δ- endotoxins are further processed to two fragments of about 45 kd and 20 kD each (Beltrao & Silva-Filha, 2007; Yamagiwa *et al.*, 2004; Komano *et al.*, 1998). The Cry11Aa is highly toxic to *Aedes* and *Culex* while being moderately toxic to *Anopheles* (Revina *et al.*, 2004; Otieno-Ayayo *et al.*, 2008). This toxin also is cleaved to two fragments of about 38 and 30 kD (Yamagiwa *et al.*, 2002; Yamagiwa *et al.*, 2004; Dai & Gill, 1993; Revina *et al.*, 2004; Beltrao & Silva-Filha, 2007). The Cyt1Aa has low insecticidal activity against all three species (Otieno-Ayayo *et al.*, 2008). But, it is the most important δ- endotoxin in *Bti* as it not only enhances the activities of Cry4Aa and Cry11Aa, but also delays or prevents the onset of resistance among the target insects towards the Cry toxins (Wirth *et al.*, 2005). Other Cry toxins like Cry10Aa has synergistic activities with Cry4Ba and Cyt1Aa (Hernandez-Soto *et al.*, 2009). Cyt2Ba is synergistic with Cry4Aa, Cry4Ba and Cry11Aa (Manasherob *et*

al., 2006). Unfortunately, Cyt1Ca is not detected in any strains. The transcripts for its gene were found but is probably too unstable to be translated (Stein *et al.*, 2006; Itsko *et al.*, 2007; Manasherob *et al.*, 2006).

2.8.5 Limitations of *Bti* based insecticides

Currently, *Bti* is used for commercial applications as bio-insecticides against the *Dipteran* insects. But, by economical standards, it is not yet the perfect strategy for the bio-control of these insects. Despite their numerous advantages over the alternate chemical products, they are still not able to completely replace the chemical insecticides in the market due to their limitations. The most important limitation to consider is its low persistence. The low persistence is due to ingestion by non-target organisms, weakening due to natural factors like rain and sunlight, encapsulation and incapacitation by surrounding organic matter, unavailability of the toxins throughout the water bodies due to protein sinking, etc (Ben-Dov, 2014).

2.9 Non-*Bacillus thuringiensis israelensis* strains

Bt var israelensis is not the only subspecies that can produce insecticidal proteins against *Dipteran* insects. Many research groups have isolated other subspecies that form spherical crystals but are phylogenetically different to the *Bt israelensis*. *Bt jegathesan* (*Btj*) was isolated by Sun *et al.* (2013) that had high insecticidal activities against mosquitoes. *Bt kenyae* (*Btk*) and *Bt entomocidus* (*Bte*) were isolated from Mexico, which also displayed the presence of spherical crystals containing four main δ- endotoxins usually found in *Bti*. These proteins were also encoded by the mega-plasmid of the same size as pBtoxis of *Bti*. These subspecies had comparable insecticidal activities against the *Dipteran* insects. In 1996, four strains of *Bt* were isolated from belonging to the subspecies *Bt canadensis*, *Bt thompsoni*, *Bt jegathesan* and *Bt malaysiensis*. These four isolates were found to have the same combinations of

δ- endotoxins as well as equally high insecticidal activity compared to *Bti* (Ragni *et al.*, 1996). Elluech *et al.* (2014) found that 10 of their *Bt* isolates had similarity to be included under *Bt* subspecies, but they differed from the other known subspecies, including *israelensis*. These isolates had spherical crystals, but their protein patterns and plasmid patterns differed very much from the reference strain *Bti* H14. Comparing their insecticidal activities with the reference strain showed that they were equally or more effective than the reference against *Dipteran* insect (*C capitata*). Another interesting study involved isolation of six strains of *Bt* from Brazil, which showed high insecticidal activities against *A aegypti*, but only one of them showed the presence of the five δ- endotoxin genes that one would expect including *Cry4A, Cry4B, Cry10A, Cry11A* and *Cry11B* (Soares-da-Silva *et al.*, 2015). This shows that the other five strains probably display novel combinations of δ- endotoxins that needs to be explored. Moreover, a non-insecticidal *Bt* strain isolated by Jung *et al.* (2006) showed spherical crystals that had cytolytic activities. The gene responsible was sequenced and was found to have homology with Cry31A toxin. This strain had specific cytotoxic effect on human cancer cells and no effect on the normal human cells. Guan *et al.* (2014) found that *Bt sichuansis* showed spherical crystals and had a novel δ- endotoxin gene *cry69Aa1*. This gene when isolated and cloned into acrystalliferous strain, formed crystals of δ- endotoxins and had high insecticidal activities against *Culex quinquefaciatus*.

Some δ- endotoxins are toxic to more than one family of insects. One such example is Cry2 protein. Cry2 δ- endotoxin is toxic to Lepidopteran as well as Dipteran insects (Hofte & Whiteley, 1989). Cry2 is usually expressed by *Bt* strains like var. *kurstaki*, that acts against Lepidopteran insects. Cry2 protein is encoded by an ORF3 gene that forms a part of a 3 ORF operon. The ORF2 is a regulatory gene that encodes

for a regulatory protein. This regulatory protein aids the translation of ORF3 gene to form the Cry2 protein. Also, once the Cry2 protein is translated, ORF2 protein helps the former to form the cuboidal crystal (Staples *et al*., 2001).

In addition to the presence of novel δ- endotoxins, these non-*Bti* subspecies may also harbour other accessorial essential proteins. In 1977, a new subspecies *Bt pakistani* (*Btp*) expressed and released chitinase enzyme at a very high concentration. Although at that time, the importance was given only to the enzyme for industrial degradation, today we recognize the additive effect it has on the insecticidal activities (de Barjac *et al.*, 1977).

2.10 Current research on *Bt*

As it is evident so far, the different ecologies and climatic conditions affect the diversity of the *Bt* strains. Different research groups in new ecologies continuously carry out large-scale screenings worldwide. Apart from these, mainly the research focuses on modification of the known and novel δ- endotoxin proteins at genetic and molecular levels to increase their insecticidal activities. Also, many groups are focusing on heterologous expression of these δ- endotoxin genes in other organisms. Different combinations of δ- endotoxin genes are cloned together in the carrier organisms to evaluate their efficiency.

Sansinenea *et al.* (2015) isolated melanin producing local strain of *Bt*, which gives the δ- endotoxins protection against sunlight when applied on the fields. This will help resolve the issue of low persistence of δ- endotoxins in nature due to UV light degradation. Other factors that affect the persistence and recycling of the applications in the field are organic matter content and the presence of susceptible insects (Duchet *et al.*, 2014; Tetreau *et al.*, 2012).

The different types of formulations of *Bti* in different ecologies affect the efficiency of the applications. For example, it was found that the breeding grounds like tree holes would have better control of the mosquitoes if the formulations are in granular instead of liquid form (Harwood *et al.*, 2015). Earlier, another group had reported the same by experimentally proving that the backpack application (granular form) was more effective than source reduction (surfactant in monolayer film) in urban areas. This was especially beneficial because the backpack applications were also more cost effective that the latter (Sun *et al.*, 2014). The specific receptors on the gut epithelia that the Cry toxics bind to are fairly understood today. For instance, the Cry4Ba toxin has two specific isoforms of aminopeptidase N (Aroonkesorn *et al.*, 2015). On the other hand, Farajollahi *et al.* (2013) validated the efficacy and persistence of both granulated and pellet formulations for up to 4 weeks.

Large-scale screenings are being undertaken in new ecologies worldwide. In Saudi Arabia, out of 157 *Bt* strains isolated, one strain was found to have good protein production and an enhanced insecticidal activity when compared to the reference *Bti* H14 (El-kersh *et al.*, 2014). Elleuch *et al.* (2014) isolated 10 *Bt* strains producing spherical crystals but had entirely different plasmid patterns compared to H14. They found that three of these strains produced novel δ- endotoxins that had moderate toxicity to *C capitate,* which till then was only susceptible to the exotoxins of *Bt.* As these exotoxins are toxic to vertebrates, they cannot be used for field applications. The mosquitocidal strains were also isolated from excreta of the birds and showed good insecticidal activities against *Culex, Anopheles* and *Aedes* species (Poopathi *et al.*, 2014). A soil isolate from China also showed comparable insecticidal activities that could be an alternative to the H14 reference strain (Zhang *et al.*, 2012).

Once efficient genes have been isolated, they are cloned. The next step is

heterologous expression of these genes in suitable organisms that can over-express particular toxin proteins. Many study groups have utilized different organisms and found good candidates for this purpose: aquatic bacterium *Asticcacaulis excentricus* (Armengol *et al.*, 2005), *E coli* (Khasdan *et al.*, 2001).

Apart from the δ- endotoxins, *Bt* strains may produce many other proteins that have synergistic or additive effects on their insecticidal activities. The production of chitinase was found to enhance the insecticidal activity of *Bt kurstaki* (Driss *et al.*, 2011). The helper gene *p20* from *Bti* was found to enhance the stability of the Cyt proteins as well as Cry4A and Cry11A proteins, thereby increasing their insecticidal activity (Elleuch *et al.*, 2015). The expression of other δ- endotoxins also enhances the insecticidal activity and prevents the development of resistance among the target insects (Park *et al.*, 2005). On the other hand, mutagenesis is used to select new mutants that have high insecticidal activities or higher persistence. Ghribi *et al.* (2004) mutated the local strains randomly with UV and nitrous oxide. They could obtain new strains that had higher insecticidal activities. The same group (Ghribi *et al.*) in 2005 improved the *Bt* strains by heat and high concentration of NaCl. And, in 2006, they improved the strains by changing their growth conditions (aeration) to overcome catabolite repression (Ghribi *et al.*, 2006). On molecular levels, DNA shuffling and *in silico* procedures are also being tested for increasing the activities (Lucena *et al.*, 2014).

Another major focus of research in this field depends on understanding the triggers and patterns of possible development of resistance. Despres *et al.* (2014) reported that the gene expressions triggered in the insects due to biological and chemical insecticides are exactly the opposite. Hence, they suggested an integrated method of management where the resistance development will be very difficult in such cases. Known Cry genes are usually amplified and cloned using specific primers by

PCR amplification. But, now mass spectroscopy has been found to be a better method to identify rare and novel toxins (Zhang *et al.*, 2014).

The commercial production of the bio-insecticides can replace the chemical alternatives only when the production is extremely economical. Apart from other limitations, it is essential to find good, cheap, highly productive media for the growth and sporulation of *Bti*. Different study groups have suggested such media: mushroom substrate by solid state fermentation (Wu *et al.*, 2014), chicken feather-based media (Poopathi & Archana, 2012), pigeon pea and soya bean flour as carbon and nitrogen sources (Devidas *et al.*, 2014), sugarcane processed bagasse (Poopathi *et al.*, 2013), starch and soybean media (Khedher *et al.*, 2011).

2.11 Current market status of *Bt* insecticides

For a long time, the biocontrol agents could not compete with the chemical pesticides because of its limitations. Growing evidences for harmful effects of the synthetic chemical control agents and the development of resistance among the insects have brought about a decline in the development of new chemical pesticides. Slowly, but steadily the scenario is changing in the market for insecticides. According to the BCC research LLC report (CHM029E, 2014), the market for insecticides will increase globally at a rate of 6.3% by 2019 and reach $ 80 billion. Out of this, the major contribution will be due to the bio-insecticides forming a 4% increment. This change in scenario can be witnessed in the form of the new mergers and acquisitions formed between the agricultural development and bio-insecticide production companies (Luca, 2015). Currently, commercial products based on Bti insecticidal endotoxins are available; like mosquito dunks, Aquabac, Bonide, Microbe lift, etc. *Bt* continues to be the “star” of biopesticides and many formulations, needing the search for novel *Bt* toxins. Many research groups from different continents are in constant search for *Bt*

strain diversity and particularly δ- endotoxins with high insecticidal activities. In this PhD, we are contributing to the worldwide search by screening for novel biopesticides and other activities based on *Bt* strains isolated from Qatar.

CHAPTER 3: MATERIALS AND METHODS

3.1. Materials

3.1.1. Strains studied

Table 1. List of important strains studied with reference strains

[Non-*Bti* strains listed are only representatives of the group of strains in their class; number of strains represented by each representative is mentioned in brackets next to it]

***Bti* strains** (Jaoua *et al*., unpublished)	**Non-*Bti* representative strains** (Jaoua *et al*., unpublished)	**Reference strains**
*QBT*205	*QBT*3 (4)	*Bacillus thuringiensis israelensis* H14
*QBT*213	*QBT*6 (7)	*Bacillus thuringiensis israelensis* 4Q7
*QBT*214	*QBT*34 (19)	*Bacillus thuringiensis kurstaki* HD1
*QBT*215	*QBT*39 (12)	*Bacillus cereus*
*QBT*216	*QBT*41 (29)	*Bacillus subtilis*
*QBT*217	*QBT*43 (33)	*Pseudomonas*
*QBT*218	*QBT*56 (28)	
*QBT*220	*QBT*81 (2)	
*QBT*221	*QBT*99 (2)	
*QBT*222	*QBT*212 (10)	
*QBT*223	*QBT*240 (48)	
*QBT*224	*QBT*320 (16)	
*QBT*225	*QBT*418 (9)	
*QBT*226	*QBT*555 (202)	

***Bti* strains** (Jaoua *et al*., unpublished)	**Non-*Bti* representative strains** (Jaoua *et al*., unpublished)	**Reference strains**
*QBT*227	*QBT*674 (1)	
*QBT*228		
*QBT*229		
*QBT*230		
*QBT*608		

3.1.2. Insect larvae studied

1. *Culex pipiens*

2. *Aedes aegypti Bora Bora*

3.1.3. Cell lines

NCIH1975 [H1975, H1975] (ATCC® CRL5908^{TM}) – Adherent lung cancer epithelial cell lines

3.1.4. Media compositions

1) Luria Bertini medium

   Tryptone – 10 g/l

   Yeast extract – 5 g/l

   Sodium Chloride (NaCl) – 5 g/l

   pH – 7

   Agar – 15 g/l

2) T3 medium

Tryptone – 3 g/l

Tryptose – 2 g/l

Yeast extract – 1.5 g/l

Sodium hydrogen phosphate (Na_2HPO_4) – 1.4 g/l

Sodium dihydrogen phosphate (NaH_2PO_4) – 1.2 g/l

Manganese sulphate ($MnSO_4.7H_2O$) – 0.002 g/l

Magnesium sulphate ($MgSO_4.7H_2O$) – 0.02 g/l

pH – 6.8

Agar – 15 g/l

3) Glucose based medium

Glucose – 15 g/l

Glycerol – 5 g/l

Ammonium sulphate – 5.4 g/l

Calcium carbonate – 20 g/l

Monopotassium phosphate (KH_2PO_4) – 1 g/l

Dipotassium phosphate (K_2HPO_4) – 1 g/l

Manganese sulphate ($MnSO_4$) – 0.01 g/l

Magnesium sulphate ($MgSO_4$) – 0.3 g/l

Ferrous sulphate ($FeSO_4$) – 0.01 g/l

pH – 7

4) Soya-meal starch based medium

Starch – 15 g/l

Soyameal – 25 g/l

Calcium carbonate – 20 g/l

Monopotassium phosphate (KH_2PO_4) – 1 g/l

Dipotassium phosphate (K_2HPO_4) – 1 g/l

Manganese sulphate ($MnSO_4$) – 0.01 g/l

Magnesium sulphate ($MgSO_4$) – 0.3 g/l

Ferrous sulphate ($FeSO_4$) – 0.01 g/l

pH – 7

5) Blood agar medium

Peptone – 5 g/L

Beef extract – 3 g/L

Sodium chloride (NaCl) – 5 g/L

Blood – 50 ml/L

Agar – 15 g/L

pH – 7

6) Cell line medium

RPMI-1640

10% Fetal Bovine Serum

100 µg/ml Streptomycin

100 IU/ml Penicillin

3.1.5. Primers used

Table 2. List of primers used in the study

Gene	Primers	Sequence	Reference
16s rRNA	Rib73	5′-AGAGTTTGATCCTGGCTCAG	
	Rib74	5′-AGGAGGTGATCCAGCCGCA	
cry4A,	Dip1A	5'CAAGCCGCAAATCTTGTGGA	Carozzi *et*
cry4B	Dip1B	5'ATGGCTTGTTTCGCTACATC	*al*., 1991
cry4B	Dip2A	5'GGTGCTTCCTATTCTTTGG	
	Dip2B	5'TGACCAGGTCCCTTGATTAC	
cry4	Cry4-1	5'GGTGCTTCCTATTCTTTGG	Carozzi *et*
	Cry4-2	5'ATGGCTTGTTTCGCTACATC	*al*., 1991
cyt1A	Cyt1A1	5'GTTGTAAGCTTATGGAAAAT	Zghal *et al*.,
	Cyt1A2	5'TTAGAAGCTTCCATTAATA	2008
cyt2	Cyt2-1	5'AATACATTTCAAGGAGCTA	Guerchicoff
	Cyt2-2	5'TTTCATTTTAACTTCATATC	*et al*., 1997
cry11	Cry11-1	5'TTAGAAGATACGCCAGATCAAGC	Bravo *et al*.,
	Cry11-2	5'CATTTGTACTTGAAGTTGTAATCCC	1998
cry10	Cry10-1	5'ATATGAAATATTCAATGCTC	Porcar *et al*.,
	Cry10-2	5'ATAAATTCAAGTGCCAAGTA	1999
cyt1C	Cyt1c-1	5'CAAAATCTACGGGAGCAAGG	Nair *et al*.,
	Cyt1c-2	5'GGAAGGATCCCTTTGACTTTT	2018 (a)
cry2	Cry2A	5`GTTATTCTTAATGCAGATGAATGGG	Ben Dov *et*
	Cry2B	5`CGGATAAAATAATCTGGGAAATAGT	*al*., 1997
cry2	Cry2-1	5’ACTATTTGTGATGCGTATAATGTA	Ben Dov *et*
	Cry-2	5’AATTCCCCATTCATCTGC	*al*., 1997

Gene	Primers	Sequence	Reference
cry11	Cry11-1	5`CCGAACCTACTATTGCGCCA	Bravo *et al.*,
	Cry11-2	5`CTCCCTGCTAGGATTCCGTC	1998
p19	P19-1	5'GCAGGAGGAACATCACCATT	Nair *et al.*,
	P19-2	5'GGATTTGCTGAGCAGGTCAT	2018b
p20	P20-1	5'TGACGAGGAAACAGAGTATACGA	
	P20-2	5' TGAAAGGTTAAACGTTCCGATT	
parasporin	PS1-1	5′ AGCACCTAATGATGATAGAGGAA	Ammons *et*
1	PS1-2	5′ CCCAGATTCAAATAATAACCAAGA	*al.*, 2016
parasporin	PS2-1	5′	
2		GATGGTATTGCATTAAATAATGAAAC	
	PS2-2	5′ TTCTCCACCAATTTCAAAGACT	
parasporin	PS3-1	5′ ATACAAGATGTGAGGAAATGATGA	
3	PS3-2	5′ GTATGGCTCAGCTCAATTTGA	
parasporin	PS4-1	5′ ACTAGTCAGCCTATAATCAGAACGA	
4	PS4-2	5′ ACTATTCCAGTACCAGTGTAACC	
parasporin	PS5-1	5′ TCAACGCCACAATTAACAAATA	
5	PS5-2	5′ TCCCTTGTATAGTTGCCTTTGT	
parasporin	PS6-1	5′ TGTTTACTATGTGAAAGGTGGAGA	
6	PS6-2	5′ CAATAGTGGTTCCTATTGGACC	

Gene	Primers	Sequence	Reference
	F1	5’GAAGAAGGGCCAATAGATGG	
	R1	5’AATTTGATCCGTCCTGATGC	
	F2	5’GGGAAAGAATATGCAGGGAAA	
	R2	5’TCAAACGTCGGTTTGTGAAA	
	F3	5’TCGGTTGTACCCCATACACA	
Plasmid	R3	5’CCATTCCACGAAAAAGGAGA	Designed
mapping	F4	5’AAGTAAAGCTGGGGCCTTGT	for the study
	R4	5’GTCAGATCCTTGCCAAAAG	
	F5	5’GCCAAATCAACCACTGAAAAA	
	R5	5’CCTGTGTGACCAGGACCTTT	
	F6	5’GGATAAACCAAGGGCGTTTT	
	R6	5’TTTTCCATAGCGTGAAACCA	

3.1.6. Databases accessed:

1) National Center for Biotechnology Information (NCBI)
2) UniProt

3.1.7. Softwares used:

1) ImageJ
2) Probit analysis
3) Tree viewer
4) MS Excel
5) Spectrum Mill
6) ExPasy tool

7) SWISS-MODEL

8) Primer3 plus

3.2. Methodology

3.2.1. Qatari *Bt* strains collection, culture conditions, revival and storage

More than 700 *Bt* isolates collected from Qatar soil form the *Bt* collection. This PhD project is a part of this broader study of isolation and characterization of Qatari *Bt* isolates. This project concentrates on 441 Qatari *Bt* isolates that produce spherical crystals. The spherical crystal producing strains are known to be toxic to Dipteran insects, which are the target insects for this study. The collection is stored in the form of spore-crystal mixture in 25% glycerol at -80^{0}C.

3.2.1.1. Revival of the strains

Spore-crystal mixture of each strain was taken from -80^{0}C freezer, transferred on Luria Bertini (LB) agar plate by four quadrant streaking technique and incubated at 30^{0}C overnight. A pure isolated colony was then streaked on a new LB agar plate and incubated at 30^{0}C overnight to get a revived pure culture of each strain.

3.2.1.2. Culture conditions and storage

To obtain fresh culture of each strain, the latter was inoculated on LB agar plates by four-quadrant method of streaking and incubated at 30^{0}C overnight. For liquid cultures, a single isolated colony from the LB agar plate was inoculated into LB broth and incubated at 30^{0}C in a shaker overnight at 150 rpm. The strains that produced spherical crystals were stored again separately in the spore-crystal form. To obtain the spores and crystals, isolated colony of each strain from LB agar plate was transferred to the T3 sporulation medium. The T3 agar plates were incubated at 30^{0}C for 96 hours to allow complete sporulation of the culture (Molina *et al*., 2010). The spore-crystal mixtures

were transferred with a loop from the plate to 30% glycerol prepared with LB broth and stored at -80^{0}C.

3.2.2. Characterization of the *Bt* strains producing spherical crystals

3.2.2.1. Based on Crystal morphology

441 *Bt* isolates producing spherical crystals were studied first based on their crystal morphology. Completely sporulated cultures of each strain were used for the following staining and microscopy procedures.

A. Wet mounting under light microscope:

Sporulated cultures from T3 medium were used to study the crystal forms under the microscope. Wet mounts were performed at 1000x magnification under light microscope. The samples that show spherical crystals were tested for solubilisation test at high pH. To confirm that the spherical structures seen under light microscope are indeed protein crystals, 0.5 M NaOH was added to the sides of the slides, still under the microscope. The complete disappearance of the spherical forms after 3-4 minutes of incubation confirmed that the crystals were present before the alkaline solubilisation.

B. Endospore staining and light microscopy:

The spore-crystal mixture was stained by Schaeffer-Fulton method, one of the differential endospores staining techniques. By using steam treatment, the usually impermeable endospores were stained with malachite green. The crystals were counter-stained with saffranine. The imaging was performed with light microscope at 1000x (oil immersion) (Gorashi *et al.*, 2012; Kassogue *et al.*, 2015).

C. Scanning electron microscopy:

For better resolution and understanding of the crystal structures, the samples were imaged with scanning electron microscope. The samples were observed by FEI Nova NanoSem 450 Scanning Electron Microscope. The images obtained were then edited and further studied using the software ImageJ. The dimensions of each spore and crystal were measured using this software.

3.2.2.2. Based on protein patterns of the solubilized δ- endotoxin crystals

Protein patterns were studied for all 441 samples using denaturing SDS-PAGE technique (Laemmli, 1970). The protein patterns of each isolate were studied and were classified based on the similar patterns. A representative was chosen from each group.

A. Preparation of SDS-PAGE gels:

Upper and lower acrylamide gels were prepared as listed in the **table** 3 below. The upper stacking gel was a 3% gel and the lower resolving gel was a 10% gel (Guz *et al.*, 2005).

Table 3. Preparation of stacking and resolving gels for SDS-PAGE gels

Components	**3% acrylamide gel**	**10% acrylamide gel**
30% Acrylamide and 0.8% Bis-acrylamide	3.46 ml	0.835 ml
Tris-HCl	1.4 ml (3M, pH 8.8)	0.333 ml (1M, pH 6.8)
20% SDS	0.05 ml	0.05 ml
10% Ammonium persulphate	0.05 ml	0.025 ml
TEMED	0.006 ml	0.006 ml
Water	5.4 ml	3.75 ml
Total volume	10.366 ml	4.99 ml

B. Preparation of sample:

The spore-crystal mixture was obtained from the completely sporulated culture of each strain by centrifugation at 10000 rpm for 5 min. The pellets obtained were washed thrice in cold distilled water. The resuspended pellets were then mixed with half volume of the boiling buffer containing 1 M Tris-HCl, 20% SDS, β-mercaptoethanol, 60% glycerol and 1% bromophenol blue. This sample was then boiled for 5 min. After boiling, the samples were loaded in to the wells of the SDS-PAGE gels. A broad range protein marker was also loaded in the first well of each gel as ladder to identify the size of the protein bands obtained from the samples.

C. Electrophoresis:

The gel was run in a tank buffer containing 0.025 M Tris-HCl (pH 8.5), 0.1 M Glycine and 0.1% SDS. The gel was run at 100 mA till the loading dye reaches the bottom of the gel (about 3 hours).

D. Staining, de-staining and imaging:

The gel was stained with a staining solution containing 0.25% Coomassie brilliant blue in ethanol, glacial acetic acid and water in 5:1:4 ratio. The gels were left on a shaker at low speed overnight. De-staining was performed with a de-staining solution of ethanol, glacial acetic acid and water in the ratio of 5:7:88 (Ammouneh *et al.*, 2011). De-staining solution was changed after 2 hours and the gels were left in de-staining solution overnight at low speed on a shaker. The next day, de-staining solution was changed again, and the gels were imaged with a white background.

3.2.2.3. Based on plasmid patterns by gel electrophoresis

The plasmid patterns of the 441 *Bt* strains were studied and the isolates were classified based on their patterns. A representative strain was chosen from each group.

A. Plasmid isolation:

The total plasmid DNA from each of the 441 isolates was obtained by alkaline lysis and alcohol precipitation method. A pure isolated colony was spread on a fresh LB agar plate and incubated overnight at 30^0C. The cells were harvested from $1/4^{th}$ part of the plate into 1 ml distilled water. The cells were then separated by centrifugation at 5000 rpm for 3 min. The pellet was resuspended in Solution AL containing Tris-HCl, Glucose, EDTA-Na_2 and 4 mg/ml lysozyme (added just before use). The mixture was then incubated at 37^0C for half hour so that lysozyme can lyse the cell membrane. Solution B containing potassium acetate and glacial acetic acid is added and the samples

are transferred to ice. A freshly prepared alkaline solution C containing NaOH and SDS was added to the samples before incubating them back on ice for 5 min. After separating the cell debris by centrifugation, the supernatant containing nucleic acids and some proteins was added to an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) buffer. This separates the proteins from the desired nucleic acids. After centrifugation, the upper phase containing the nucleic acids was transferred to 2.5 V absolute ethanol and incubated at -20^0C overnight. After precipitating by ethanol, the nucleic acids were washed with 70% ethanol to discard any salts. Pure nucleic acids were then treated with RNase to remove the RNA molecules from the plasmids. The pellets containing pure plasmids were re-suspended in Tris-EDTA solution of 8 pH and stored at -20^0C (Faguntes *et al.*, 2011; Sambrook *et al*., 1989).

B. Gel preparation and tank buffer:

For the study of plasmid patterns, an agarose gel of 1% was prepared with 0.5% TAE buffer containing 0.4 mM tris-acetate and 0.01 mM EDTA-Na_2. The gel was stained using 0.5 µg/ml Ethidium bromide. 0.5% TAE buffer was used as tank buffer as well.

C. Electrophoresis and imaging:

5 µl of the plasmid sample, mixed with 2 µl of 6x loading dye and 5 µl of distilled water, was loaded in each well. A 1 kb plus DNA ladder was always loaded in the first well of each gel. The gel was run at 25 V for 10 min and then 50V till the tracking dye reaches near the bottom of the gel (about one and half hour). The gels were studied and imaged under Ultra-violet light (Reyes-Ramirez & Ibarra, 2008).

3.2.2.4. Phylogenetic studies

16s rRNA sequences were analysed by amplification of conserved regions using Polymerase Chain Reaction (PCR) technique. The PCR amplifications were performed with Universal primers Rib73 and Rib74.

A. DNA isolation:

The total DNA was isolated using the heat shock method. Single isolated colony of the *Bt* strain from a fresh overnight LB agar plate was resuspended in 250 µl of sterile distilled water. The resuspended cells are then incubated at -80^{0}C for 20 min and then immediately boiled for 10 min. The cell debris was separated from the nucleic acids by centrifugation at 10000 rpm for 45 seconds. The nucleic acids in the supernatant was transferred to a sterile Eppendorf for further use.

B. Polymerase chain reaction:

The PCR was run with total DNA isolated and universal primers (Lane *et al.*, 1991) used for the amplification of a (~) 1.5 kb conserved region of 16s rRNA gene. The PCR was performed with a 25 µl reaction mixture containing 2.5 µl of 13 µM forward (Rib73) and reverse (Rib74) primers each, 12.5 µl 2x Thermo fisher PCR master Mix, 5 µl of molecular water and 2.5 µl of isolated total DNA. The PCR cycle consisted of one 5 min step of denaturation (at 95^{0}C), which was followed by 35 cycles involving denaturation for five min (at 95^{0}C), annealing for one min (at 50^{0}C) and polymerization of one and half minutes (at 72^{0}C) (Awad *et al.*, 2007, Punina *et al.*, 2013).

C. Electrophoresis and gel purification of DNA:

The PCR products were loaded on 1.2% agarose gel (prepared as discussed in section 3.2.3). The gel was run for 30 min at 25 V and at 50V for one hour. The gel was observed and imaged under the UV light. The bands of interest were cut from the gel.

The desired PCR product of about 1.5 kb was purified from the gel using a gel purification kit from Qiagen, Germany. The eluted PCR product was used for DNA sequencing.

D. DNA sequencing and phylogenetic studies:

PCR amplified products were sequenced with 3700 DNA sequencer and *Taq* DyeDeoxy Terminator cycle sequencing kit (Elleuch *et al.*, 2015). PCR amplified products were sequenced with 3700 DNA sequencer and *Taq* DyeDeoxy Terminator cycle sequencing kit (Elleuch *et al.*, 2015). Tree viewer program was used to form a dendrogram based on the sequences (Thompson *et al.*, 1997; Felsenstein, 1993; Elleuch *et al.*, 2015).

3.2.3 Study of insecticidal activity

3.2.3.1. Qualitative insecticidal bioassay

The 19 *Bti* strains and the 14 Non-*Bti* like strains were tested for their insecticidal activity along with *Bti* H14 strain as positive control and a crystalliferous *Bti* 4Q7 strain as negative control.

A. Insect rearing:

For the qualitative study, the eggs of *Culex pipiens complex* were collected locally in water with organic matter stored outdoors. The eggs collected were then transferred to distilled water and allowed to transform to larval stages till 3rd instar. The 3rd instar larvae were then used for the bioassay.

B. Isolation and purification of spore-crystal mixture:

The *Bt* strains were inoculated on LB plates and incubated overnight at 30^{0}C. Single isolated colony from each agar plate was transferred to a pre-culture of 3 ml LB broth and incubated overnight at 30^{0}C with 150 rpm. After 16 hours, 500 µl of preculture was

transferred to a second pre-culture of 50 ml LB broth and incubated overnight at the same conditions as above. The O D of the second preculture was taken at 600 nm of the light spectrometer. Accordingly, the amount of pre-culture to be added to sporulation media was calculated such that the sporulation media starts at an O D of 0.1. The sporulation culture was incubated at the same conditions as above for 96 hours, until complete sporulation. The spore-crystal mixture was obtained by centrifugation. The pellet of spores, crystal and cell debris was washed thrice with sterile cold 1 M NaCl solution and thrice with sterile cold distilled water. The washed pellets were resuspended in 10 ml cold sterile distilled water. This purified spore-crystal mixture was then used for qualitative bioassay.

C. Experimental design of the insecticidal bioassay:

For each of the *Bt* strains tested, three 50 ml centrifuge tubes were filled with 10 ml water and five 3rd instar larvae. From the resuspended spore-crystal mixture, 500 µl was added to each of the centrifuge tubes. The test tubes were incubated at 26^{0}C with 12:12 light and dark photoperiod. As negative control, three replicates were left untreated. After 24 hours, the number of live and dead larvae were counted for each of the strains (WHO, 2005; Tetreau *et al.*, 2013).

3.2.3.2. Quantitative insecticidal bioassay

The 19 *Bti* strains were tested for their insecticidal activity along with *Bti* H14 strain as positive control and a crystalliferous *Bti* 4Q7 strain as negative control.

A. Insect rearing:

In this study, the Dipteran insect *Aedes aegypti Bora Bora* was used for the insecticidal bioassay. The eggs of *A aegypti* were obtained from *Laboratoire de Lutte contre les Insectes Nuisibles* (LIN), Montpellier, France on filter papers. These eggs were then

transferred to water and allowed to develop into 3rd instar larvae by incubation at 26^0C (± 2) with a 12:12 hours light & dark photoperiod.

B. Isolation and purification of spore-crystal mixture:

The procedure was as mentioned in section 3.2.3.1.B

C. Quantification of protein content:

Protein concentration of spore-crystal mixture of each strain was estimated by Bradford's method (Bradford, 1976). 100 µl of spore crystal mixture was incubated in sterile NaOH solution with the final concentration of 50 mM for 2 hours. Once the crystals were completely dissolved, the spores were separated from the solution by centrifugation. The solubilized protein for each sample was then used for calculating the optical density (O D) by spectrophotometer. The protein concentration was then extrapolated on a standard graph of O D versus protein concentration of Bovine serum albumin (BSA). The non-crystal producing strain 4Q7 was used as a control for all the proteins that could be present in the solution, other than the δ- endotoxins. The protein concentration obtained from 4Q7 was subtracted from the concentrations of all strains to obtain the actual δ- endotoxin protein concentrations for each strain.

D. Experimental design of insecticidal bioassay:

The quantitative bioassays were performed by the standard protocols of WHO (WHO, 2005). The 3rd instar larvae were added to 100 ml water in plastic cups. Each concentration was triplicated, and the larvae were exposed to the spore-crystal mixture at 27 (± 2) oC with 12:12 light and dark photoperiod. As negative control, three replicates were left untreated. The number of dead and live larvae were counted after 24 hours. The bioassay was first performed with different concentrations of δ-endotoxins from reference H14 and 4Q7. The probable LC_{50} concentration for H14

strain was estimated. The 19 *Bti* strains were tested at this concentration to investigate their efficiency against *Aedes* larvae. The *Bti* strains were then grouped into three classes based on the following categories: efficiency lower than H14, efficiency resembling H14 and efficiency higher than H14. The third group with higher efficiency were tested for more concentrations to calculate their actual LC_{50} values.

E. *Statistical analysis:*

The values from Bioassay were used to calculate the LC_{50} values of each *Bt* strain tested along with H14 using Probit analysis software. The 95% fiducial limit range was also calculated for each. This range was used to draw graphs to compare the efficiency of local strains with each other and the reference.

3.2.3.3. Histological studies

After performing the bioassay, the live and dead larvae were collected separately and observed under the light microscope by wet mounting to understand the probable cause of death (Knaak & Fiuza, 2005). The live larvae from the negative control were also checked under the microscope.

3.2.4. Exploration of insecticidal proteins in *Bti* strains

3.2.4.1. Identification of insecticidal protein genes and helper genes by PCR

A. Plasmid isolation:

The total plasmid DNA from each of the 19 *Bti* isolates was obtained by alkaline lysis and alcohol precipitation method. A pure isolated colony was spread on a fresh LB agar plate and incubated overnight at 30^0C. The cells were harvested from $1/4^{th}$ part of the plate into 1 ml distilled water. The cells were then separated by centrifugation at 5000 rpm for 3 min. The pellet was resuspended in Solution AL containing Tris-HCl, Glucose, EDTA-Na_2 and 4 mg/ml lysozyme (added just before use). The mixture was

then incubated at 37^0C for half hour so that lysozyme can lyse the cell membrane. Solution B containing potassium acetate and glacial acetic acid is added and the samples are transferred to ice. A freshly prepared alkaline solution C containing NaOH and SDS was added to the samples before incubating them back on ice for 5 min. After separating the cell debris by centrifugation, the supernatant containing nucleic acids and some proteins was added to an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) solution. This separates the proteins from the desired nucleic acids. After centrifugation, the upper phase containing the nucleic acids was transferred to 2.5 V absolute ethanol and incubated at -20^0C overnight. After precipitating by ethanol, the nucleic acids were washed with 70% ethanol to remove any salts. The pure nucleic acids were then treated with RNase to remove the RNA molecules from the plasmids. The pellets containing pure plasmids were re-suspended in Tris-EDTA solution of 8 pH and stored at -20^0C (Faguntes *et al.*, 2011; Sambrook *et al*., 1989).

B. Polymerase chain reaction:

The PCR was run with plasmid DNA isolated. The primers used for the detection of each of the genes encoding insecticidal proteins and helper proteins were either adopted from published articles or designed specifically for this study. The list of primers and the associated details are given in the **table 2**. The PCR was performed with a 25 µl reaction mixture containing 2.5 µl of 13 µM forward and reverse primers each, 12.5 µl 2x Thermo fisher PCR master Mix, 5 µl of molecular water and 2.5 µl of isolated total DNA. The PCR cycle consisted of one 5 min step of denaturation (at 95^0C), which was followed by 35 cycles involving denaturation for five min (at 95^0C), annealing for time depending on expected PCR product (at temperatures appropriate for each primer set) and polymerization of one and half minutes (at 72^0C) (Awad *et al.*, 2007, Punina *et al.*, 2013). All PCRs were performed with *Bti* H14 as positive control for *Bti* genes and *Bti*

4Q7 as negative control for *Bti* genes. *Btk* HD1 was used as positive control for *Btk* genes and negative control for *Bti* genes.

C. Electrophoresis and gel imaging:

The PCR products were loaded on 1.2% agarose gel (prepared as discussed in section 3.2.3). Every gel had one kb plus ladder in the first well. The gel was run for 30 min at 25 V and at 50V for one hour. The gel was observed and imaged under the UV light.

3.2.4.2. Exploration of insecticidal proteins in Non-Bti representatives

A. Plasmid isolation:

The total plasmid DNA from each of the 14 Non *Bti* representative isolates was obtained by alkaline lysis and alcohol precipitation method. A pure isolated colony was spread on a fresh LB agar plate and incubated overnight at 30^0C. The cells were harvested from $1/4^{th}$ part of the plate into 1 ml distilled water. The cells were then separated by centrifugation at 5000 rpm for 3 min. The pellet was resuspended in Solution AL containing Tris-HCl, Glucose, EDTA-Na_2 and 4 mg/ml lysozyme (added just before use). The mixture was then incubated at 37^0C for half hour so that lysozyme can lyse the cell membrane. Solution B containing potassium acetate and glacial acetic acid is added and the samples are transferred to ice. A freshly prepared alkaline solution C containing NaOH and SDS was added to the samples before incubating them back on ice for 5 min. After separating the cell debris by centrifugation, the supernatant containing nucleic acids and some proteins was added to an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) solution. This separates the proteins from the desired nucleic acids. After centrifugation, the upper phase containing the nucleic acids was transferred to 2.5 V absolute ethanol and incubated at -20^0C overnight. After precipitating by ethanol, the nucleic acids were washed with 70% ethanol to remove

any salts. The pure nucleic acids were then treated with RNase to remove the RNA molecules from the plasmids. The pellets containing pure plasmids were re-suspended in Tris-EDTA solution of 8 pH and stored at -20^{0}C (Faguntes *et al.,* 2011; Sambrook *et al*., 1989).

B. Polymerase chain reaction:

The PCR was run with plasmid DNA isolated for all 14 Non *Bti* representative isolates. The primers used for the detection of each of the genes encoding insecticidal proteins and helper proteins were either adopted from published articles or designed specifically for this study. The list of primers and the associated details are given in the **table 2**. The PCR was performed with a 25 µl reaction mixture containing 2.5 µl of 13 µM forward and reverse primers each, 12.5 µl 2x Thermo fisher PCR master Mix, 5 µl of molecular water and 2.5 µl of isolated total DNA. The PCR cycle consisted of one 5 min step of denaturation (at 95^{0}C), which was followed by 35 cycles involving denaturation for five min (at 95^{0}C), annealing for time depending on expected PCR product (at temperatures appropriate for each primer set) and polymerization of one and half minutes (at 72^{0}C) (Awad *et al.,* 2007, Punina *et al.,* 2013). All PCRs were performed with *Bti* H14 as positive control for *Bti* genes and *Bti* 4Q7 as negative control for *Bti* genes. *Btk* HD1 was used as positive control for *Btk* genes and negative control for *Bti* genes.

C. Electrophoresis and gel imaging:

The PCR products were loaded on 1.2% agarose gel (prepared as discussed in section 3.2.3). Every gel had one kb plus ladder in the first well. The gel was run for 30 min at 25 V and at 50V for one hour. The gel was observed and imaged under the UV light.

D. Sequencing of novel genes:

The novel genes were amplified and purified from the gel using techniques discussed in section 3.2.4. Full δ- endotoxin genes amplified using PCR were sequenced by Sanger sequencing. The gene sequences were then compared to the other similar δ-endotoxin protein gene sequences on the NCBI database.

3.2.4.3. Protein profiling to identify insecticidal proteins

A. Purification of crystal proteins:

The *Bt* strains were inoculated on LB plates and incubated overnight at 30^0C. Single isolated colony from each agar plate was transferred to a pre-culture of 3 ml LB broth and incubated overnight at 30^0C with 150 rpm. After 16 hours, 500 ul of preculture was transferred to a second pre-culture of 50 ml LB broth and incubated overnight at the same conditions as above. After 16 hours, 1 ml of this pre-culture was added to 49 ml of T3 sporulation broth. The sporulation culture was incubated at the same conditions as above for 96 hours, until complete sporulation. The spore-crystal mixture was obtained by centrifugation. The pellet of spores, crystal and cell debris was washed thrive with sterile cold 1 M NaCl solution and thrice with sterile cold distilled water. The washed pellets were resuspended in 10 ml cold sterile 50 mM Na_2CO_3 to solubilize the crystal proteins. After complete solubilization, the spores were removed by centrifugation at 10,000 rpm for 10 minutes.

B. Trypsin activation:

The proteins were digested as per the protocol described by Huang *et al*. (2012), with slight modifications. The total proteins were reduced to 3 µg/µl by added 1 µl of 1 M DTT and incubating at 37^0C for two and half hours. The solution was alkylated by 5 µl of 1 M iodoactamide and incubating at room temperature in dark for 40 minutes. The

proteins were then precipitated using six volumes of cold acetonitrile-Acetone-Acetic acid in the ratio of 50:50:0.1 (V/V/V). The precipitation process took 12 hours. The pellets obtained were washed thrice with cold 100% alcohol and resuspended in 50 mM NH_4HCO_3 along with trypsin in the ratio trypsin:protein of 1:100 (Wt/Wt). After four hours of incubation in a shaker, trypsin was added again in the ration trypsin:protein of 1:50. This activation process was continued overnight. Formic acid was then added the next day to quench trypsin activity. The entire mixture was then filtered to remove unwanted larger molecules. Then the filtrate was lyophilized. Each sample was triplicated.

C. Protein profiling:

Mass spectrometry for the samples were performed using LTQ XL Mass spectrometer couple to nano-ionization source and an autosampler. To trap the peptides, two parallel enrichment columns were used. A total of 50 µg sample was introduced into the SCX colum on the HPLC system. A reverse column was also applied in the HPLC system. A series of increasing concentrations of NH_4HCO_3 solutions were used for the first dimension of separation in Liquid chromatography. These solutions eluted the remaining peptides in a gradual manner from SCX column. Once the peptides were trapped and desalted completely using the exchanged enrichment columns, secondary separation commenced using RP column. The peptides that eluted from RP column then moved to MS online. The buffers used were Elution buffer A containing 0.1% FA in molecular water and Elution buffer B containing 1% FA in acetonitrile. The elution buffers used in the first 5 minutes contained 98% A and 2% B, followed by only 40% of B for 45 minutes, followed by 90% B for 30 minutes and going back to 98% A and 2% B for last 20 minutes. The data collected during the scan used a full range of 300 to 2000 m/z. This was followed by a positive mode of MS/MS analysis and intervening

scans of the strongest ions from the earlier scan.

D. Data analysis:

The raw files were checked against the databases downloaded from UniProt Protein Knowledgebase (https://www.uniprot.org/; accessed on 19th January 2019) in the form of FASTA files. The software Spectrum Mill was used to check and analyse the data. For the search parameter: the mass tolerance was set to 1.5 Da and the fragmentations ions were set at 1.0 Da; only y and b fragments were used; The target false discovery rate was adjusted to 1% by using a target and decoy database. When three or more unique peptides were recognized, the protein was "identified".

3.2.5. Evaluation of cytolytic activities

3.2.5.1. Evaluation of haemolytic activity

A. Sample preparation:

The 19 *Bti* strains and 14 Non-*Bti* representatives were inoculated on a fresh LB plates and incubated overnight at 30^0C. the well isolated colony from each strain was picked by a sterile toothpick and was used as sample for haemolytic assay.

B. Media composition:

Blood agar was used for this assay. Nutrient rich LB agar was prepared as discussed before. The temperature of the melted agar medium was maintained at around 50^0C. To this media, fresh human blood water added to get a final 5% blood in the media. The Blood agar was then poured in petri plates and allowed to solidify for use.

C. Experimental design:

The Blood agar plates were divided into grids and each box was assigned to a single strain. The strains were transferred from the fresh LB plate to Blood agar plate using sterile toothpick. Once inoculated, the blood agar plates were incubated at 30^0C

overnight. The next day, the plates were checked for zone of clearance around the *Bt* strain colonies.

3.2.5.2. Evaluation of anticancer activity

A. Isolation and activation of protein:

The proteins were isolated using the same protocol discussed in the section 3.5.2. The proteins were activated using 15µg/µl Trypsin by adding 15 µl to 500 µl of purified and solubilized crystal proteins (Vilchez *et al.*, 2004). The activation procedure was done after quantifying the protein content as discussed below.

B. Quantification of proteins:

Protein concentration of each strain was estimated by Bradford's method (Bradford, 1976). The solubilized protein for each sample was used for calculating the optical density (O D) by spectrophotometer. The protein concentration was then extrapolated on a standard graph of O D versus protein concentration of Bovine serum albumin (BSA).

C. Cell lines and experimental conditions:

For the cytolytic assay, NCIH1975 [H1975, H1975] (ATCC® CRL5908™) was used; which is an adherent lung epithelial cancer cell line. The cell lines were obtained from ATCC in RPMI-1640 medium. The cells were maintained in the same media supplemented with 10% Fetal Bovine serum (FBS), 100 µg/ml of Streptomycin and 100 IU/ml of Penicillin. The cells were grown and maintained at 37^0C with 5% humidity in a CO_2 incubator. The experiments were also conducted in the same media and conditions.

D. Experimental design:

The cytotoxicity assay was performed by the MTT protocol, also called as cell proliferation assay (Heiss *et al*, 1997). 5000 cells were added to each of the 96 wells of microtiter plate. The cells were allowed to attach overnight and then the media was replaced with fresh media containing appropriate concentrations of *Bt* crystal proteins. The *Bt* crystal proteins from each strain was diluted to get three different concentrations: 0.15 µg/ml, 0.35 µg/ml and 0.5 µg/ml. Along with these a 0.0 µg/ml concentration (untreated) was also used for each strain. Each of these concentrations were triplicated. And each concentration was tested for 24 hrs, 48 hrs and 72 hrs.

After the treatment, 100 µl MTT reagent was added to each well and incubated again for three hours. Then 20 µl of DMSO reagent was added to each well and incubated for 20 minutes. The purple formazan dye formed and solubilized in DMSO was quantified using spectrophotometer (Correa *et al.*, 2012).

E. Statistical analysis:

The viability was estimated by comparing the O D of the untreated wells to that of the treated wells (Correa *et al.*, 2012). The three readings were considered for each concentration and the standard errors and deviations were calculated using MS excel.

3.2.5.3. Investigation of anticancer Parasporin protein genes

The strains showing anticancer activity were tested for the presence of Parasporin protein genes. The plasmid DNA was isolated by alkaline lysis and alcohol precipitation method. The PCR amplifications were performed using the primers listed below in table 2. The presence of all six families of Parasporins were checked.

3.2.5.4. Investigation of novel Cyt protein

The *Bti* strain showing the highest activity was investigated further on molecular level by comparing its Cyt protein with that of the reference H14.

A. Amplification and sequencing of cyt1A gene:

The whole *cyt1A* gene was amplified by PCR as discussed in section 3.4.1. The whole gene was sequenced by Sangers sequencing method. The gene sequence was compared to the *cyt1A* gene of *Bti* H14, as available on the NCBI database.

B. Prediction of amino acid sequence:

The gene sequence was translated into amino acid sequence using the ExPasy tool provided by the website of Swiss Institute of Bioinformatics. The amino acid sequence of Cyt1A protein of Qatari *Bti* was compared to the amino acid sequence of the Cyt1A protein of reference H14, as available on NCBI database.

C. Protein modelling and structural homology studies:

To understand the consequences of differences of amino acids between the local *Bti* and reference *Bti*, the amino acid sequences were used to model the protein. The software used for the same was SWISS-MODEL. The structures deduced from the software were checked for differences in conformation. SWISS-MODEL was also used to check the changes in the polarity and charges due to changes in amino acids.

3.2.6. Evaluation of protein production capacity

The four strains, showing higher insecticidal activity than the reference, were analysed for their protein production capacity.

3.2.6.1. Study of protein production using glucose-based media

A. Media composition and culture conditions:

The protein production was tested in a new sporulation medium called Glucose based medium as it is known to increase the level of crystal protein production. The glucose-based medium composition was as mentioned in Materials section 3.1. The glucose, glycerol and calcium carbonate were autoclaved separately and added to autoclaved media later. The four strains along with the references H14 and 4Q7 were inoculated on LB plates and incubated overnight at 30^0C. Single isolated colony from each agar plate was transferred to a pre-culture of 3 ml LB broth and incubated overnight at 30^0C with 150 rpm. After 16 hours, 500 ul of preculture was transferred to a second pre-culture of 50 ml LB broth in 500 ml Erlenmayer's flask and incubated overnight at the same conditions as above. The O D of the second preculture was taken at 600 nm of the light spectrometer. Accordingly, the amount of pre-culture to be added to sporulation media (50 ml in 500 ml Erlenmayer's flask) was calculated such that the sporulation media starts at an O D of 0.1. The sporulation culture was incubated at the same conditions as above for 120 hours, until complete sporulation. 1 ml of completely sporulated culture was transferred to four eppendorfs, each. One tube was used for estimation of cell biomass and the remaining three were used for estimation of protein production per ml (Ghribi *et al.*, 2007).

B. Estimation of protein production:

The three eppendorfs with sporulated culture was centrifuged at 10000 rpm for 10 min to obtain the pellet of spore-crystal mixture. The pellet was washed thrice with sterile cold 1 M NaCl and thrice with sterile cold distilled water. The washed pellet was then resuspended in 1 ml of 50 mM NaOH incubated at room temperature for 2 hours till the crystals are completely solubilized. Once the crystals were completely dissolved, the

spores were separated from the solution by centrifugation. The solubilized protein for each sample was then used for calculating the optical density (O D) by spectrophotometer. The protein concentration was then extrapolated on a standard graph of O D versus protein concentration of Bovine serum albumin (BSA).

C. Estimation of cell biomass:

One remaining Eppendorf from above was used for the estimation of cell biomass by serial dilution and cfu count method. The culture was serially diluted till 10^{-7}. The last four dilutions were used for the cfu count. 100 µl from each dilution was spread on LB agar plate and incubated at 30^0C overnight. Each dilution was also triplicated. The colony forming units were counted for each plate after 16 hours of incubation.

D. Estimation of protein yield:

The protein yield per spore was calculated by dividing the protein production values by the cell biomass values for each strain. To obtain the real value of δ- endotoxins produced by each spore, the value obtained for the reference 4Q7 was subtracted from them. This ensured that the readings due to other spore associated proteins were removed and the values represented the true concentration of δ- endotoxin proteins.

E. Statistical analysis:

All the tests were replicated thrice, and the values were used to calculate the standard error. Histograms were drawn using Tree viewer software for each to compare the protein yield of the local strains and that of the reference H14.

3.2.6.2. Study of protein production using soya meal starch based media

A. Media composition and culture conditions:

The protein production was tested in an alternative sporulation media called Soya bean and starch-based media as it has cheaper and complex sources of carbon and nitrogen.

The Soya bean and starch-based media composition was as mentioned in Materials section 3.1. The four strains along with the references H14 and 4Q7 were inoculated on LB plates and incubated overnight at 30^0C. Single isolated colony from each agar plate was transferred to a pre-culture of 3 ml LB broth and incubated overnight at 30^0C with 150 rpm. After 16 hours, 500 ul of preculture was transferred to a second pre-culture of 50 ml LB broth and incubated overnight at the same conditions as above. The O D of the second preculture was taken at 600 nm of the light spectrometer. Accordingly, the amount of pre-culture to be added to sporulation media was calculated such that the sporulation media starts at an O D of 0.1. The sporulation culture was incubated at the same conditions as above for 120 hours, until complete sporulation. 1 ml of completely sporulated culture was transferred to four eppendorfs, each. One tube was used for estimation of cell biomass and the remaining three were used for estimation of protein production per ml.

B. Estimation of protein production per millilitre:

As discussed in section 3.8.1

C. Estimation of cell biomass:

As discussed in section 3.8.1

D. Estimation of protein yield per spore:

As discussed in section 3.8.1

E. Statistical analysis:

As discussed in section 3.8.1.

3.2.7. Investigation of plasmid structural stability

3.2.7.1. Study of possible deletion of plasmid in Bti strains by mapping

A. Designing overlapping primers:

Published p*Bt*oxis plasmid sequence was used to design the primers that can amplify regions that overlap each other along the plasmid. The region under study extended from *cry4* gene to *cyt1C* gene. The primers studied are listed in Table 2. The reference strain H14 was also tested along with the 19 local *Bti* strains.

B. Polymerase chain reactions:

The PCR cycles were run as per the conditions mentioned in section 3.2.4. Different combinations of forward and reverse primers were used, and amplifications of various regions were carried out. This was necessary to find exactly which part of the plasmid was missing.

C. Gel electrophoresis and mapping:

The PCR products were run on a 1.2% agarose gel stained with Ethidium bromide and imaged under Ultra violet light. The mapping was done by analysing the PCR product obtained using each set of primers and “walking” through the region of interest on the pBtoxis plasmid (Figure 7).

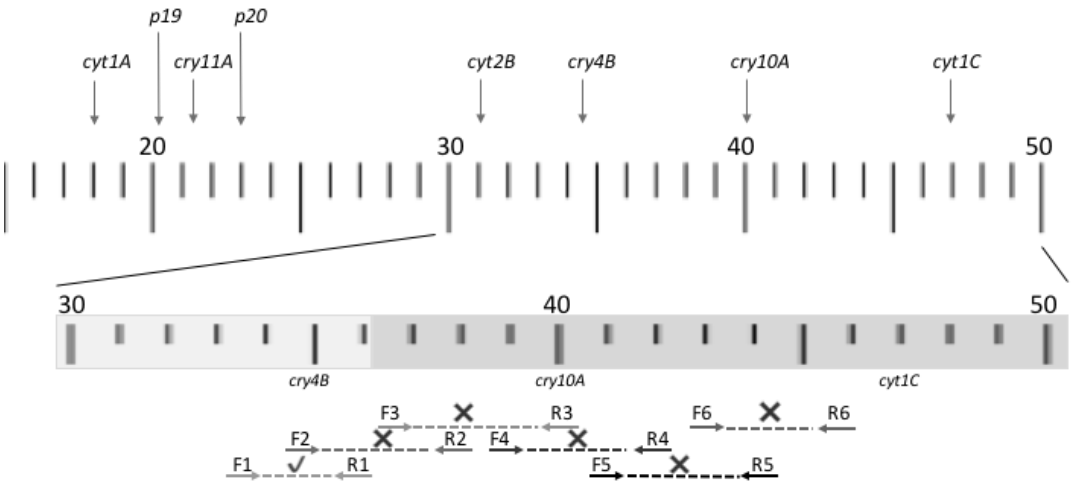

Figure 8. A representation of the section of pBtoxis plasmid that was studied. The position of each primer designed and the expected PCR products are represented by arrows and dotted lines, respectively.

3.2.7.2. Study of effect of plasmid curing on protein production capacity

A. Plasmid curing:

The plasmid curing was performed with two local *Bti* strains *QBT*217 and *QBT*220. A single isolated colony of these two strains were inoculated in 2 ml of LB broth and incubated at 42^0C overnight at a rpm of 200. After 24 hours, 100 µl of this culture was transferred to 2 ml of fresh LB broth. These steps were continued for 7 days.

B. Isolation of new clones:

On the 8^{th} day, the culture was serially diluted till 10^{-5} and the last dilution was spread on LB agar plates. After overnight incubation of these plates at 30^0C, each of the colonies obtained were transferred to a separate LB agar plate. These were considered the new clones of the original *Bti* strains.

C. Investigation of retaining pBtoxis after curing by PCR:

Each clone was tested for the presence of pBtoxis plasmid by amplifying the *cry4* gene by PCR and running gels to check the results. The clones that gave positive expected PCR products for this amplification were considered for protein production experiments.

D. Evaluating the change in protein production capacity:

The protein production experiments were performed with clones of interest using the methodology discussed in section 3.8.1. The controls used in the experiments were *Bti* H14, 4Q7, original *QBT*217, original *QBT*220, acrystalliferous clones of *QBT*217 and *QBT*220 obtained by plasmid curing. The change in protein production was calculated by comparing the production capacities of original *Bti* strains and the newly obtained clones.

CHAPTER 4.1 RESULTS: CHARACTERIZATION OF QATARI *BT* STRAINS PRODUCING SPHERICAL CRYSTALS

4.1.1. Introduction

In this PhD project, 441 *Bt* strains producing spherical crystals were explored. The first part of the characterization included morphological studies of the parasporal crystals produced by the *Bacillus thuringiensis israelensis* (*Bti*) strains. This was performed using three approaches: Wet mounting to ensure the crystal shape, Endospore staining to differentiate between crystals and spores and scanning electron microscopy at higher magnification to identify the different morphologies observed among the strains producing spherical crystals. Once the different crystal morphologies of the parasporal crystals were identified, the crystal proteins were analysed by SDS-PAGE. The different protein sizes observed were used to group the strains with similar crystal content into classes. Similarly, the plasmid DNA was isolated from each strain and the plasmid patterns were also used to group the strains into classes with similar plasmid patterns. This is a novel approach of characterization of such big collections of *Bt*. Instead of relying on one category to characterize the *Bt* strains, all three categories mentioned above were taken into consideration to achieve an efficient classification system. This efficiency of classification ensures that the representatives taken from each class would truly represent the whole. A true representative will be a good candidate to investigate at molecular levels as done in next chapters. All the *Bacillus thuringiensis israelensis* (*Bti*) strains and one representative from each class of Non *Bti* spherical crystal producing strains were hence selected and used for other studies described in the next chapters. The strains that showed interesting results were used for ribotyping and phylogenetic studies. We have published parts of the results from this chapter as an original article in Frontiers in Microbiology journal: Nair *et al*., 2018 (b). Article is attached as Appendix B.

4.1.2. Results

A. Evaluation of morphology of parasporal crystals

Wet mounting

Wet mounting was performed for all 441 strains to ensure that the strains analysed are producing spherical crystals. As reference, *Bti* strain H14 was used alongside. The strains when observed after 48 hours of culturing in sporulation medium, showed spores and crystals still encapsulated in vegetative cells. After 72 hours, the cultures showed spores and crystals released and absence of any vegetative cells (complete sporulation). A completely sporulated culture of each strain was mounted on a slide with a drop of water and observed under oil immersion lens of the light microscope with a coverslip. The elliptical shaped spores and spherical crystals could be seen under the microscope (figure 9). Compared to the reference, Non-*Bti* strains showed the presence of larger crystals (figure 9c). To ensure that the spherical structures are protein crystals and the elliptical structures are spores, the endospore staining was performed.

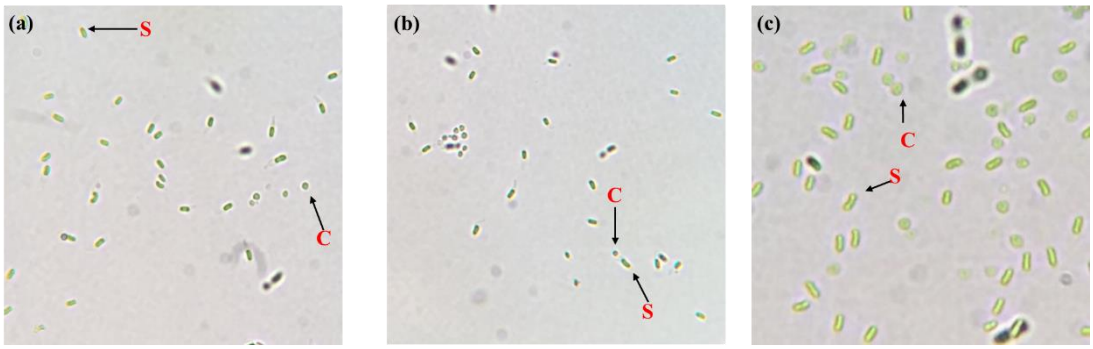

Figure 9. Microscopical images of spores and crystals observed under light microscope [(a): spores and crystals of reference strain *Bti* H14; (b): spores and crystals of *QBT*225 (resembled that of reference); (c): spores and crystals of *QBT*446 (with larger crystals); s: elliptical spore; c: spherical crystal]

Endospore staining

Endospore staining was performed for the *Bt* strains to confirm that the elliptical structures were spores and the spherical structures were protein crystals. The malachite green dye under steam used for endospore staining gave the spores and crystals, a green colour. And the destaining step removed the green colour from crystals while the malachite green got trapped under the spore; allowing the spore only to retain the green colour. A counter stain of safranin coloured the crystals pink while the endospores green (figure 10). It was clear once again that the Non-*Bti* strain had crystals that were larger and not exactly smooth and round (figure 10c). To understand the different morphologies, it was essential to check the crystals at higher magnification.

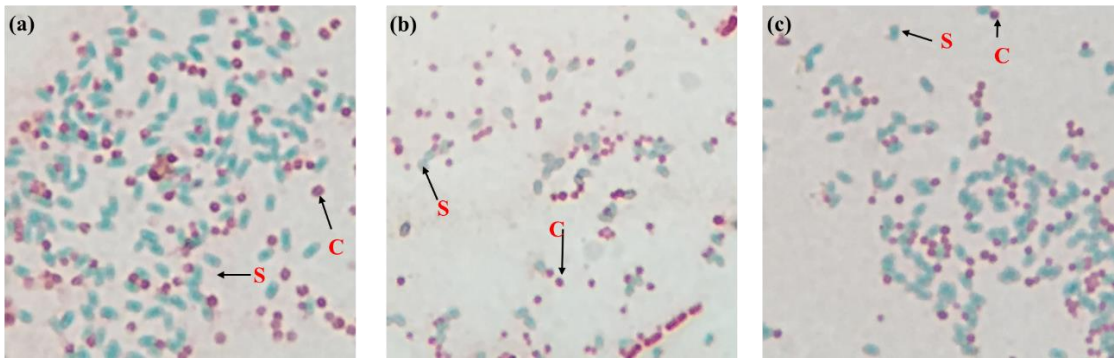

Figure 10. Microscopical images of spores and crystals of *Bt* strains stained with endospore staining technique

[(a): spores and crystals of *QBT*446 (with larger crystals); (b): spores and crystals of reference strain *Bti* H14; (c): spores and crystals of *QBT*225 (resembled that of reference); s: elliptical spore; c: spherical crystal]

Scanning Electron Microscopy

The Scanning Electron Microscopy (SEM) of the completely sporulated cultures of the *Bt* strains was performed by mounting the spore-crystal mixture on aluminium stubs and coating it with gold. The surface and shape of the spores and crystals could be seen in the two-dimensional pictures taken on the SEM. Among the 441 strains, five different morphologies were observed: smooth spherical (figure 11b), spherical with undulated surface (figure 11c), spherical but deflated balloon (figure 11d), spherical concave (figure 11e) and spherical pointy edged (figure 11f). The smooth spherical crystals were observed for 19 *Bt* strains and they resembled that of the reference strain H14. *Bt* strains producing spherical crystals with undulated surface were the maximum among the collection of *Bt* (table 4). The rare morphologies observed were the ones with spherical concave and spherical with pointy edge. Only two strains showed the presence of crystals with each of these two morphologies (table 4).

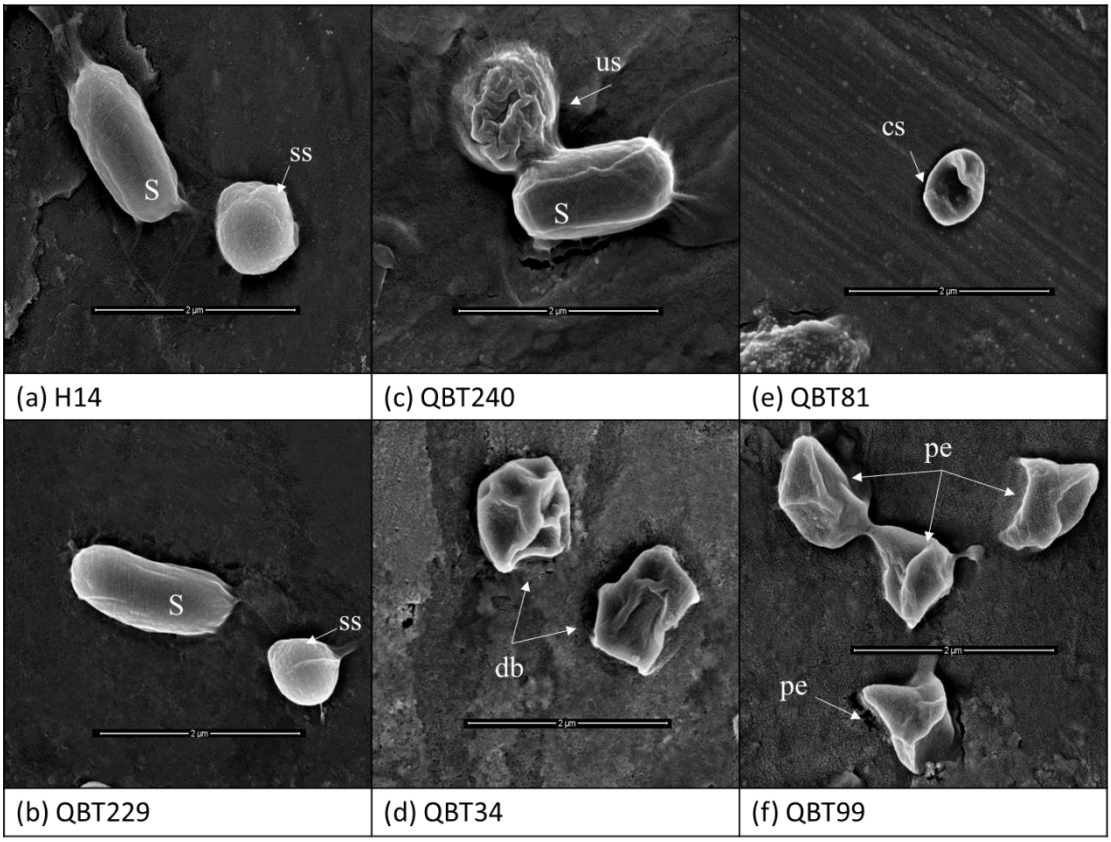

Figure 11. Crystal morphologies observed among the *Bt* strains studied (Nair *et al*., 2018b)

[Scanning electron microscopy images of the crystal morphologies; S: spores; ss: smooth spherical; us: spherical undulated; cs: spherical concave; db: spherical deflated balloon; pe: spherical pointy edged]

Table 4. Distribution of *Bt* strains based on parasporal crystal morphologies (Nair *et al*., 2018b)

Crystal morphologies	**Number of *Bt* strains**
Smooth spherical	19
Spherical undulated	326
Spherical deflated balloon	92
Spherical concave	2
Spherical pointy edged	2
Total strains	**441**

B. Analysis of protein patterns of parasporal crystal proteins

The parasporal crystal proteins from each *Bt* strain were solubilized and run on SDS-PAGE gels to separate the different δ- endotoxins based on their sizes. Based on the content of crystals and the type of patterns formed as per the different sized proteins, the strains were grouped into classes. Among the 441 *Bt* strains studied, 15 different protein patterns were seen (figure 12). They were named Prot1 to Prot15. Prot1 consisted of 19 *Bt* strains that had similar protein patterns like the reference H14. The 19 strains were considered as *Bti* strains. The main protein sizes seen in this *Bti* pattern included 130 kD, 65 kD, 45 kD and 27 kD. The different protein sizes forming the remaining 14 patterns are listed in the table 5. The Prot10 pattern was seen the most among the *Bt* strains analysed. Prot14 and Prot15 patterns were the rarest; seen among only two strains each. The distribution of different protein patterns seen among the 441

strains tested are listed in the **table 5**.

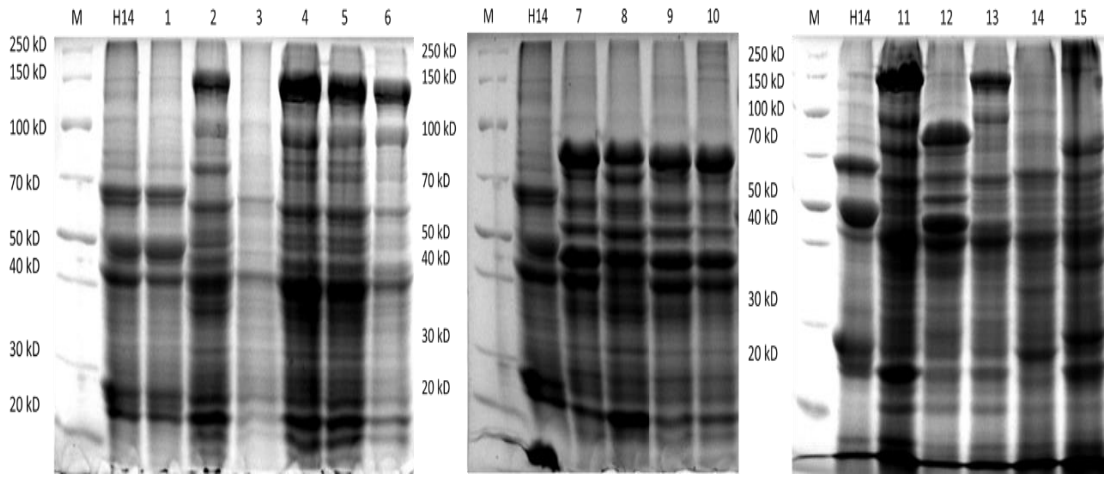

Figure 12. Crystal protein patterns observed among the *Bt* strains analysed in the study (Nair *et al*, 2018b)

[M: broad range protein marker; H14: the crystal protein pattern for reference strains H14; Lanes 1 to 15: crystal protein patterns Prot1 to Prot15]

Table 5. Distribution of *Bt* strains based on its δ- endotoxin content of the parasporal crystals

Protein pattern	Number of *Bt* strains	Protein sizes (kD)
Prot1	19	130, 65, 45, 27
Prot2	4	130, 90, 75, 65, 50, 40, 25
Prot3	7	100, 65, 40
Prot4	19	180, 140, 90, 80, 60, 45, 25
Prot5	12	140, 90, 80, 60, 45, 25
Prot6	10	130, 90, 60, 40, 22
Prot7	48	100, 80, 60, 45, 40
Prot8	16	80, 60, 45, 40, 35
Prot9	9	150, 80, 60, 45, 40, 27
Prot10	203	180, 150, 80, 60, 45, 40, 27
Prot11	29	130, 90, 75, 55, 40, 27, 22
Prot12	33	85, 65, 55, 45, 30, 27, 22
Prot13	28	130, 100, 65, 45, 27, 22
Prot14	2	65, 45, 35, 27
Prot15	2	230, 150, 85, 70, 40, 27, 25
Total strains	**441**	

C. Analysis of plasmid patterns of the *Bt* strains

The plasmid samples for all the 441 strains were run. The different plasmids of each strains separated on the gel forming unique patterns. These patterns were used to group the strains with similar plasmid contents into one class. Among the *Bt* strains tested, six

patterns were observed (figure 13). They were named Plas1 to Plas6. Most common plasmid pattern observed among the *Bt* strains was Plas4 with 286 strains showing similar plasmid content. Plas5 was the rarest pattern seen, with only two strains showing this plasmid pattern (table 6). All the plasmid patterns observed and the number of strains showing these patterns are listed in table 6. The Plas1 pattern observed for 19 strains resembled that of the reference H14. Hence, these 19 strains were considered as *Bti* strains.

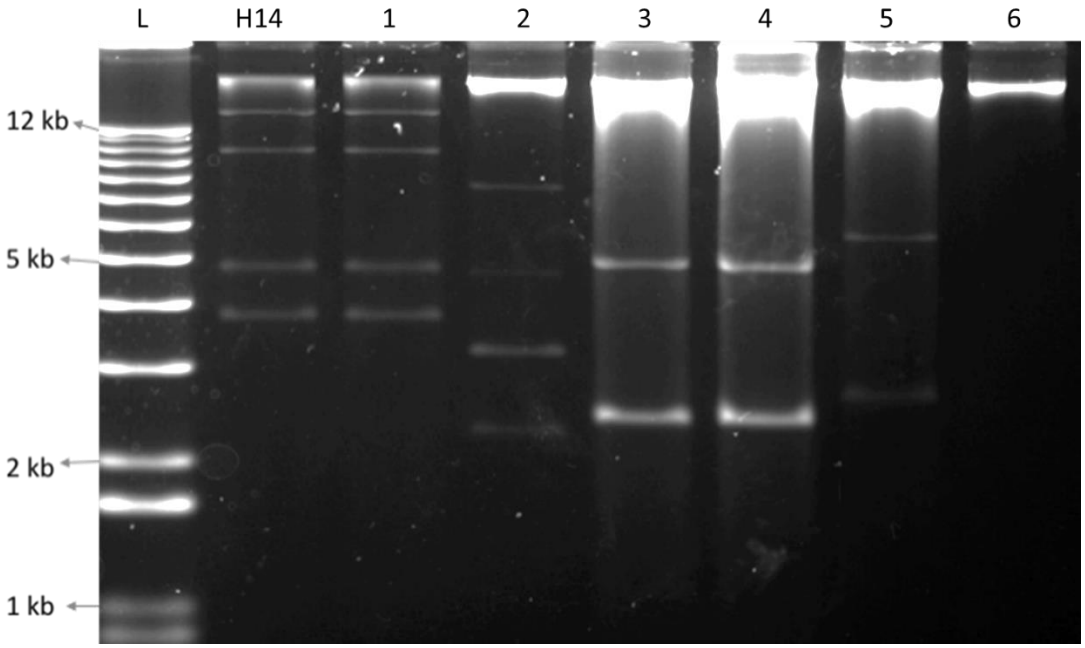

Figure 13. Plasmid patterns observed among the *Bt* strains analyzed

[L: 1 kb plus linar DNA marker; H14: the plasmid pattern for the reference strains H14; Lanes 1 to 6: Plasmid patterns Plas1 to Plas6]

Table 6. Distribution of plasmid patterns observed among the *Bt* strains analyzed

Plasmid patterns	**Number of *Bt* strains**
Plas1	19
Plas2	7
Plas3	33
Plas4	286
Plas5	2
Plas6	94
Total *Bt* strains	**441**

D. Phylogenetic analysis of the *Bt* strains

Analysis by sequencing and dendrogram study

After characterizing the *Bt* strains based on their crystal morphology, δ- endotoxin content and plasmid patterns, phylogenetic studies were performed for a few strains for two reasons: confirming their identity as *Bt* and check their relationship with other *Bt* strains from the NCBI database. It was seen that the Non *Bti* strains also are very close to the references *Bti* and *Bacillus thuringiensis kurstaki* (*Btk*). But the dendrogram showed that the Non *Bti* strains are very close to other *Bacillus* species like *anthracis* and *subtilis* also. This is common as phylogenetically *thuringiensis, anthracis, subtilis* and *cereus* are very closely related (figure 14). The main characteristic that differentiates *thuringiensis* is the presence of parasporal crystals during sporulation. The Dendrogram confirmed that the Non-*Bti* strains do belong to the *Bt* group despite having different plasmid and protein patterns.

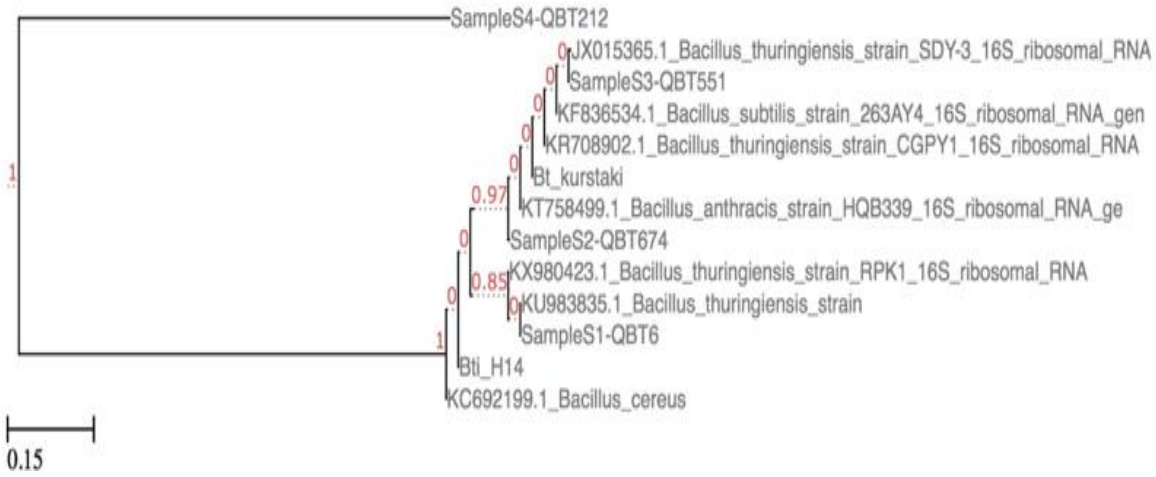

Figure 14. Dendrogram showing the phylogenetic relationship between the Qatari *Bt* strains and references

[Nucleotide sequence of 1.5 kb region of the 16s rRNA gene from Qatari Non-*Bti* strains compared with that of reference strains from NCBI database; reference strains selected include *Bt*, *Btk*, *cereus*, *subtilis* and *anthracis*]

Analysis by Amplified ribosomal DNA restriction analysis (ARDRA)

A second method of phylogenetic analysis was performed using the ARDRA technique. The 16s rRNA gene was amplified and cut using a restriction enzyme Sau3A. The Sau3A restriction enzyme has a four-nucleotide target sequence: GATC. Small targets ensure that more fragments are formed when cut. For references, *Bacillus cereus, Bacillus subtilis, Pseudomonas, Bacillus thuringiensis israelensis* and *Bacillus thuringiensis kurstaki*. When the patterns were compared, it was clear that the *Bti* and Non *Bti* strains resembled that of the *Bti* and *Btk* and *cereus* (figure 15). It confirmed that despite the differences seen, the strains are *Bt*.

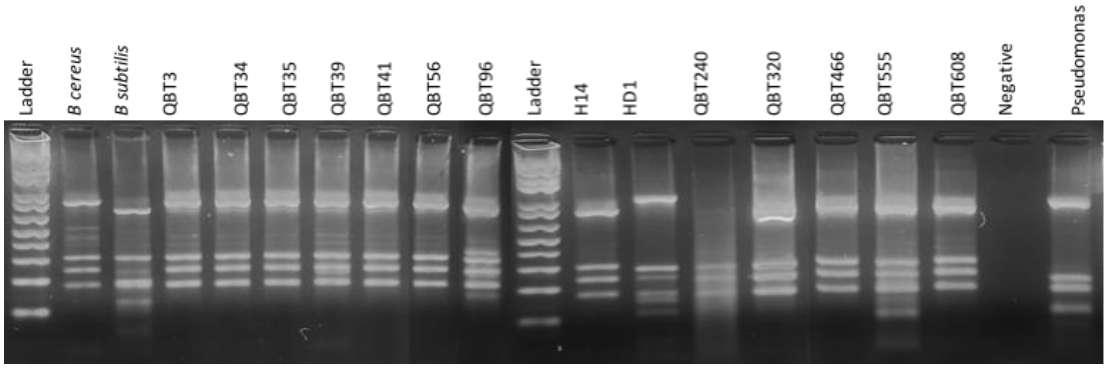

Figure 15. Patterns of DNA fragments formed by ARDRA

[Ladder: 1 kb plus linear DNA marker; *QBT*3, *QBT*34, *QBT*35, *QBT*39, *QBT*41, *QBT*56, *QBT*96, *QBT*240, *QBT*320, *QBT*466, *QBT*555: Qatari Non *Bti* strains; *QBT*608: Qatari *Bti* strain]

4.1.3. Discussion

Isolation and characterization of *Bt* strains was done for the first time in Qatar and a huge collection of diverse *Bt* strains has formed a part of this study. Huge collections like this needs an efficient system of characterization as it is not possible to analyse each of the 700 *Bt* strains from Qatar for every property. Hence, it is necessary to classify the collection into groups that can be truly represented by a representative strain. The goal of this PhD work was to identify *Bt* strains with insecticidal activity towards Dipteran insects, and it is known that *Bt* strains with spherical parasporal crystals are normally insecticidal to Dipteran insects. Hence, this study focused on 441 *Bt* strains that showed the production of spherical crystals during sporulation. A collection of 441 *Bt* strains with spherical crystals is still huge when the objectives of this project included many detailed molecular analyses. Hence, it was essential to classify the collection further into groups and choose true representatives for the molecular analyses. Crystal morphologies and plasmid patterns are usually used for

classification, but the protein pattern of the parasporal δ- endotoxins were included in this objective to see the actual variety among the collection. The classification based on crystal morphology only divided the collection into five groups (table 4) and that for the plasmid patterns divided the collection into six groups (table 6). On the other hand, when the protein patterns were considered, the collection was divided into 15 groups (table 5). A representative was chosen from each of these 15 groups considering the crystal morphology and the plasmid patterns also. The classification has been summarized in the table 7.

The first group of strains represented by *QBT*229 had one crystal morphology with smooth spherical shape (figure 11b) and one plasmid pattern Plas1 (figure 12) and all had one protein pattern Prot1 (figure 13). These characteristics resemble that of the reference *Bti* strain H14. It was concluded that these 19 strains are Qatari *Bti* strains. The second crystal morphology observed was spherical crystal with undulated surface. This is the most common morphology found. This group of strains have three different plasmid patterns among them and seven different protein patterns among them. The third type of crystal morphology observed was deflated balloon shaped. This group of strains had just one plasmid pattern but five protein patterns among them. The fourth type of crystal morphology observed was the spherical with one concave face. This type of strains had one protein pattern and one plasmid pattern among them. The last type of crystal morphology observed was the pointy edged crystals. Like the last group, these strains also displayed just one protein and one plasmid pattern among them. It is interesting to note that Plas6 plasmid pattern was seen among two strains with two different crystal shapes: spherical deflated balloon and spherical concave. This proves that the plasmid pattern, which is usually considered a representative of the crystal morphology, is not always so. Once again, it justifies the use of protein pattern for the

right classification of huge collections of *Bt*.

Some of the Non-*Bti* strains were used for amplifying the 1.5 kb region of their 16s rRNA gene. The sequence, of which, was compared with that of the references. Despite the novel plasmid patterns and crystal morphologies observed, the collection of *Bt* strains producing the spherical crystals truly belong to the parent group of *Bacillus cereus*. The crystals they produce during the sporulation stage distinguishes them as *thuringiensis* from the other species like *anthracis*, *subtilis* and *cereus*.

Table 7. Summary of the classification & distribution of Qatari *Bt* strains producing spherical crystals

[Family representatives and their respective plasmid patterns, protein patterns and crystal morphology has been listed; Plas1 to Plas6 plasmid patterns are shown in figure 13; Prot1 to Prot15 protein patterns are shown in figure 12; the SEM images of the crystal morphologies mentioned are shown in figure 11]

Family representative	**Crystal morphology**	**Plasmid pattern**	**Protein pattern**	**Number of *Bt* strains**
*QBT*229	Smooth spherical	Plas1	Prot1	19
*QBT*6	Spherical undulated	Plas2	Prot3	7
*QBT*43		Plas3	Prot12	33
*QBT*212		Plas4	Prot6	10
*QBT*240		Plas4	Prot7	48
*QBT*320		Plas4	Prot8	16
*QBT*418		Plas4	Prot9	9
*QBT*555		Plas4	Prot10	203
*QBT*3	Spherical deflated balloon	Plas6	Prot2	4
*QBT*34		Plas6	Prot4	19
*QBT*39		Plas6	Prot5	12
*QBT*41		Plas6	Prot11	29
*QBT*56		Plas6	Prot13	28
*QBT*81	Spherical concave	Plas6	Prot14	2

Family representative	Crystal morphology	Plasmid pattern	Protein pattern	Number of *Bt* strains
*QBT*99	Spherical pointy edged	Plas5	Prot15	2
			Total *Bt* strains	**441**

4.1.4. Conclusion

The characterization and classification of the *Bt* strains producing spherical crystals were completed in this part of the project. The categories used for classification of the 441 spherical crystal producing *Bt* strains were crystal morphology, plasmid patterns and crystal protein patterns (Nair *et al*., 2018b). The *Bt* strains were divided into 15 groups based on protein patterns, with 1st group of 19 *Bti* strains and 14 groups of Non-*Bti* strains. A true representative was chosen from each of these 14 groups, considering their plasmid patterns and crystal morphologies. Phylogenetic studies by 16s rRNA sequencing and ARDRA confirmed the identity novel *Bt* profiles as belonging to the *Bacillus thuringiensis* species. All the analyses performed hereafter in this study were done with 19 *Bti* strains and 14 Non *Bti* representative strains.

CHAPTER 4.2 RESULTS: EXPLORATION OF THE INSECTICIDAL ACTIVITIES OF QATARI *BTI* STRAINS AGAINST *AEDES AEGYPTI* AND *CULEX PIPIENS* LARVAE

4.2.1. Introduction

In this part of the study, the insecticidal activities of the Qatari *Bt* strains producing spherical crystals were studied against the 3rd instar larvae of Dipteran insects like *Culex pipiens* and *Aedes aegypti*. Two groups of *Bt* strains were tested for their insecticidal activity. The first group consisted of 19 Qatari *Bti* strains (*QBti*); characterized as such based on their crystal morphology, plasmid and protein patterns, insecticidal protein genes content, etc (Refer Chapter 4.1). Based on these characteristics, the 19 *Bti* are the most important strains which were expected to be possible candidates for bio-pesticide production. The second group consisted of 14 Non *Bti* representatives that were also chosen based on the aforementioned characteristics (refer Chapter 4.1). As reference, the *Bti* strain H14 was used as positive control as it has been widely studied and characterized by many research groups. *Bti* H14 is also considered one of the best *Bti* available today in the market to produce bio-pesticides. As negative control, *Bti* 4Q7 strain was used. This is an acrystalliferous clone of *Bti* H14. In the qualitative bioassay experiments, the Dipteran larvae were exposed to high concentrations of the crystal proteins from each strain of the two groups. This was carried out in order to screen the strains in terms of their insecticidal potentials. In the quantitative bioassay, the LC_{50} of *QBti* strains were determined using H14 as positive control strain. For the histological studies, the live and dead larvae were collected after the bioassay experiments and observed by wet mounting to identify the possible tissues affected by the *Bt* insecticidal proteins.

4.2.2. Results

A. Qualitative insecticidal bioassay

After treating the 3rd instar larvae of the *Culex pipiens* for 24 hours with high doses of insecticidal spore-crystal mixture of *Bt* strains, it was found that some strains killed all five larvae while some couldn't kill any. Then there were some strains that could kill 1 or 2 or 3 larva(e). The *QBti* strains and the positive reference H14 killed all five strains as expected (table 8). None of the Non *Bti* strains could kill all five larvae. Among the Non *Bti* strains, *QBT*3, *QBT*39, *QBT*43, *QBT*81, *QBT*240 and *QBT*418 could kill none of the larvae; like the negative control reference strain 4Q7. *QBT*34 and *QBT*56 could kill 2 and 3 larvae, respectively. Other Non-*Bti* strains including *QBT*6, *QBT*41, *QBT*99, *QBT*212, *QBT*320 and *QBT*555 could kill only one larva out of the five (table 9).

Table 8. Qualitative bioassay to screen *QBti* strains for their insecticidal activities

[Each test performed with five larvae]

	Insecticidal activity (No. of dead and viable larvae)	
***Bti* Strains**	**Dead**	**Viable**
H14	5	0
*QBT*205	5	0
*QBT*213	5	0
*QBT*214	5	0
*QBT*215	5	0
*QBT*216	5	0
*QBT*217	5	0
*QBT*218	5	0
*QBT*220	5	0
*QBT*221	5	0
*QBT*222	5	0
*QBT*223	5	0
*QBT*224	5	0
*QBT*225	5	0
*QBT*226	5	0
*QBT*227	5	0
*QBT*228	5	0
*QBT*229	5	0
*QBT*230	5	0
*QBT*608	5	0
4Q7	0	5

Table 9. Qualitative bioassay to screen Non-*Bti* strains for their insecticidal activities [Each test performed with five larvae; Dead and viable larvae numbers mentioned for each strain tested]

	Insecticidal activity (No. of dead and viable larvae)	
Non *Bti* Strains	**Dead**	**Alive**
H14	5	0
***QBT*3**	0	5
***QBT*6**	1	4
***QBT*34**	2	3
***QBT*39**	0	5
***QBT*41**	1	4
***QBT*43**	0	5
***QBT*56**	3	2
***QBT*81**	0	5
***QBT*99**	1	4
***QBT*212**	1	4
***QBT*240**	0	5
***QBT*320**	1	4
***QBT*418**	0	5
***QBT*555**	1	4
4Q7	0	5

B. Quantitative insecticidal bioassay

Determination of protein concentrations of each strain for Bioassay against Aedes Aegypti

In order to assess the larvicidal properties of the studied strains, crystal protein concentrations were determined by calculating the protein concentration of a sample from the spore crystal mixture. The sample was solubilized completely, spores separated by centrifugation, and the protein concentration was determined by Bradford's method. The estimated protein concentrations of each strain are listed in the table 10. The spore-crystal mixture of each strain was diluted as per requirements of the bioassay experiments.

Table 10. Estimation of protein concentration in spore-crystal mixture of *Bt* strains

[δ- endotoxin protein concentrations in µg/l; the estimated value was calculated by Bradford's method and subtracting the value obtained for negative control reference strain 4Q7]

Strains	**Actual δ-endotoxin content (µg/l)**
4Q7	0
***QBT*215**	996246.663
***QBT*224**	996246.663
***QBT*227**	1058746.66
***QBT*213**	1162916.66
***QBT*222**	1204580
***QBT*230**	1204580
***QBT*205**	1246250

Strains	Actual δ-endotoxin content (µg/l)
***QBT*229**	1350413.33
***QBT*214**	1392080
***QBT*220**	1496246.66
***QBT*225**	1517080
***QBT*223**	1579580
***QBT*221**	1600413.33
***QBT*228**	1662913.33
H14	1700416.66
***QBT*226**	1808746.66
***QBT*218**	1829580
***QBT*216**	2121246.66
***QBT*217**	2767080
***QBT*608**	2850413.33

Determination of the lethal concentration (50) of reference Bti H14

Among the different concentrations of - δ- endotoxin tested for the reference H14, all concentrations below 0.01 µg/l could not kill any larvae and all concentrations above 0.25 µg/l killed all the larvae. The concentration of 0.1 µg/l of - δ- endotoxin proteins from H14 could kill about 50% of the larvae (figure 16). The concentration of 0.1 µg/l was considered as the estimated LC_{50} value for the reference H14.

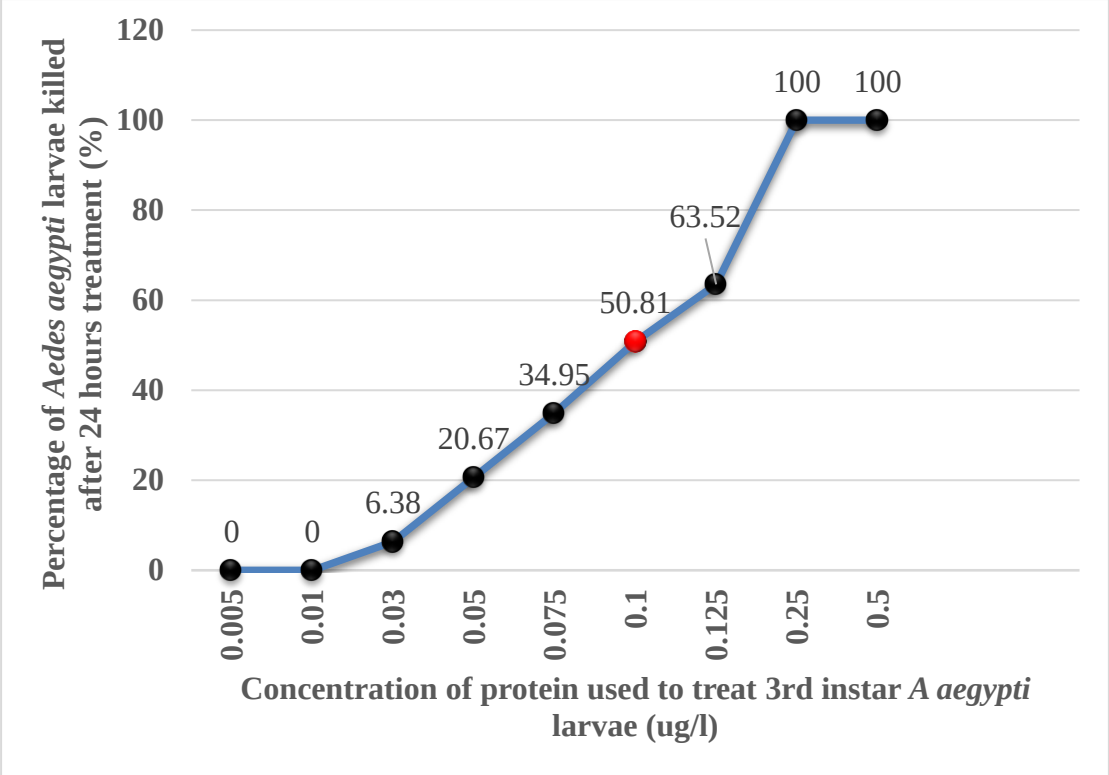

Figure 16. Determination of LC_{50} value of the δ- endotoxins of reference strain H14 against *Aedes aegypti*

[The graph represents the concentrations of δ- endotoxins tested against the percentage of larvae killed by respective concentrations of δ- endotoxins; the red dot represents the ~50% larvae killed at the concentration of 0.1 µg/l of δ- endotoxins]

Evaluation of efficiency of local strains against Aedes aegypti larvae

When tested at a - δ- endotoxin concentration of 0.1 µg/l, *QBti* strains showed different insecticidal activities against *Aedes aegypti* larvae. Based on their efficiency, the *QBti* strains were divided into three groups. The first group consisted of *QBT*205, *QBT*222, *QBT*224, *QBT*227, *QBT*226, *QBT*213 and *QBT*214. The strains belonging to this group could kill only less than 40% larvae. The second group consisted of *QBT*223, *QBT*230, *QBT*215, *QBT*229, *QBT*216, *QBT*225, *QBT*228 and *QBT*608. The insecticidal activities

of the strains of this group are similar to that of H14, killing 50% of the larvae. The third group consisted of *QBT*221, *QBT*217, *QBT*218 and *QBT*220; able to kill more than 60% of the larvae. The three different groups, thus formed, are shown in the figure 17.

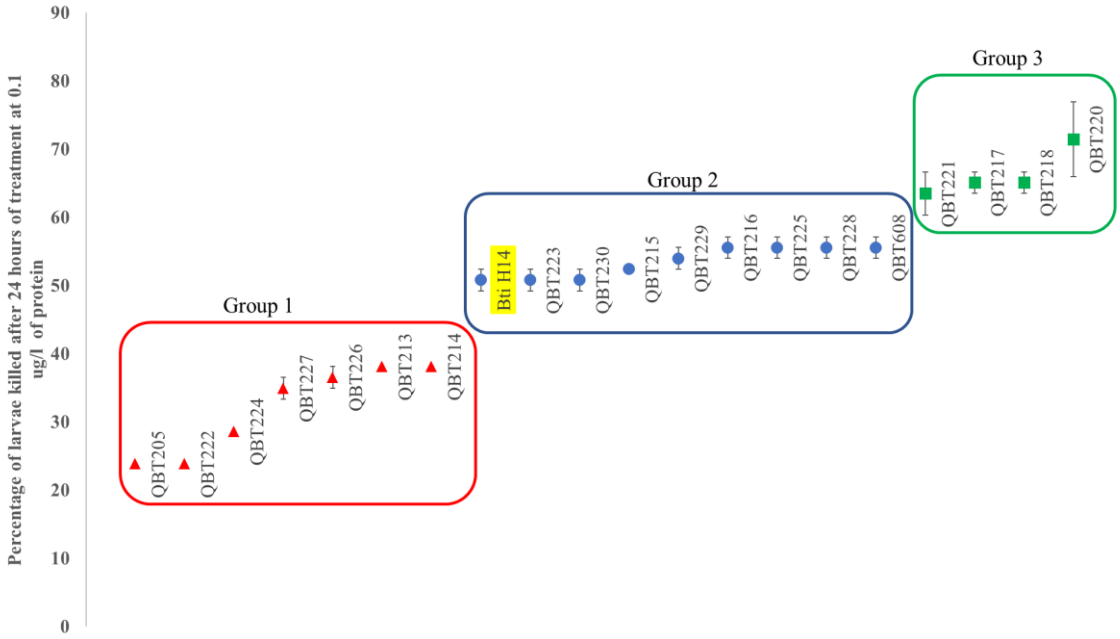

Figure 17. Study of the toxicity efficiencies of *QBti* strains against *Aedes aegypti* larvae [The graph represents the percentage of larvae killed by the reference and the *QBti* strains at the δ- endotoxin concentration of 0.1 µg/l; error bars are plotted with the standard errors calculated based on three readings for each strain; Group 1 (red box) consists of *QBti* strains that could kill only 40% or less larvae; Group 2 (blue box) consists of *QBti* strains and reference that could kill 50% of larvae; Group 3 (green box) consists of *QBti* strains that could kill more than 60% of larvae]

Determination of LC_{50} of the most insecticidal QBti strains

When tested at more concentrations of - δ- endotoxins, the Group 3 strains could not kill as larvae at or below 0.005 µg/l and killed all the larvae at or above 0.25 µg/l. These strains killed about 50% of larvae at a concentration of 0.075 µg/l of - δ- endotoxins. At other concentrations of 0.03 µg/l, 0.05 µg/l, 0.1 µg/l and 0.125 µg/l, they killed about 19%, 38%, 66% and 86%, respectively. Among these Group 3 strains, the percentage of larvae killed by *QBT*220 was slightly higher as shown in the table 11.

Table 11. Study of toxicity of Group 3 *QBti* strains against *Aedes aegypti* larvae

Strains	**Concentrations (µg/l)**	**Larvae killed (%)**			**Average larvae killed % (+/- Error)**
		I	II	III	
***QBT*217**	0.005	0	0	0	0 +/- 0
	0.03	14	19	19	18 +/- 1.59
	0.05	33	33	38	35 +/- 1.59
	0.075	52	57	52	54 +/- 1.59
	0.1	67	62	67	65 +/- 1.59
	0.125	86	86	86	88 +/- 0
	0.25	100	100	100	100 +/- 0
***QBT*218**	0.005	0	0	0	0 +/- 0
	0.03	19	19	19	19 +/- 0
	0.05	38	38	38	38 +/- 0
	0.075	57	52	52	54 +/- 1.59

Strains	Concentrations (µg/l)	Larvae killed (%)			Average larvae killed % (+/- Error)
		I	II	III	
QBT218	0.1	62	67	67	65 +/- 1.59
	0.125	81	86	81	83 +/- 1.59
	0.25	100	100	100	100 +/- 0
***QBT*220**	0.005	0	0	0	0 +/- 0
	0.03	19	19	24	21 +/- 1.59
	0.05	43	43	38	41 +/- 1.59
	0.075	57	57	67	60 +/- 3.17
	0.1	62	81	71	71 +/-5.5
	0.125	86	95	86	89 +/- 3.17
	0.25	100	100	100	100 +/- 0
***QBT*221**	0.005	0	0	0	0 +/- 0
	0.03	14	19	19	18 +/- 1.59
	0.05	29	33	38	33 +/- 2.75
	0.075	52	48	48	49 +/- 1.59
	0.1	67	67	57	64 +/-3.17
	0.125	81	81	86	83 +/-1.59
	0.25	100	100	100	100 +/- 0

Statistical analysis

The actual LC_{50} value was calculated for the reference and the group 3 strains with the data from table 11. Probit analysis software showed that the LC_{50} value of the reference H14 was 0.095 µg/l. The LC_{50} value for the group 3 strains were: 0.065 µg/l for *QBT*217, 0.066 µg/l for *QBT*218, 0.06 µg/l for *QBT*220 and 0.068 µg/l for *QBT*221 (table 12). The fiducial limit at 95% confidence was calculated and the same has been shown in the figure 18.

Table 12. Probit analysis of the insecticidal activities of Group 3 *QBti* strains and reference H14 against *Aedes aegypti* larvae

[The output for the LC_{50} calculated for Group 3 *QBti* strains and reference from Probit analysis software; lower and upper values represent the fiducial range for each strain at 95% confidence level]

STRAINS	PROBIT ANALYSIS OUTPUT			
H14	LD/LC	LD/LC	95% Fiducial CI	
	(%)	ug/l	Lower	Upper
	LD50	**0.094797**	**0.0675826**	**0.1329701**
***QBT*217**	LD/LC	LD/LC	95% Fiducial CI	
	(%)	ug/l	Lower	Upper
	LD50	**0.065119**	**0.0492225**	**0.0861484**
***QBT*218**	LD/LC	LD/LC	95% Fiducial CI	
	(%)	ug/l	Lower	Upper
	LD50	**0.065488**	**0.0484001**	**0.088608**
***QBT*220**	LD/LC	LD/LC	95% Fiducial CI	
	(%)	ug/l	Lower	Upper
	LD50	**0.060121**	**0.0452774**	**0.0798318**
***QBT*221**	LD/LC	LD/LC	95% Fiducial CI	
	(%)	ug/l	Lower	Upper
	LD50	**0.068275**	**0.0507161**	**0.091914**

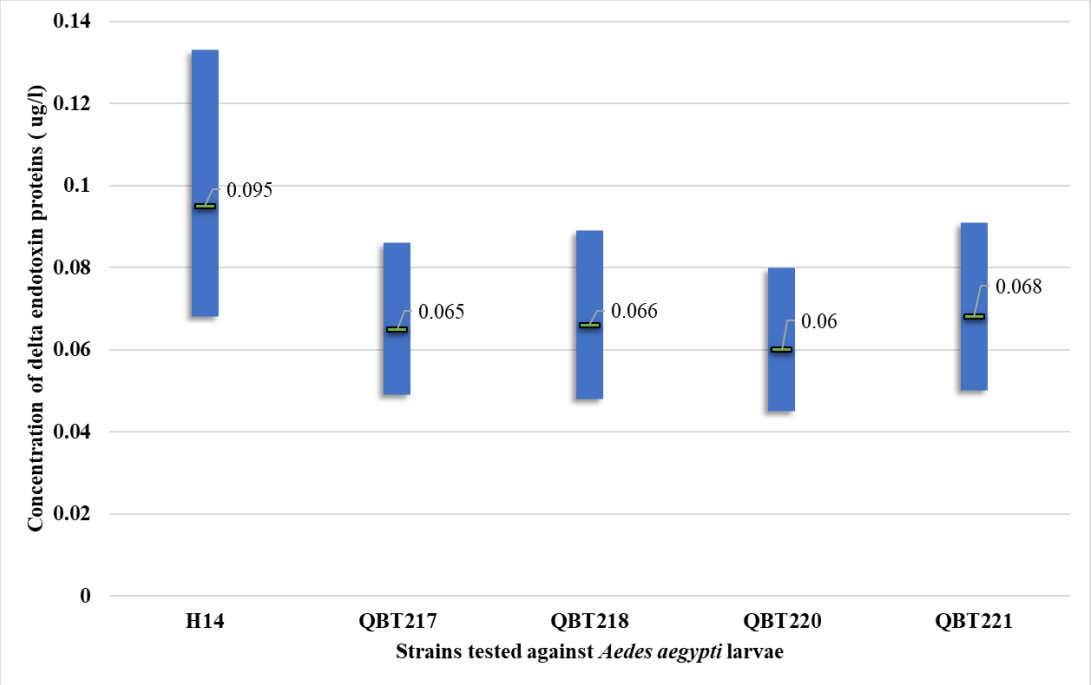

Figure 18. Comparison between the insecticidal activities of *QBti* strains and reference H14 based on probit analysis

[The LC_{50} values of the Group 3 *QBti* strains and reference H14; the bars represent the range of LC_{50} values for each strain, the black line and the associates values show the calculated LC_{50} values]

C. Histological study of larvae exposed to *QBti* insecticidal proteins

In this study, the effect of *QBti* insecticidal proteins on larvae were analysed. It was seen that the dead larvae had shrunk in size and that all the dead larvae had a broken gut, compared to the intact structure found in untreated larvae. The break was always found to be between the anterior and posterior midgut in dead larvae. It shows that the insecticidal δ- endotoxins do bind to the larval midgut causing a disruption in its membrane. The loss of integrity of the midgut membrane wall causes starvation and eventual death of the larvae treated with *Bti* crystals.

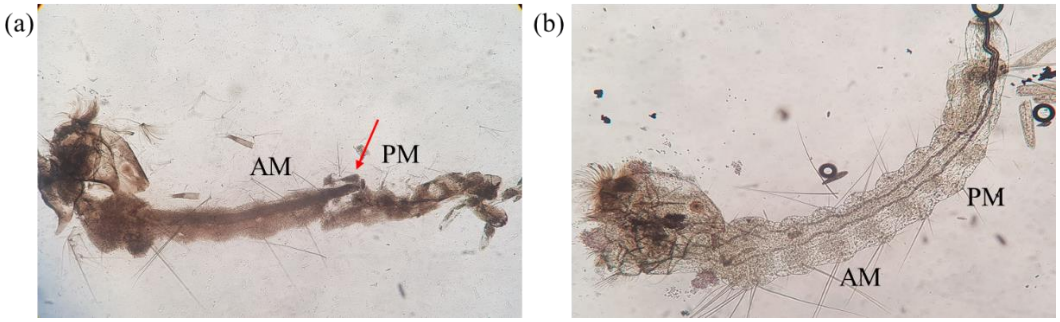

Figure 19. Microscopic observation of the effect of *QBti* δ- endotoxins on the morphology of larval midgut

[The microscopical observation of dead and live larvae after treating with the - δ-endotoxin and spore mixture of *Bt*; (a) shows the dead larvae and (b) shows the untreated larvae collected after 24 hours of treatment with δ- endotoxins; AM is the anterior midgut and PM is the posterior midgut of the larvae; the red arrow represents the region where all the dead larvae showed a break in their midgut tube]

4.2.3. Discussion

The two groups of *Bt* strains tested for qualitative bioassay were the Qatari *Bacillus thuringiensis israelensis* strains (*QBti*) and the Qatar Non-*Bti* strains. This selective bioassay had three goals: (1) main aim was to select the strains that have insecticidal activity against Dipteran insects, (2) consequently also to confirm the characterization of 19 strains as *Bti* based on their protein and gene content and (3) compare the LC_{50} of the different strains. To see the slightest insecticidal activity that the strains could possess, very high concentrations of δ- endotoxins were used for the treatment (40 µg/ml to 150 µg/ml). Like the reference strain, the *QBti* killed all the larvae tested. This was expected as the *QBti* strains had the molecular characteristics of the reference *Bti* and carried genes encoding insecticidal proteins (Chapter 4.1). The Non-*Bti* strains, on

the other hand, could not kill all the larvae even at high concentrations of their insecticidal proteins confirming that the δ- endotoxin forming crystals do not belong to the same family as the *QBti* strains. Therefore, the target of these Non-*Bti* strains could be other insect families. The quantitative bioassay was carried out with the *QBti* strains only as they showed promising results in the selective qualitative bioassay.

The quantitative bioassay is usually carried out with all the tested strains at different concentrations and then narrowed down to the LC_{50} concentration. As a huge number of strains were to be tested, a new approach was used to group the strains based on their efficiency. From research articles a rough range of LC_{50} value of the reference strains H14 is available (Elleuch *et al*., 2015). But, the culture conditions and the instruments used may give us varied results in our lab. So, to standardize the results, the bioassay was first carried out with different concentrations of δ- endotoxins produced by H14 alone. The concentration that killed almost 50% of the tested larvae was considered as the estimated LC_{50} value for H14. As the reference could kill 50% of larvae at 0.1 µg/l, a strain that is as efficient as H14 should be able to kill 50% of the larvae as well. On the other hand, a strain more efficient than H14 should be able to kill more than 50% of the larvae. Similarly, a strain less efficient than H14 will not be able to kill 50% of the larvae. As the goal of the study is to find the strains that are more efficient than H14, this strategy could narrow down the number of strains to be studied further. As shown in the figure 18, the strains were grouped into 3 groups. The Group 3 strains were of interest as they proved to be more efficient than H14; killing more than 60% of the larvae tested. The four strains *QBT*217, *QBT*218, *QBT*220 and *QBT*221 were chosen as candidate strains for further studies. To calculate the LC_{50} value of these strains, it was essential to repeat bioassays for them at more concentrations above and below 0.1 µg/l. From the second set of bioassays, it was found that the Group 3 strains had their

LC_{50} values below 0.075 µg/l, as they killed 50% of the larvae at this concentration. Probit analysis software was used to calculate the exact LC_{50} values for the reference as well as Group 3 strains. The results from the software showed that the LC_{50} value for H14 was 0.095 µg/l (~ estimated was 0.1 µg/l) and that for Group 3 strains were between 0.06 µg/l to 0.068 µg/l. Lower LC_{50} values among the *QBti* strains confirmed once again that they were more efficient as insecticidal *Bt* against *Aedes aegypti* larvae. There are still controversies regarding which pathway fits the best to explain the mode of action of the Cry proteins (Adang *et al*., 2014; Vachon *et al*., 2012). Two different models have been suggested: pore-formation model and cell signal transduction model. But it is accepted that Cry proteins recognize the proteins on the brush border of the midgut of larvae. The larval midgut epithelial layers loose integrity due to either of the two models that leads to insect death (Soberon *et al*., 2009; Adang *et al*, 2014). The break in the midgut is expected after the treatment with insecticidal - δ- endotoxin. When the treated live and dead larvae were observed under the microscope, the expected break in the larval midgut were visible for all the dead larvae. On the contrary, the live larvae had intact alimentary tract.

4.2.4. Conclusion

In this study, 441 *Bt* strains that produce spherical crystals were characterized to identify 19 *Bti* strains that were expected to have insecticidal activity against the 3rd instar larvae of Dipteran insects. Out of these 19 *Bti* strains, it was shown that four *Bti* strains were more efficient against *Aedes aegypti* larvae compared to the reference H14. Their efficiency was confirmed when they displayed low LC_{50} values, much lower than that of H14. These *QBti* strains were hence recognized as candidate strains for bio-insecticide production against *Aedes aegypti* mosquitoes but most probably against other Dipteran insects as well. These strains were then tested for their δ- endotoxin

production capacity, which is discussed in Chapter 4.5. Ultimately, the aim is to not only find a highly insecticidal strain, but also to find one that can produce the efficient insecticidal proteins at a much higher rate than the reference strain H14.

CHAPTER 4.3 RESULTS: EXPLORATION OF THE δ ENDOTOXINS OF QATARI *BACILLUS THURINGIENSIS* STRAINS

4.3.1. Introduction

The objective of this part of the work was to identify δ-endotoxins synthesized by the *Bt* strains isolated from Qatar. The first method used for the identification of δ-endotoxins was to amplify whole or part of the δ-endotoxins coding genes by PCR. As the goal of the project was to identify the *Bt* strains having insecticidal activities against Dipteran insects, the PCR amplifications were done first using primers specific to genes encoding δ-endotoxins targeting Dipteran larvae, like *cry4*, *cry2*, *cry11*, *cyt1A*, *cry10*, *cyt2A*, *cyt1C*. The other important accessory protein genes were screened in a second phase, including *p19* and *p20*. The PCR screenings were performed with 19 *Bti* strains and 14 Non *Bti* representative strains. In the second part, a different strategy was used for identifying the possible δ-endotoxins: protein profiling. Here the crystal proteins from Non *Bti* strains were solubilized, digested and run on liquid chromatography coupled with mass spectrometer (LC-MS). The data from these profiling was compared to *Bt* database on UniProt and similar proteins were identified. The protein profiling was performed for Non *Bti* strains, as they failed to amplify most of the *Bti* δ-endotoxins coding genes. Protein profiling was added to the project to analyse Non *Bti* to overcome the main limitation of the PCR screenings: the inability to distinguish between the absence of gene and differences in the gene sequences. In both cases, PCR gives a negative result with no amplification. This chapter is hence divided into two sections: PCR screenings and Protein profiling.

4.3.2. Results

A. Prediction of δ- endotoxin proteins by PCR

Screening of Bti strains

The 19 *Bti* strains were screened for the presence of Dipteran specific δ endotoxin genes and accessory protein genes. The *Bti* strains H14 and acrystalliferous strains 4Q7 were used as positive and negative controls, respectively. It was found that the Qatari *Bti* strains gave the expected amplifications for PCRs run with primers specific to genes like *cry4*, *cry11*, *cyt1A*, *cyt2A*, *p19* and *p20*. The amplifications obtained for these genes were the same as the ones obtained with reference strain H14. Unlike H14, Qatari *Bti* strains failed to give any amplifications for the two genes *cry10* and *cyt1C*. Also, as expected Qatari *Bti* strains did not give any amplifications for PCRs run with primers specific to *cry2* gene.

Table 13. Primers and expected PCR products

Gene	Primer pairs	Sequences	Tm	Expected product size
cry4A, cry4B	Dip1A	5' CAAGCCGCAAATCTTGTGGA	60	800 bp
	Dip1B	5' ATGGCTTGTTTCGCTACATC	58	
cry4B	Dip2A	5' GGTGCTTCCTATTCTTTGG	56	1293 bp
	Dip2B	5' TGACCAGGTCCCTTGATTAC	60	
cry4	Dip2A	5' GGTGCTTCCTATTCTTTGG	56	2609 bp
	Dip1B	5' ATGGCTTGTTTCGCTACATC	58	

Gene	Primer pairs	Sequences	Tm	Expected product size
cyt1A	Cyt1A1	5' GTTGTAAGCTTATGGAAAAT	52	701 bp
	Cyt1A2	5' TTAGAAGCTTCCATTAATA	48	
cyt2	Cyt2-1	5' AATACATTTCAAGGAGCTA	50	471 bp
	Cyt2-2	5' TTTCATTTTAACTTCATATC	48	
cry11	Cry11-1	5' TTAGAAGATACGCCAGATCAAGC	66	304 bp
	Cry11-2	5' CATTTGTACTTGAAGTTGTAATCCC	68	
cry10	Cry10-1	5' ATATGAAATATTCAATGCTC	50	614 bp
	Cry10-2	5' ATAAATTCAAGTGCCAAGTA	50	
cyt1C	Cyt1C1	5' CAAAATCTACGGGAGCAAGG	60	1320 bp
	Cyt1C2	5' GGAAGGATCCCTTTGACTTTT	60	
cry2	Cry2-1	5`GTTATTCTTAATGCAGATGAATGGG	68	701 bp
	Cry2-2	5`CGGATAAAATAATCTGGGAAATAGT	66	
p19	P19-1	5’GCAGGAGGAACATCACCATT	60	291 bp
	P19-2	5’GGATTTGCTGAGCAGGTCAT	60	
p20	P20-1	5’TGACGAGGAAACAGAGTATACGA	66	704 bp
	P20-2	5’TGAAAGGTTAAACGTTCCGATT	60	
cry2A	Cry2A-1	5' ACTATTTGTGATGCGTATAATGTA	62	600 bp
	Cry2A-2	5' ACTATTTGTGATGCGTATAATGTA	52	
cry2	KNCry2F	5’ATGAATAATGTATTGAATAGTGGAAGAA	70	1902 bp
	KNCry2R	5’TTAATAAAGTGGTGGAAGATTAGTTGG	72	

Gene	Primer pairs	Sequences	Tm	Expected product size
ORF2	KNORF2-1	5'TGCTAAAATATCATTTTCC	50	758 bp
	KNORF2-2	5'CTACTTACAACCACAATCAC	56	

Screening of Non-Bti strains

Among the Non-*Bti* strains, all the Non- *Bti* failed to amplify any of the *Bti* δ-endotoxins and accessory proteins coding genes. Interestingly, one Non *Bti* strains *QBT*674 gave the expected amplification for primers specific to *cry2* gene. The *Bacillus thuringiensis kurstaki HD1* (*Btk* HD1) producing Dipteran specific Cry2 protein was used as reference. *QBT*674 was further analysed as this was a novel finding.

*QBT*674 is a spherical crystal forming Non-*Bti* strain. It has an undulated surface on its crystal (figure 20). When compared to the references *Bti* and *Btk*, it has protein (figure 21a) and plasmid profiles which were very different (figure 21b). The primers used for amplifying the whole gene of *cry2* was used for PCR amplifications. *QBT*674 and reference strain HD1 gave the correct expected amplification. The *ORF2* gene associated with *cry2* function was analysed with the primers amplifying the complete gene of *ORF2*. Reference strain HD1 gave the correct expected amplification but not in case of QBT674.

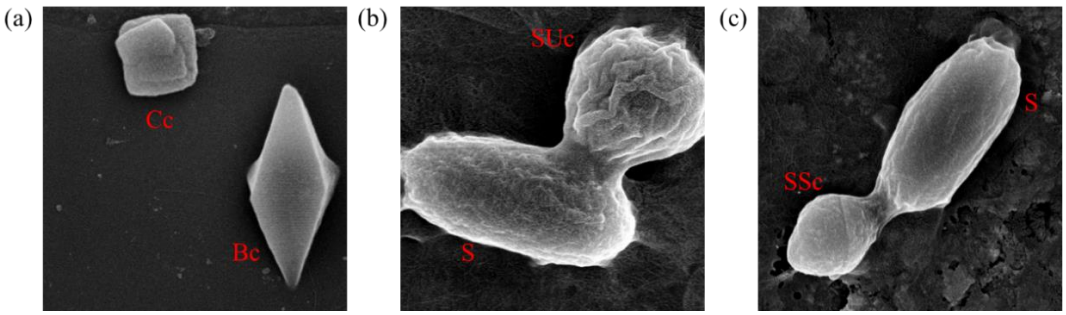

Figure 20. Micrographs of crystals and spores produced by Qatari *Bt* strain *QBT*674 [SEM images of crystal morphology of (a): *Btk* HD1; (b): *QBT*674; (c): *Bti* H14; Cc: cuboidal; Bc: bipyramidal; SUc: spherical undulated; SSc: Spherical smooth; S: spore]

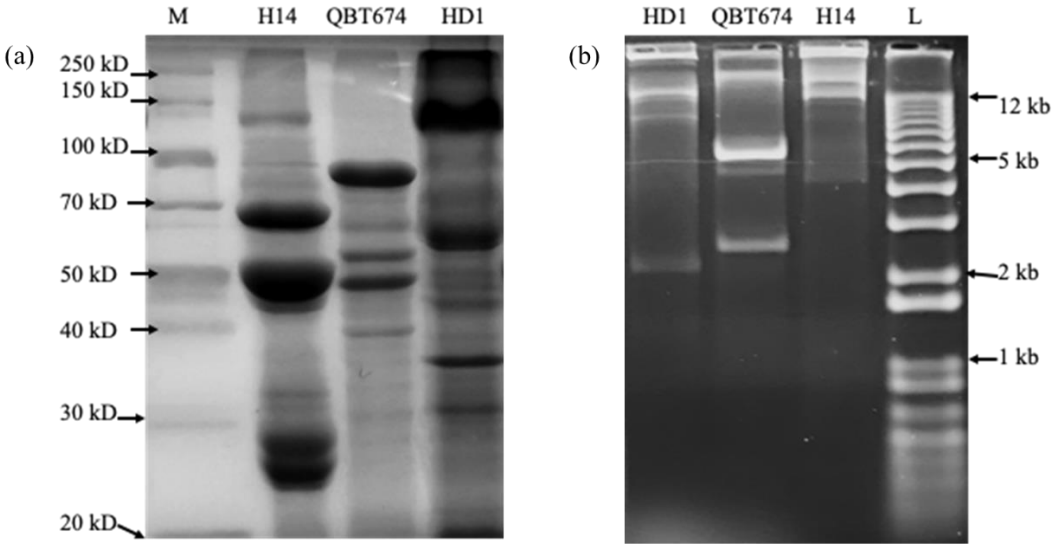

Figure 21. Comparison between *QBT*674 and reference strains on molecular level [(a): protein pattern formed by SDS-PAGE of parasporal crystals; (b): plasmid pattern on agarose gel; M: broad range protein marker; L: 1 kb plus linear DNA marker; H14: reference strain *Bti* H14; HD1: reference strain *Btk* HD1; *QBT*674: Qatari Non *Bti* strains *QBT*674]

Table 14. Identification of *Bt* strains *cry* genes by PCR

Genes	***cry4***	***cry2***	***cry11***	***cry10***	***cyt1A***	***cyt1C***	***cyt2A***	***p19***	***p20***
				***Bti* Strains**					
H14	✓	✗	✓	✓	✓	✓	✓	✓	✓
HD1	✗	✓	✗	✗	✗	✗	✗	✗	✗
4Q7	✗	✗	✗	✗	✗	✗	✗	✗	✗
***QBT*205**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*213**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*214**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*215**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*216**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*217**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*218**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*220**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*221**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*222**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*223**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*224**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*225**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*226**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*227**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*228**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*229**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*230**	✓	✗	✓	✗	✓	✗	✓	✓	✓
***QBT*608**	✓	✗	✓	✗	✓	✗	✓	✓	✓
				Non *Bti* strains					
***QBT*3**	✗	✗	✗	✗	✗	✗	✗	✗	✗
***QBT*6**	✗	✗	✗	✗	✗	✗	✗	✗	✗
***QBT*34**	✗	✗	✗	✗	✗	✗	✗	✗	✗
***QBT*39**	✗	✗	✗	✗	✗	✗	✗	✗	✗
***QBT*41**	✗	✗	✗	✗	✗	✗	✗	✗	✗
***QBT*43**	✗	✗	✗	✗	✗	✗	✗	✗	✗
***QBT*56**	✗	✗	✗	✗	✗	✗	✗	✗	✗

Genes	*cry4*	*cry2*	*cry11*	*cry10*	*cyt1A*	*cyt1C*	*cyt2A*	*p19*	*p20*
***QBT*81**	✕	✕	✕	✕	✕	✕	✕	✕	✕
***QBT*99**	✕	✕	✕	✕	✕	✕	✕	✕	✕
***QBT*212**	✕	✕	✕	✕	✕	✕	✕	✕	✕
***QBT*240**	✕	✕	✕	✕	✕	✕	✕	✕	✕
***QBT*320**	✕	✕	✕	✕	✕	✕	✕	✕	✕
***QBT*418**	✕	✕	✕	✕	✕	✕	✕	✕	✕
***QBT*555**	✕	✕	✕	✕	✕	✕	✕	✕	✕
***QBT*674**	✕	✓	✕	✕	✕	✕	✕	✕	✕

B. Prediction of δ- endotoxin proteins by protein profiling

The spectral data for each sample was obtained from the nano-liquid chromatography coupled to tandem mass spectrometer. The protein identification was done from the spectral data using the software Spectrum Mill. Checking against the UniProt database for *Bti*, all the *Bti* proteins that Non *Bti* strains could have, were identified. The list of proteins identified for each of the strains tested are attached in Appendix. The relevant proteins identified are listed in the **table 15** for the reference strains H14. Among the Non *Bti* strains tested, *QBT*555 showed the presence of *Bti* proteins.

*QBT*555 is a spherical crystal producing Non *Bti* strain isolated from Qatar. It does not have a smooth spherical crystal like the reference *Bti* H14. Although spherical, the surface of the crystal is undulated (figure 22). Compared to H14, it has different proteins and plasmid pattern (Chapter 4.1). The PCR amplifications for the *Bti* strains gave negative results (**table 14**).

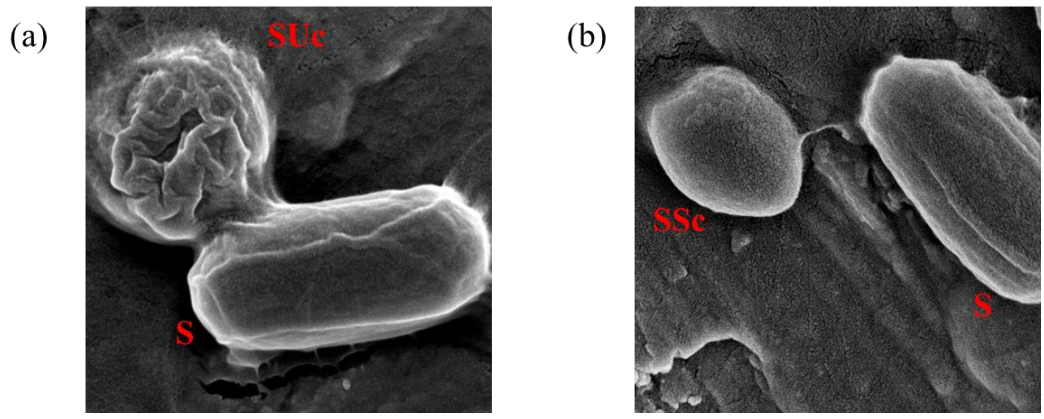

Figure 22. Micrographs of crystals and spores produced by Qatari *Bt* strain *QBT*555 [Scanning electron microscopy images showing crystal morphology of (a): crystal and spore of *QBT*555; (b): crystal and spore of reference strain *Bti* H14; SUc: spherical undulated crystal; SSc: spherical smooth crystal; S: spore]

The protein profile for the crystals of *QBT*555 showed many common proteins when compared to the database for reference strains H14 on UniProt. Most of the proteins in the list of identified protein entries included the sporulation associated proteins and enzymes like Spore coat protein GerQ, Stage 0 sporulation protein A, etc. When screened for insecticidal proteins, it was found that *QBT*555 had Cry11A protein. Among the peptides inserted to mass spectrometer, 25 peptides matched with Cry11 protein from the database with 16 peptides that were unique to Cry11A protein. It showed a high coverage of 28% to 31%. As control, the reference strains H14 was also run along with *QBT*555. H14 samples gave 56 peptides matching Cry11A protein with 35 unique peptides. H14 sample gave a coverage of 55% to 56%. In case of Cyt1A protein, H14 had 10 unique peptides out of 45 that matched, giving a coverage of 74%. On the other hand, *QBT*555 had six unique peptides out of 11, giving a coverage of 55%. Cry10Aa is another protein that was identified in *QBT*555 with three unique

peptides out of three and a coverage of 6%. H14 sample had 11 unique peptides out of 13 and a coverage of 21% for Cry10Aa protein. Apart from these insecticidal proteins, an accessory protein was also identified in *QBT*555: *pBt*152. *QBT*555 had one unique peptide out of just one peptide that matched this protein with a coverage of 3% (**table** 15). On the other hand, H14 had 8 unique peptides out of 9 that matched and a coverage of 25% (**table** 14).

Table 15. List of identified proteins from Protein profile data for reference strain H14 [No. Spectra: Number of spectra for peptides that matched with the protein; No. Unique peptides: Number of unique peptides out of all the spectra that matched with the protein; Percent coverage: The percentage of the amino acids of the whole protein that matched with all the peptides combined; Accession number: the ID for the protein from the database on UniProt; Identified protein: the list of relevant proteins that were screened]

No. Spectra	**No. Unique Peptides**	**Percent Coverage**	**Accession number**	**Identified protein**
42	25	55.5	P21256	Cry11Aa
14	10	56.3	A0A141DWM3	Cry11
45	10	74.2	P0A382	Cyt1Aa
13	11	21.7	P09662	Cry10Aa
9	8	25.2	Q8KNP4	*pBt*152
19	16	18.1	P05519	Cry4Ba
19	16	16.8	Q1RN84	Cry4BLB
4	3	24.3	O30895	Cyt2B
6	6	10	Q8KNV1	Cyt1C

Table 16. List of identified proteins from Protein profile data for Qatari strains *QBT*555 [No. Spectra: Number of spectra for peptides that matched with the protein; No. Unique peptides: Number of unique peptides out of all the spectra that matched with the protein; Percent coverage: The percentage of the amino acids of the whole protein that matched with all the peptides combined; Accession number: the ID for the protein from the database on UniProt; Identified protein: the list of relevant proteins that were screened]

No. Spectra	No. Unique Peptides	Percent Coverage	Accession number	Identified protein
18	11	28.6	P21256	Cry11Aa
7	5	31.7	A0A141DWM3	Cry11
11	6	55.4	P0A382	Cyt1Aa
3	3	6.6	P09662	Cry10Aa
1	1	2.9	Q8KNP4	pBt152

4.3.3. Discussion

The screening for δ endotoxin proteins and accessory proteins were conducted using two different techniques: PCR amplifications of the encoding genes and protein profiling of the parasporal crystals by LC/MS. Different primers that can amplify part or whole of the genes were used to screen the *Bt* strains for the presence of the encoding genes. Among the Qatari *Bti* strains, all the important Dipteran specific δ endotoxin coding genes were present including the *cry4A/4B*, *cry11*, *cyt1A* and *cyt2B.* They also showed the presence of accessory protein coding genes like *p19* and *p20*. Despite resembling the reference strain in every way, Qatari *Bti* strains lacked the *cry10* and

cyt1C genes. This showed the possible gene instability among the *Bti* strains from Qatar. This instability was studied further, and the details are described in Chapter 4.6. Among the Non *Bti* strains, PCR screenings showed that they probably do not have the Dipteran specific insecticidal protein encoding genes. This was expected as the Non *Bti* groups of strains were very different compared to the reference H14, in every aspect tested. But, it was interesting to see that one Non *Bti* strain *QBT*674 gave expected amplification of *cry2* gene. Cry2 protein is expressed by *Btk* strains that produce cuboidal crystals along with bipyramidal crystals during sporulation. Cry2 protein, that has insecticidal activity against Dipteran insects, is known to be responsible for the crystallization of itself and other proteins into cuboidal shape. Cry2 protein is part of an operon of three ORFs and the protein itself is encoded by the ORF3. The ORF2 of this operon encodes for a protein that is essential for the cuboidal crystal formation. When checked for the presence of this ORF2 in *QBT*674, it gave negative results. In summary, *QBT*674 has the ORF3 gene encoding Cry2 protein but not ORF2 encoding the regulatory protein for cuboidal crystal formation. Because of this, *QBT*674 produces only spherical crystals with undulated surface, but no cuboidal crystal. This is the first report of a spherical crystal producing strain to show the presence of *cry2* gene.

Apart from *QBT*674, the possible presence of any δ endotoxins could not be detected in any other Non *Bti* strains. Hence, a new approach was tested in the form of LC/MS protein profiling. In these screenings, it was found that a Non *Bti* strain *QBT*555, with all the characteristics very different than H14, showed the presence of *Bti* δ endotoxin proteins and accessory proteins. To evaluate the data for *QBT*555, the reference H14 was also tested and compared to its own database of proteins on UniProt. In this technique, the parasporal crystals were solubilized and digested with Trypsin to get many peptides of all the proteins that are present in the crystals. The peptides were

separated by size and then charge, and they were sent into mass spectrometer. Each peptide was given a spectrum by the spectrometer. At the end, each sample for each strain had data in the form of 1000s of spectra. These spectra were then compared to the database of spectra of the proteins from the reference strain *Bti* on UniProt website. The software Spectrum Mill then gave out the data in the form of excel sheets where the list of protein identified were given. Along with the list, it showed the number of peptides from the samples that matched a protein of *Bti* and the percentage of the amino acid sequence of the identified protein that was covered by the amino acids from the peptides. For *QBT*555, three δ- endotoxins and one accessory protein were identified: Cry11, Cry10, Cyt1A and pBt152.

The absence of Cry4A/4B protein could explain its lack of insecticidal activity against Dipteran larvae (Chapter 4.2). Cry4A/4B is one of the biggest and most toxic δ-endotoxins that act against the Dipteran larvae. Also, Cry4A/4B is responsible for the crystallization of itself and other proteins into spherical shape. Possible absence of this protein could mean that *QBT*555 possesses some other crystallization protein that forms the spherical crystal.

pBt152 is a hemagglutinin protein that is also essential for the spherical crystal formation. Only the Cry4A/4B protein is not enough to form the spherical crystals among the *Bti* strains. It can be postulated that the accessory protein pBt152 is present to help the other crystallization protein that *QBT*555 possesses.

4.3.4. Conclusion

The Qatari *Bt* strains producing spherical crystals were screened for the Dipteran specific δ endotoxin proteins and the associated accessory proteins. It was found that the Qatari *Bti* strains have the essential insecticidal proteins and accessory protein, except Cry10 and Cyt1C. The absence of these proteins in all the Qatari *Bti* strains shows that this a signature plasmid instability among the Qatari *Bti* strains. Among the

Non *Bti* strains, a novel *Bt* strain *QBT*674 was found that produces spherical crystals only during the sporulation stage but does have the *cry2* gene and the regulatory gene ORF2 that is necessary for the formation of cuboidal crystal. As no other Dipteran specific δ- endotoxins could be detected by PCR screenings, protein profiling was conducted for the Non *Bti* strains. It was found that *QBT*555, which did not give any amplifications for the PCRs used to screen all the *Bti* δ- endotoxin genes, showed the presence of the *Bti* δ- endotoxins and accessory proteins in its parasporal crystals.

CHAPTER 4.4 RESULTS: INVESTIGATION OF HAEMOLYTIC & CYTOLYTIC ACTIVITY OF *BT* STRAINS

4.4.1. Introduction

Apart from its insecticidal activities, *Bt* is also known for its cytolytic activity; which is explored in this part of the study. The 19 *Bti* strains and the 14 Non *Bti* representatives were tested for their cytolytic and hemolytic activities along with the references H14 and 4Q7. This part of the study had three sections. In the first section, all the strains were tested for their hemolytic activity by analyzing their ability to break down red blood cells (RBC) on Blood agar. The hemolytic activity was evaluated based on the zone of clearance seen around the colony of the *Bt* strain due to the breakdown of RBCs. In the second section, all the strains were tested for their cytolytic activity by MTT assay against cancer cell line. The MTT assay was carried out for each strain at five different concentrations of their parasporal crystal proteins. Each test was conducted thrice for 24 hours, 48 hours and 72 hours, each. The anticancer property is attributed to Parasporins and/or Cyt1A proteins. Hence, the strains showing anticancer property were analyzed further to check the presence of *parasporin* genes and *cyt1A* gene and encoded proteins. In the third section, the strain showing the highest anticancer activity was studied intrinsically to understand the molecular and biochemical reasons for the higher activity. Along with the gene sequence, amino acid sequences were compared and then a 3D model was prepared using bioinformatic tools. The different protein moieties from the model were studied and their possible function in cytolytic activity was deduced based on the understanding from published research works. The results of this chapter have been published as part of two research articles: Nair *et al*., 2018(a) and Nair *et al*., 2018(b). Articles are attached as Appendix A and Appendix B.

4.4.2. Results

A. Hemolytic activity of *Bt* strains

When the strains were grown on Blood agar overnight, three different types of hemolytic activities were observed among the local strains. Some strains showed no hemolytic activity (**figure** 23c), while the others showed slight (**figure** 23b) or high hemolytic activity (**figure** 23a). All the Qatari *Bti* strains had high hemolytic activity along with three Non *Bti* strain representatives, viz., *QBT*6, *QBT*81 and *QBT*99. Among the other Non *Bti* strains, *QBT*43, *QBT*240, *QBT*320 and *QBT*418 showed slight hemolytic activities around their respective colonies. Other Non *Bti* strain representatives like *QBT*212, *QBT*555, *QBT*3, *QBT*34, *QBT*39, *QBT*41 and *QBT*56 showed no hemolytic activities (**table 17**).

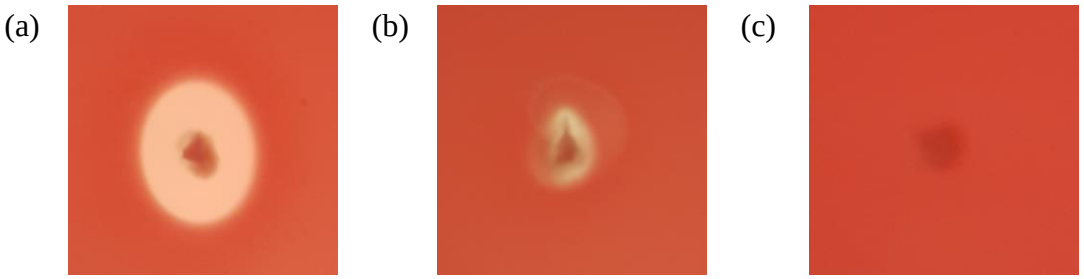

Figure 23. Types of hemolysis observed among the *Bt* strains studied for the hemolytic activity

[a: The zone of clearance observed around the highly hemolytic *Bt* strains; b: the slight zone of clearance observed around the *Bt* strains with lower hemolytic activities; c: the absence of any zone of clearance observed around the *Bt* strains with no hemolytic activities]

Table 17. Hemolytic activities of *Bti* strains, Non *Bti* representative strains and the reference strain H14

[++: indicates the high hemolytic activities (figure 23a); +: indicates the slight hemolytic activities (figure 23b); -: the absence of hemolytic activities (figure 23c)]

***Bti* strains**		**Non-*Bti* strains**	
Strains	**Hemolytic activity**	**Strains**	**Hemolytic activity**
***QBT*205**	++	***QBT*3**	-
***QBT*213**	++	***QBT*6**	++
***QBT*214**	++	***QBT*34**	-
***QBT*215**	++	***QBT*39**	-
***QBT*216**	++	***QBT*41**	-
***QBT*217**	++	***QBT*43**	+
***QBT*218**	++	***QBT*56**	-
***QBT*220**	++	***QBT*81**	++
***QBT*221**	++	***QBT*99**	++
***QBT*222**	++	***QBT*212**	-
***QBT*223**	++	***QBT*240**	+
***QBT*224**	++	***QBT*320**	+
***QBT*225**	++	***QBT*418**	+
***QBT*226**	++	***QBT*555**	-
***QBT*227**	++	**Reference**	
***QBT*228**	++	**Strains**	**Hemolytic activity**
***QBT*229**	++	**H14**	++
***QBT*230**	++		
***QBT*608**	++		

B. Cytolytic activity of *Bt* strains

The MTT assay for each of the strains was carried out in triplicate for 24 hours, 48 hours and 72 hours, each (results for 72 hours listed in **table 18**, full results attached as Appendix E). The cytotoxic activity of the strains was analyzed for anticancer activity for two of the properties: (1) the ability of the lung cancer cell lines to recover after the treatment for 24 hrs, 48 hrs and 72 hrs, and (2) the smallest concentration of the *Bt* δ-endotoxins that can inhibit the growth of maximum cancer cells. Although, the actual cytolytic assay for *Bt* is carried out only for 72 hours, in this study, 24 hours and 48 hours were also included to understand the recovery of the growing cancer cells after treatment. Among the *Bti* strains, when analyzed for cytolytic activities, four strains *QBT*205, *QBT*216, *QBT*229 and *QBT*230 showed the highest activities (**figure 24**). In other words, even after 72 hours of treatment, the number of viable cells kept on declining without recovery. Seven other *Bti* strains *QBT*213, *QBT*221, *QBT*222, *QBT*223, *QBT*224, *QBT*225 and *QBT*608 showed cytotoxic activities till 48 hours and then recovered by 72 hours (**figure 25**). The reference strain H14 and eight other *Bti* strains including *QBT*214, *QBT*215, *QBT*217, *QBT*218, *QBT*220, *QBT*226, *QBT*227 and *QBT*228 showed cytotoxic activities only for 24 hours and recovered by 48 hours (**figure 26**).

On the other hand, only two of the *Bti* strains could inhibit the growth of more than 50% of the cancer cells. At the end of 72 hours of treatment, only 49.62% of the cancer cells survived when treated with 0.5 µg/ml of δ- endotoxins from *QBT*229 (**figure** 24c). In case of *QBT*230, only 40.75% cancer cell lines survived when treated with 0.5 µg/ml of δ- endotoxins (**figure** 25d). *QBT*229 showed higher cytotoxic activities as its δ-endotoxins could inhibit about 50% of the cancer cells even at the lowest concentration tested; 0.15 µg/ml (**figure** 24c). *QBT*230, on the other hand could inhibit 50% of the cancer cells only at a concentration of 0.35 µg/ml (**figure** 24d).

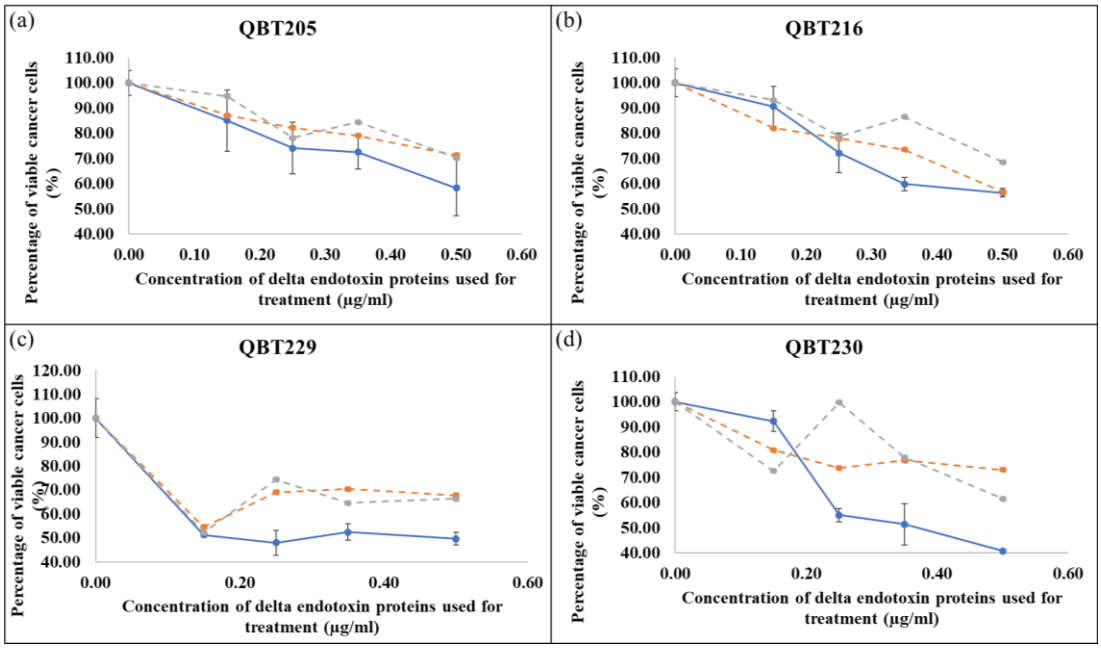

Figure 24. Cytolytic activities of *Bti* strains where lung cancer epithelial cell lines could not recover even after 72 hours of treatment

[the graph plotted with concentration of δ- endotoxins used for each treatment versus the percentage of viable cells quantified after treatment for 24 hours or 48 hours or 72 hours; blue lines indicate the cytolytic activities seen for each concentration tested after 72 hours, grey and orange dotted lines indicate the cytolytic activities of *Bt* strains after 24 and 48 hours respectively; error bars have been plotted for results obtained after 72 hours and indicate the standard errors]

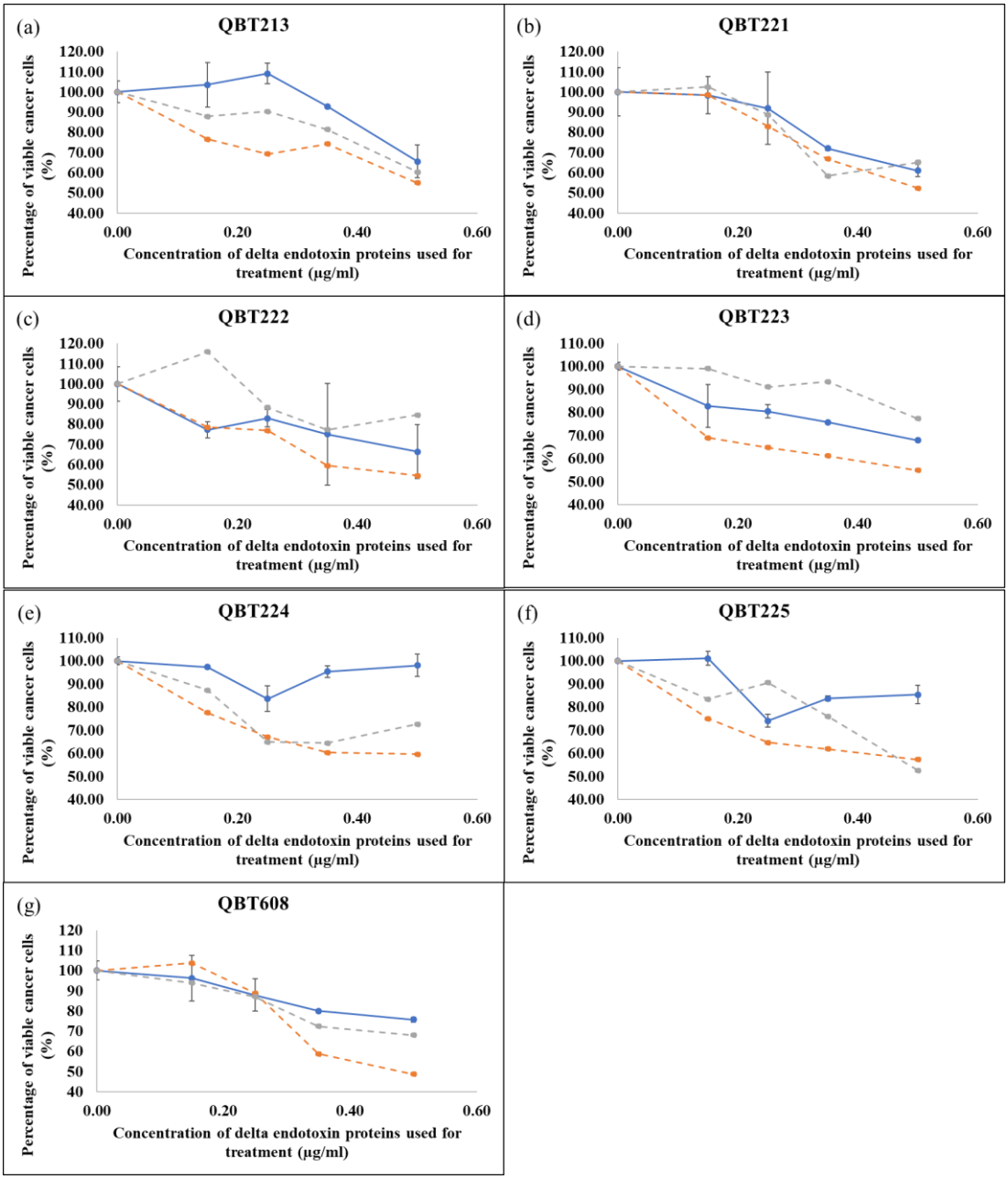

Figure 25. Cytolytic activities of *Bti* strains where lung cancer epithelial cells recovered after 48 hours of treatment

[the graph plotted with concentration of δ- endotoxins used for each treatment versus the percentage of viable cells quantified after treatment for 24 hours or 48 hours or 72 hours; blue lines indicate the cytolytic activities seen for each concentration tested after 72 hours, grey and orange dotted lines indicate the cytolytic activities of *Bt* strains after 24 and 48 hours respectively; error bars have been plotted for results obtained after 72 hours and indicate the standard errors]

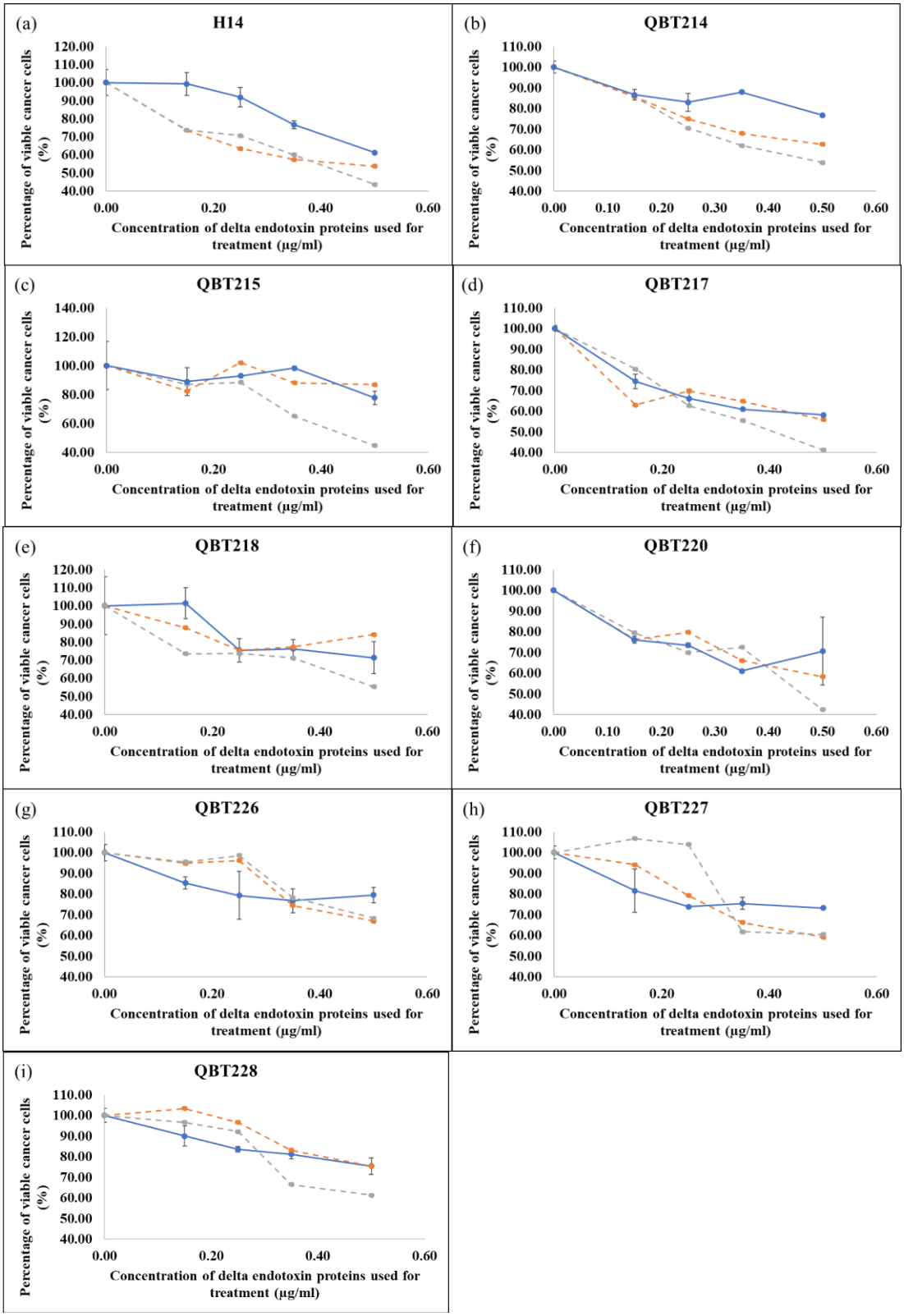

Figure 26. Cytolytic activities of reference H14 and *Bti* strains where lung cancer epithelial cells recovered after 24 hours of treatment

[the graph plotted with concentration of δ- endotoxins used for each treatment versus the percentage of viable cells quantified after treatment for 24 hours or 48 hours or 72

hours; blue lines indicate the cytolytic activities seen for each concentration tested after 72 hours, grey and orange dotted lines indicate the cytolytic activities of *Bt* strains after 24 and 48 hours respectively; error bars have been plotted for results obtained after 72 hours and indicate the standard errors]

Table 18. Cytotoxic activities shown by *Bti* strains and reference straisn *Bti* H14 after 72 hours

[Average of three percentage values of viable cells that were estimated for each *Bti* strain δ- endotoxin proteins at their five different concentrations; 0 µg/ml concentration represents untreated wells]

Strains	**Concentrations of δ- endotoxin proteins tested (µg/ml)**					
	0	**0.15**	**0.25**	**0.35**	**0.5**	
H14	100	99.33	91.89	76.73	61.28	**Percentage of viable cancer cells (%)**
***QBT*205**	100	85.03	74.07	72.46	58.27	
***QBT*213**	100	103.56	109.15	92.86	65.61	
***QBT*214**	100	86.56	82.98	87.91	76.64	
***QBT*215**	100	88.90	92.96	98.31	77.79	
***QBT*216**	100	90.58	72.15	59.81	56.34	
***QBT*217**	100	74.40	66.04	60.88	58.16	
***QBT*218**	100	101.37	75.44	76.27	71.39	
***QBT*220**	100	76.08	73.49	61.01	70.54	
***QBT*221**	100	98.38	91.92	71.98	61.04	
***QBT*222**	100	77.27	82.98	75.04	66.36	
***QBT*223**	100	82.72	80.49	75.74	67.96	

Strains	Concentrations of δ- endotoxin proteins tested (µg/ml)				
	0	**0.15**	**0.25**	**0.35**	**0.5**
***QBT*225**	100	101.07	74.09	83.73	85.32
***QBT*226**	100	85.28	79.27	76.64	79.47
***QBT*227**	100	81.51	73.76	75.38	73.20
***QBT*228**	100	89.95	83.39	81.21	75.34
***QBT*229**	100	51.30	47.98	52.44	49.62
***QBT*230**	100	92.25	54.98	51.43	40.75
***QBT*608**	100	96.209	87.878	80.028	75.747

Among the Non-*Bti* strains, none could inhibit more than 50% of the cancer cells even at the highest concentration tested for 24 hours, 48 hours or 72 hours (results for 72 hours listed in **table 19**, full results attached as Appendix). Among the Non *Bti* strains, *QBT*41 and *QBT*320 showed comparatively higher toxicity as the cancer cells could not recover after 72 hours of treatment (figure 27). In other words, the number of viable cancer cells kept on declining with time as the toxins were active even after 72 hours of treatment. Cancer cells recovered even after 24 hours when treated with δ-endotoxins from *QBT*6, *QBT*99, *QBT*212 and *QBT*555 (Figure 28). In case of *QBT*3, *QBT*34, *QBT*39, *QBT*43, *QBT*56, *QBT*81, *QBT*240 and *QBT*418, cancer cells recovered after 48 hours of treatment (figure 29).

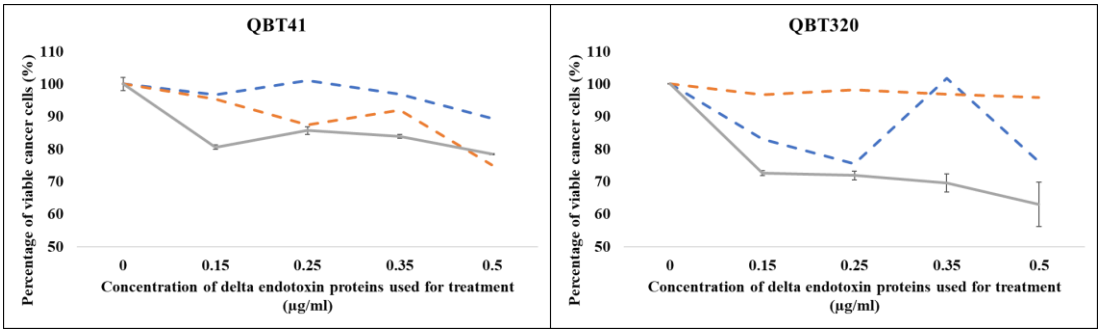

Figure 27. Cytolytic activities of Non *Bti* strains where lung cancer epithelial cells did not recover even after 72 hours of treatment

[the graph plotted with concentration of δ- endotoxins used for each treatment versus the percentage of viable cells quantified after treatment for 24 hours or 48 hours or 72 hours; grey lines indicate the cytolytic activities seen for each concentration tested after 72 hours, blue and orange dotted lines indicate the cytolytic activities of *Bt* strains after 24 and 48 hours respectively; error bars have been plotted for results obtained after 72 hours and indicate the standard errors]

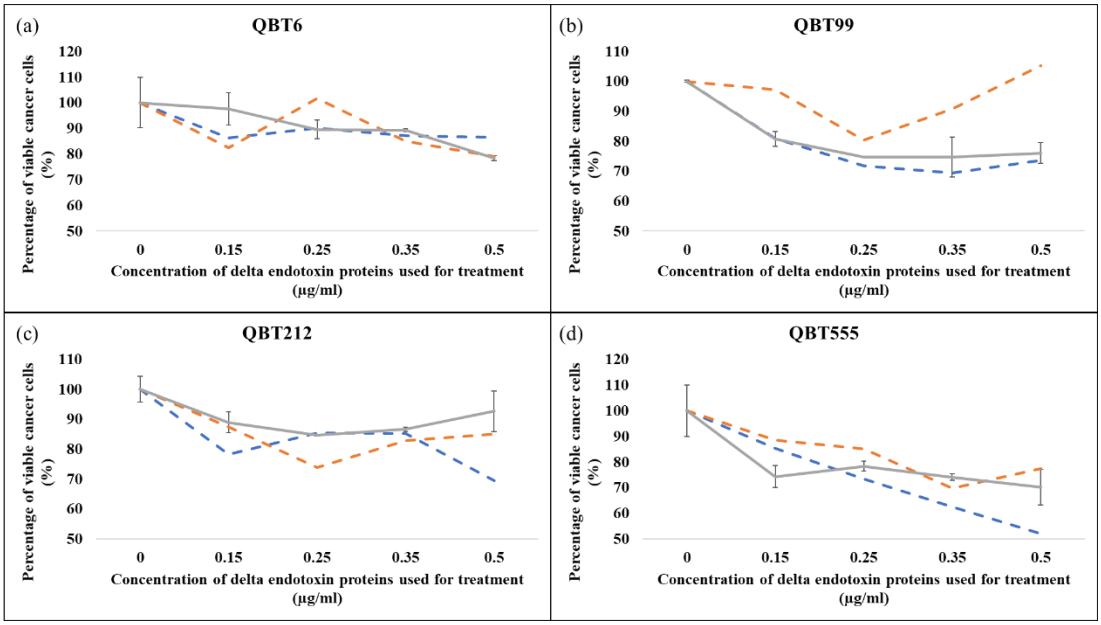

Figure 28. Cytolytic activities of Non *Bti* strains where lung cancer epithelial cells recovered after 24 hours of treatment

[the graph plotted with concentration of δ- endotoxins used for each treatment versus the percentage of viable cells quantified after treatment for 24 hours or 48 hours or 72 hours; grey lines indicate the cytolytic activities seen for each concentration tested after 72 hours, blue and orange dotted lines indicate the cytolytic activities of *Bt* strains after 24 and 48 hours respectively; error bars have been plotted for results o*Bt*ained after 72 hours and indicate the standard errors]

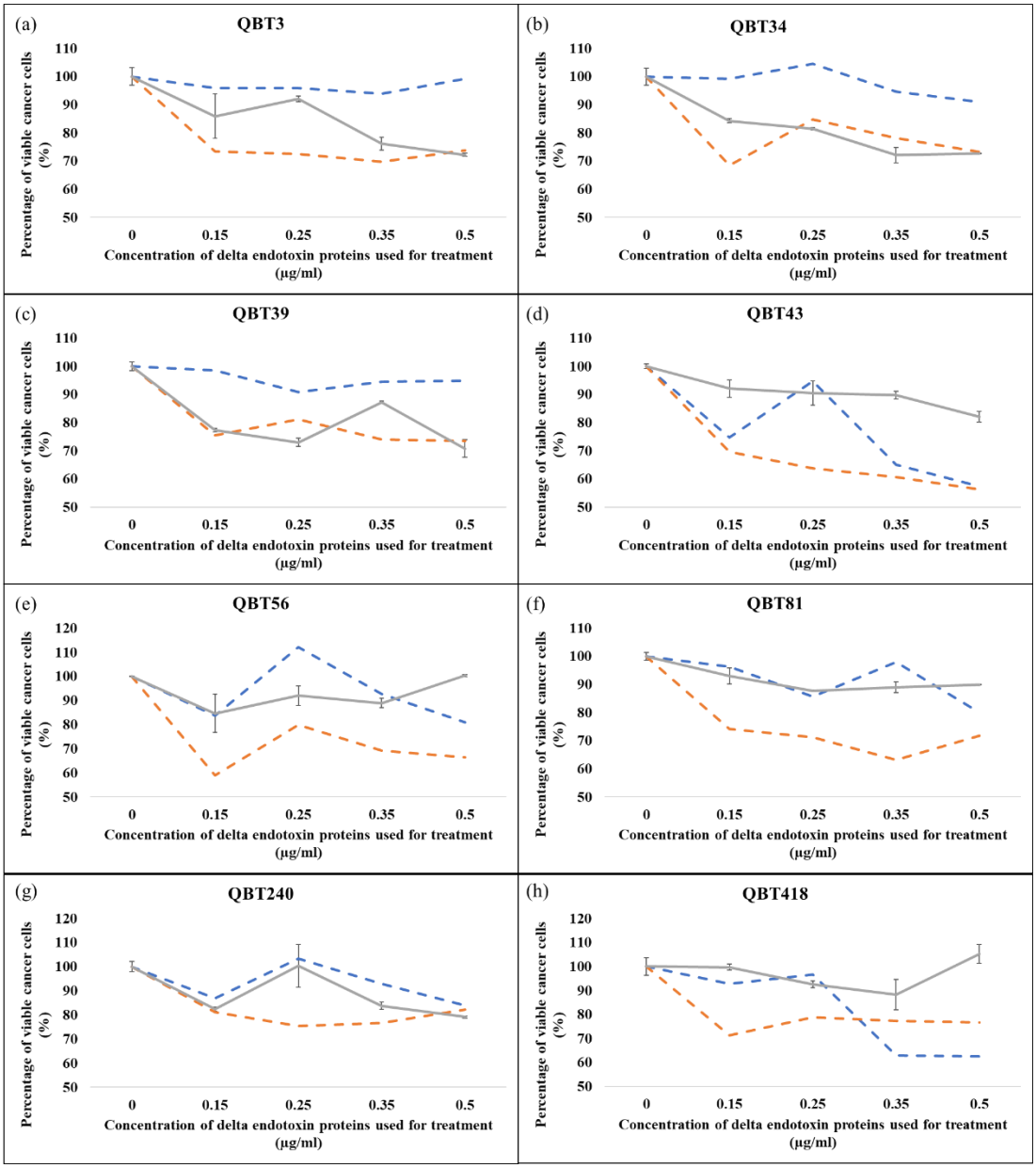

Figure 29. Cytolytic activities of reference H14 and *Bti* strains where lung cancer epithelial cells recovered after 48 hours of treatment

[the graph plotted with concentration of δ- endotoxins used for each treatment versus the percentage of viable cells quantified after treatment for 24 hours or 48 hours or 72 hours; grey lines indicate the cytolytic activities seen for each concentration tested after 72 hours, blue and orange dotted lines indicate the cytolytic activities of *Bt* strains after 24 and 48 hours respectively; error bars have been plotted for results obtained after 72 hours and indicate the standard errors]

Table 19. Cytotoxic activities shown by Non *Bti* representative strains after 72 hours [Average of three percentage values of viable cells that were estimated for each Non *Bti* strain δ- endotoxin proteins at their five different concentrations; 0 µg/ml concentration represents untreated wells]

Strains	Concentrations of δ- endotoxin proteins tested (µg/ml)				
	0	**0.15**	**0.25**	**0.35**	**0.5**
***QBT*3**	100	85.93	91.99	76.11	72.16
***QBT*6**	100	97.58	89.56	89.27	78.40
***QBT*34**	100	84.24	81.53	72.05	72.67
***QBT*39**	100	77.38	73.04	87.30	70.78
***QBT*41**	100	80.57	85.71	83.84	78.45
***QBT*43**	100	92.12	90.57	89.76	82.04
***QBT*56**	100	84.66	91.98	88.93	100.3
***QBT*81**	100	92.99	87.77	88.90	89.92
***QBT*99**	100	80.75	74.68	74.66	76.04
***QBT*212**	100	88.93	84.73	86.60	92.64
***QBT*240**	100	82.38	100.3	83.82	79.16
***QBT*320**	100	72.63	71.84	69.60	63.01
***QBT*418**	100	99.71	92.53	88.23	105.1
***QBT*555**	100	74.29	78.37	74.05	70.11

Percentage of viable cancer cells (%)

C. Parasporin genes of *Bt* strains

The PCR amplification experiments using primers specific to the Parasporin encoding genes gave negative results for all the *Bti* and Non *Bti* strains. It was deduced that the

cytotoxicity of the *Bti* strains cannot be attributed to known Parasporins. It was essential to check the Cyt1A protein.

D. Nucleotide sequence of *cyt1A* gene

To analyze the high cytotoxicity and anticancer activity shown by *QBT*229, primers were used to amplify the full-length gene of *cyt1A* by PCR. When sequenced, it was found that the nucleotide sequence of *cyt1A* gene from *QBT*229 had at least five nucleotide differences, when compared to that of *cyt1A* gene from the reference strain H14 (**figure 30**).

```
1    ATTGGATGTTAACATTGTAGCTCGCAGAATCTTGAATTGTGAAAAATAAAACTTGTTCCT
61   TTACTGCTGATACTTTAATTTCAAAACCAACTGGTACACAATACATAACGCCACCAGTTT
121  GGGCATTTTGGATTGCAAACAGGACATTGTATGTGTAATTTGTTTGATTAGCAGTTTCCT
181  TGCCCCAGAAAATCCATGCTTCATTTTTTTGAGTATTTAAATTTGTAAACGTATTTGTAA
241  CTGCTGCAGTTAATTGATCTATTACAGATCCACTTAATGCAACTCCTAATACAGTTTTTA
301  ACACTTCTAAGACTTTATTAATCATAACACTTACTTGGTTATTCTTTTGAGTTACATTTT
361  GATCAACATAACTCACTACAGCACCCATCGGTGTAATTGTGTTTGCGATTTCTAAACCTT
421  TTGCCATACTAAAGCGTAGGGCATCACCAAAATGTGTAGAAGTGGTAACTAATGTATTTT
481  GAAATGCATTTGCTAACATAATTGCTTGCAATATATAATTCGGATTATCAATTTCGTTAA
541  TAGAAAGAAGATTATTGATTTCATTTGGATCCTCAACACGTAATGTAATAACCCTTG
```

Figure 30. Nucelotide sequence of *cyt1A* full gene

[Submitted to NCBI database with accession number: MG708177]

E. Protein sequence of Cyt1A protein

The nucleotide sequence of *cyt1A* gene from *QBT*229 was translated to amino acid sequence using the ExPasy tool available on Swiss Institute of Bioinformatics website. When compared to the amino acid sequence of Cyt1A protein of the reference strain H14, five consecutive amino acid replacements were observed (**figure 31**). From the amino acid positions 225 to 229, Lysine (K) was replaced by Asparagine (N), Phenylalanine (F) by Leucine (L), Alanine (A) by Histidine (H), Glutamine (Q) by Asparagine (N) and Proline (P) by another Histidine (H).

Figure 31. Amino acid sequence alignment of Cyt1A protein from *QBT*229 and reference *Bti* (Tron.3)

[Amino acid sequence of *QBT*229 Cyt1A protein aligned with the amino acid sequence of Cyt1A protein from the reference *Bti* H14; Tron.3 is the name of the reference protein submitted to SWISS model software; red box indicates the five amino acid replacements seen in Cyt1A protein of *QBT*229 compared to H14; each set of underlined amino acids form alpha, beta or nu sheets of the protein as indicated below the underline; amino acids in blue indicate positive charge and the ones in red indicate negative charge]

F. 3D protein modeling of Cyt1A protein

A 3D model of the Cyt1A protein was drawn from the amino acid sequence using the software SWISS MODEL. Its structural homology was compared with that of the reference H14. No structural differences were noted among the two (**figure 32**). *In silico,* it was predicted that the amino acid replacements were not enough to bring about

any structural differences in the Cyt1A protein but might have changed the chemical property of the region.

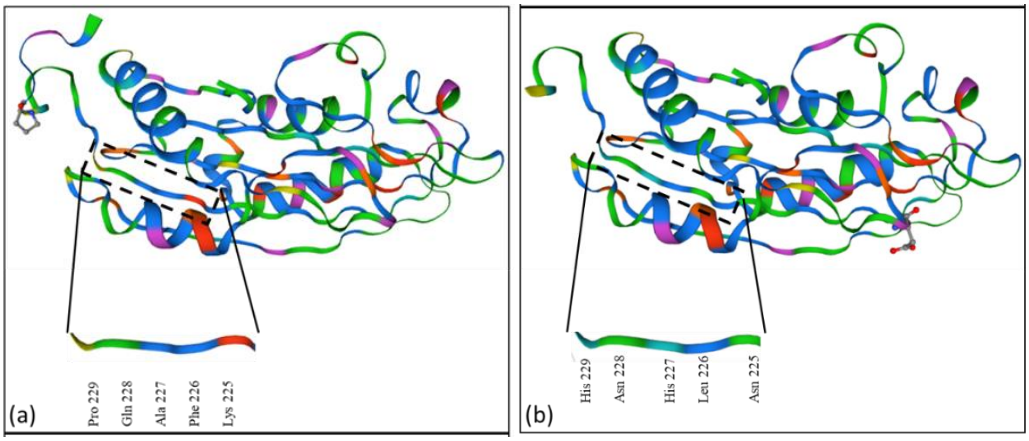

Figure 32. *In silico* 3D model of the Cyt1A protein and the region with amino acid replacements

[a: 3D model of the Cyt1A protein of reference strain H14; b: 3D model of the Cyt1A protein of local strain *QBT*229; the dotted black box indicates the section of β sheet where the amino acids have been replaced; the amino acids in this section are indicated below the section in each model]

G. Chemical homology of the novel Cyt1A protein

The chemical homology of the β8 sheet of the Cyt1A proteins from *QBT*229 and reference strain H14 was analyzed and compared. It was found that the β8 of *QBT*229 had four polar amino acids between position 225 and 229 instead of just one polar amino acid in the respective region of Cyt1A protein from the reference H14. Also, the same region of Cyt1A protein of *QBT*229 had lost two hydrophobic amino acids that were present in case of H14 (table 20). Biochemically, this β8 sheet of *QBT*229 had

become much more positively charged than that of the reference strain H14 (**figure 33**).

Table 20. Amino acids replacements observed in *QBT*229 and the consequent biochemical changes observed

[charge, polarity and hydrophobicity of each amino acid are indicated below them]

Amino Acid Positions	***Bt* subsp. israelensis H14**	**Qatari *Bt* subsp. israelensis *QBT*229**
225	Lysine (+) (Charged)	Asparagine (+) (Polar)
226	Phenylalanine (Hydrophobic)	Leucine (Hydrophobic)
227	Alanine (Hydrophobic)	Histidine (+) (Polar)
228	Glutamine (Polar)	Asparagine (+) (Polar)
229	Proline (Hydrophobic)	Histidine (+) (Polar)

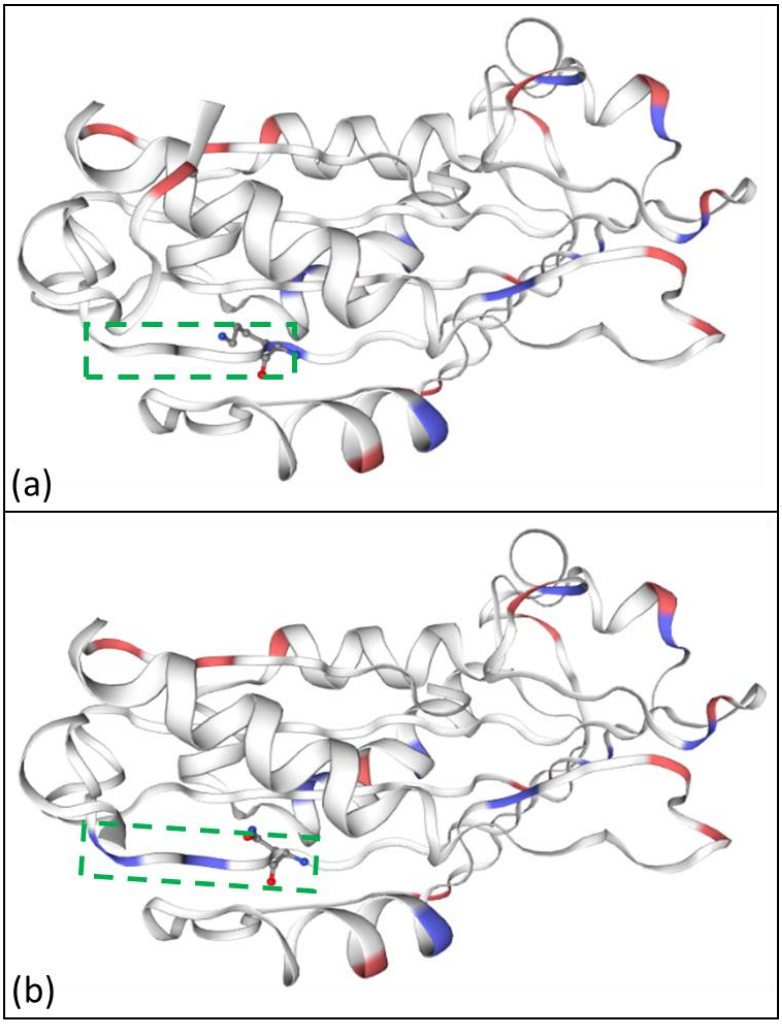

Figure 33. Chemical changes in the β-sheet of Cyt1A protein of *QBT*229 due to amino acid replacements

[green dotted box indicates the region of β sheet with the amino acid replacements; the blue color indicates positively charged regions and red color indicates negatively charged regions of the Cyt1A protein]

4.4.3. Discussion

In this part of the study, hemolytic and cytolytic activities of the *Bt* strains were studied. Analyzing the hemolytic activity, it was found that all the *Bti* strains have high hemolytic activities. In fact they gave big and clear zones around their colonies in Blood

agar; showing high breakdown of red blood cells (table 17). It was reported that *Bt* strains that have insecticidal activity and hemolytic activity do not possess anticancer cytolytic properties (Ohba *et al*., 2009). So, the *Bti* strains with high insecticidal (Chapter 4.2) and hemolytic activities, were expected to have almost no cytolytic activities. On the other hand, the Non-*Bti* representative strains showed varying degrees of hemolytic activities (table 17). Three strains *QBT*6, *QBT*81 and *QBT*99 showed similar degrees of hemolytic activities as the *Bti* strains. Like *Bti*, they were expected to have no cytolytic activities. *QBT*43, *QBT*240, *QBT*320 and *QBT*418 showed slightly less hemolytic activity. Other Non *Bti* representative strains showed no hemolytic activity at all. This last group of strains including *QBT*3, *QBT*34, *QBT*39, *QBT*41, *QBT*56, *QBT*212 and *QBT*555 with no hemolytic activity and no insecticidal activity (Chapter 4.2) were expected to have cytolytic activities.

All the *Bti* strains and the Non *Bti* representative strains were tested for their cytolytic anti-cancer activities against lung cancer epithelial cells. Among the *Bti* strains, *QBT*205, *QBT*216, *QBT*229 and *QBT*230 were found to have comparatively high cytolytic activities (figure 24). Among the Non *Bti* strains, *QBT*41 and *QBT*320 had comparatively high cytolytic activities (figure 27). They continued to inhibit the growth of cancer cells even after 72 hours of treatment. Considering the hemolytic activities, cytolytic activities and insecticidal activities, the hemolytic insecticidal *Bti* strains were expected to have no cytolytic activities. In this study, novel *Bti* strains (*QBT*205, *QBT*216, *QBT*229 and *QBT*230) were identified as having all the three properties. The Non *Bti* representative *QBT*41 was expected to have cytolytic properties (figure 27a) as it had neither hemolytic activities nor insecticidal activities against at least Dipteran insects. This strain would be a good candidate for anticancer trials.

Among the *Bt* strains tested, *QBT*229 showed the lowest LC_{50} as it could kill about 50%

of the cancer cells after 72 hours (figure 24 c) at the lowest concentration tested (0.15 µg/ml). On molecular level when compared to the reference H14, *QBT*229 had similar plasmid content, similar δ- endotoxin protein profile and showed the presence of important δ- endotoxin coding genes like *cry4* and *cyt1A* but lacked the genes *cry10* and *cyt1C* (Chapter 4.1 & 4.3). But, it had much higher cytolytic activities than H14. The anticancer parasporin encoding genes were absent in both *QBT*229 and H14. The other protein responsible for cytolytic activity is Cyt1A. The full gene nucleotide sequence was compared and there were at least five nucleotide differences among the *cyt1A* gene of *QBT*229 and H14. The translated amino acid sequences showed that there were five consecutive amino acid replacements in *QBT*229 on the β8 sheet (figure 31). A 3D model was drawn *in silico* using the amino acid sequences and structural homologies were analyzed. No structural differences were observed (figure 32). The chemical changes in this region was studied and it was found that the amino acid replacements in the β8 sheet of Cyt1A protein from *QBT*229 makes the β8 sheet more polar and positively charged; compared to that of H14 (figure 33). The β8 sheet of Cyt1A protein has two known functions: (1) interaction with the membrane to form pores to lyse the cell and (2) synergizing with other δ- endotoxins like Cry11.

The increased polarity in the β8 sheet of Cyt1A protein from *QBT*229 enhances its cytolytic activities in terms of both its functions. The overall increase in polarity enhances its ability to interact with the polar heads of the lipid molecules in the cell membrane. The increased interaction between Cyt1A protein and the cell membrane will in turn increase its pore forming and consequent cell lysis abilities.

On the other hand, the synergism between Cyt1A protein and Cry11 protein depends on the interaction between following amino acids: Lysine (+), Glutamate (+) and Lysine (+) at the positions 198, 204 and 225 of the Cyt1A protein and Serine (polar) and

Glutamate (+) at position 259 and 266 of Cry11 protein. Cry11 protein requires specific receptors for its insecticidal activities; which are absent on cancer cell lines. Hence, Cry11 protein doesn't affect the cancer cell lines unless there is Cyt1A protein to synergize the Cry11 proteins and make it bind to the membrane (Perez *et al*., 2005). The replacement of Lysine at position 225 of β8 sheet of Cyt1A protein from *QBT*229 with an Asparagine molecule makes it more polar and hence increases its interaction with the polar Serine molecule at position 266 of Cry11 molecule. To summarize, the five amino acids that are replaced in the β8 sheet of Cyt1A protein from *QBT*229 enhances its ability to form pores in the cell membrane and its ability to recruit Cry11 protein to the membrane. Overall, this novel Cyt1A protein of *QBT*229 gives it a much higher anti-cancer property towards lung cancer epithelial cell lines.

4.4.4. Conclusion

Among the *Bt* strains studied in this project, all *Bti* strains are highly hemolytic strains like the reference strain H14. Among the Non-*Bti* representative strains, *QBT*6, *QBT*81 and *QBT*99 were also highly hemolytic, *QBT*43, *QBT*240 and *QBT*320 were slightly hemolytic and the remaining were not hemolytic at all. When tested for anticancer property, *Bti* strain *QBT*229 had the highest cytolytic activity. On molecular analyses, it was found that Cyt1A protein of *QBT*229 had five consecutive amino acids replaced in its β8 sheet. These replacements gave the β8 sheet more polarity; enhancing the ability of Cyt1A protein to form pores in the cell membrane and recruit Cry11 protein to the membrane. The presence of novel Cyt1A protein explains the high anti-cancer property of *QBT*229 when compared to the reference strains H14. These findings are very important and might have many applications in the treatment of lung cancer.

CHAPTER 4.5 RESULTS: EVALUATION OF PROTEIN YIELD OF HIGHLY INSECTICIDAL *BTI* STRAINS

4.5.1. Introduction

In this part of the study, four Qatari *Bti* strains were chosen from the collection of *Bt* strains: *QBT*217, *QBT*218, *QBT*220 and *QBT*221. These strains were chosen based on their high insecticidal activity against *Aedes aegypti Bora Bora* larvae (Chapter 4.2). The reference *Bti* strain H14 was used as positive control as it has been widely studied and characterized by many study groups. *Bti* H14 is also considered as one of the best *Bti* available today in the market to produce bio-pesticide. As negative control, *Bti* 4Q7 strain was used. This is an acrystalliferous clone of *Bti* H14 having lost its insecticidal activities. In the first part of the study, the strains were grown in glucose-based medium that is known to be appropriate for δ- endotoxin production. The objective of the study was to estimate the production of δ- endotoxin per millilitre and per spore, at the end of complete sporulation. In the second part of the study, the glucose-based medium was modified, and alternate cheap and complex sources of nitrogen and carbon were provided. This was done to estimate the yield of δ- endotoxins in other sources that can be used for possible scale-up in the future and to compare between the local *Bti* and reference, the stability of crystal production in alternative media.

4.5.2. Results

A. Protein yield in Glucose based media

Standard curve of Bovine Serum Albumin

The concentrations of Bovine Serum Albumin (BSA) used and the associated O D obtained for each of the concentrations are listed in the table 21. A standard curve for BSA protein was obtained as shown in figure34**.** The linear slope trendline for the values were drawn using MS excel as shown in the figure 34. The regression (r^2) value obtained for the slope was 0.9914 (very close to 1), showing that the standard curve for

the BSA was statistically accurate to be used for Bradford's method. The slope equation obtained for this curve was y = 0.0089x + 0.0107. Where, x represents the concentration of protein and the y represents the O D. This equation was used to extrapolate the protein content for each strain based on their O D values.

Table 21. Calibration of concentrations of standard protein Bovine Serum Albumin (BSA) using a spectrophotometer at 595 nm

Concentration of BSA (ug/ml)	**O D at 595 nm**
3	0.028
5	0.055
10	0.102
15	0.157
30	0.27

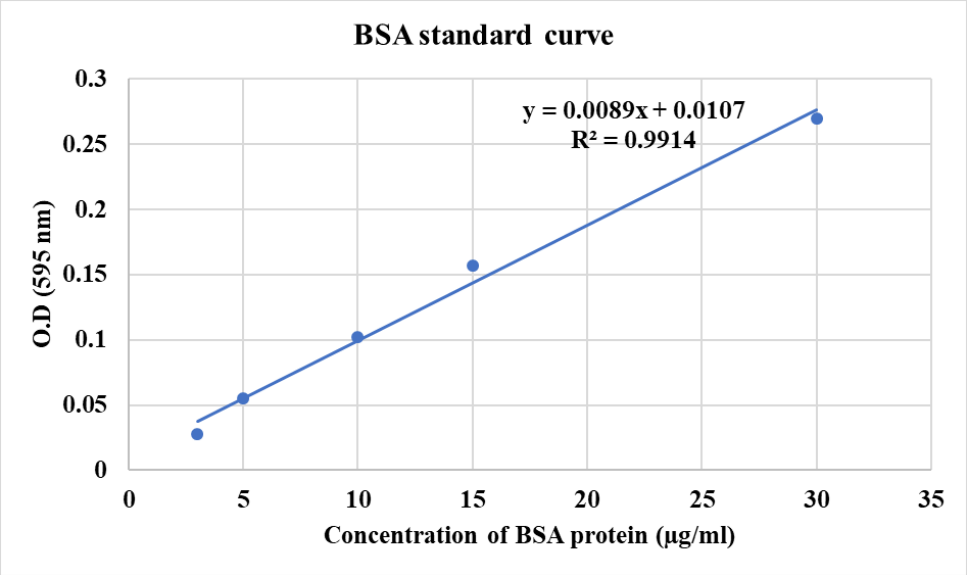

Figure 34. The standard curve for Bovine Serum Albumin protein

Estimation of protein production capacity

The reference strain H14 produced about 35.56 µg of protein per ml. This was the highest amount of protein production seen among the strains tested (**figure 35**). The Group 3 strains produced lesser proteins per ml. *QBT*217 produced 26.76 µg/ml, *QBT*218 produced 23.39 µg/ml, *QBT*220 produced 20.58 µg/ml and *QBT*221 produced 19.46 µg/ml of proteins. The negative control strain 4Q7 produced 3.17 µg/ml of proteins (**table 22**).

Table 22. Determination and statistical analysis of the protein production capacity in glucose based medium

[Positive control is *Bti* strain H14; Negative control is *Bti* strain 4Q7; standard error for each value mentioned with +/- in brackets]

Strains	**Protein concentration (µg/ml)**			**Average protein concentration (µg/ml)**
	I	**II**	**III**	
H14	33.315	35.562	37.809	**35.562 +/- 1.3**
4Q7	1.854	3.539	4.101	**3.165 +/- 0.7**
***QBT*217**	23.764	27.697	28.820	**26.760 +/- 1.5**
***QBT*218**	20.393	22.640	27.135	**23.390 +/- 2**
***QBT*220**	18.146	20.955	22.640	**20.581 +/- 1.3**
***QBT*221**	18.146	19.831	20.393	**19.457 +/- 0.7**

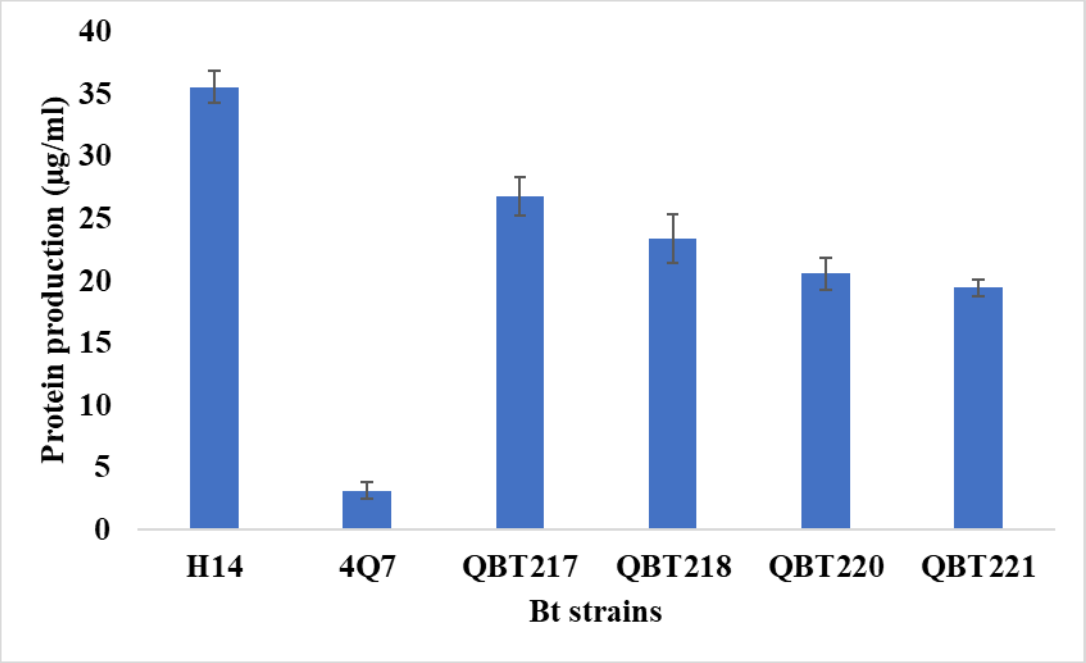

Figure 35. Comparing the protein production capacity (µg/ml) of Qatari *Bti* strains with references

[*Bti* H14 is the positive control; *Bti* 4Q7 is the negative control; Qatari *Bti* strains: *QBT*217, *QBT*218, *QBT*220, *QBT*221; error bars are standard error values plotted for positive and negative values]

Estimation of cell biomass of Bti strains

Contrary to the protein production per ml, the same strains showed a different trend when compared for their cell biomass. The reference H14 had 166.7 x 10^5 cfu/ml and the negative control 4Q7 had 536.7 x 10^5 cfu/ml. Among the *QBti* strains, *QBT*217 had the lowest cell biomass with 100 x 10^5 cfu/ml, followed by *QBT*220 with 176.7 x 10^5 cfu/ml, *QBT*218 with 636.7 x 10^5 cfu/ml and *QBT*221 with 860 x 10^5 cfu/ml (**figure 36**).

Table 23. Determination and statistical analysis of the cell biomass of *Bti* strains in glucose based medium

[Positive control is *Bti* strain H14; Negative control is *Bti* strain 4Q7; standard error for each value mentioned with +/- in brackets]

Strains	**Cell biomass (10^5 cfu/ml)**			**Average 10^5 cfu/ml**
	I	II	III	
H14	150	160	190	**166.67 +/- 12.02**
4Q7	530	590	490	**536.67 +/-29.06**
***QBT*217**	80	100	120	**100 +/- 11.6**
***QBT*218**	600	6500	660	**636.67 +/- 18.6**
***QBT*220**	150	170	210	**176.67 +/- 17.6**
***QBT*221**	820	880	880	**860 +/- 20**

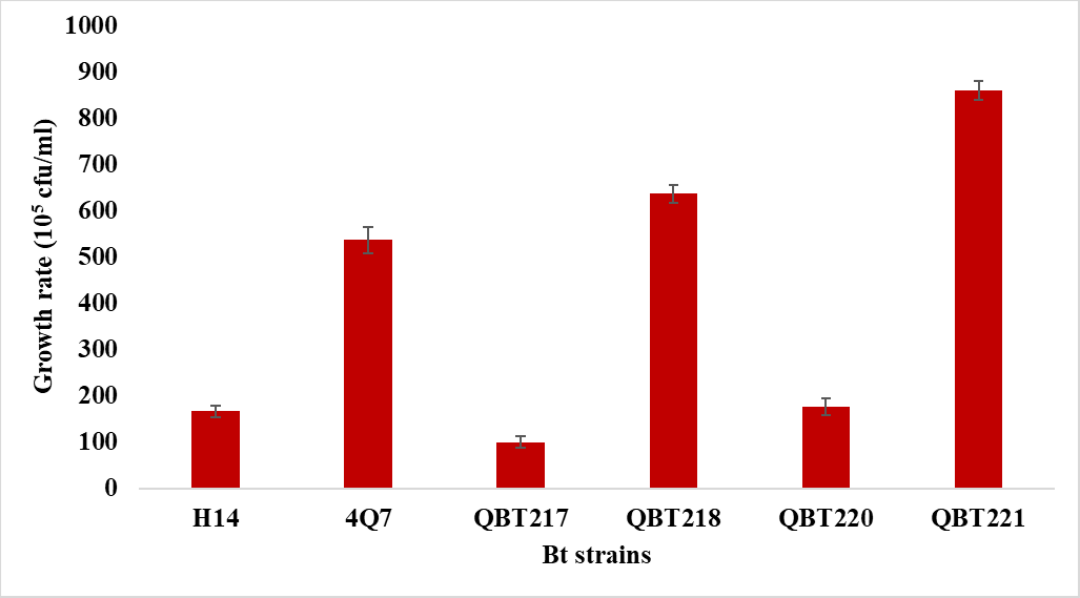

Figure 36. Comparing the cell biomass (10^5 cfu/ml) of Qatari *Bti* strains with references in glucose based medium

[*Bti* H14 is the positive control; *Bti* 4Q7 is the negative control; Qatari *Bti* strains: *QBT*217, *QBT*218, *QBT*220, *QBT*221; error bars are standard error values plotted for positive and negative values]

Estimation of protein yield of Bti strains per spore

The highest yield of δ- endotoxins was seen with *QBT*217 producing 265.4 ng δ-endotoxin proteins per 10^5 cells (**figure 37**). Comparatively, the reference had a lower protein yield. H14 could only produce 208.5 ng per 10^5 cells. Other local strains gave very low protein yield with *QBT*218 giving only 36.6 ng per 10^5 cells and *QBT*221 giving only 22.6 ng per 10^5 cells. Even though not as good as *QBT*217, *QBT*220 had comparatively good yield with 111 ng per 10^5 cells (**table 24**).

Table 24. Determination and statistical analysis of protein yield for Qatari *Bti* strains with references in glucose-based medium

[Positive control is *Bti* strain H14; Negative control is *Bti* strain 4Q7; standard error for each value mentioned with +/- in brackets]

Strains	**Protein production (µg/ml)**	**Cell biomass (10^5 cfu/ml)**	**δ endotoxin content (ng/10^5 cfu)**
H14	35.562 +/- 1.3	166.67 +/- 12.02	**208.50** +/- 7.73
4Q7	3.165 +/- 0.7	536.67 +/-29.06	**0.00** +/- 1.41
***QBT*217**	26.760 +/- 1.5	100 +/- 11.6	**265.44** +/- 16.66
***QBT*218**	23.390 +/- 2	636.67 +/- 18.6	**30.69** +/- 2.25
***QBT*220**	20.581 +/- 1.3	176.67 +/- 17.6	**111.40** +/- 4.81
***QBT*221**	19.457 +/- 0.7	860 +/- 20	**16.66** +/- 0.3

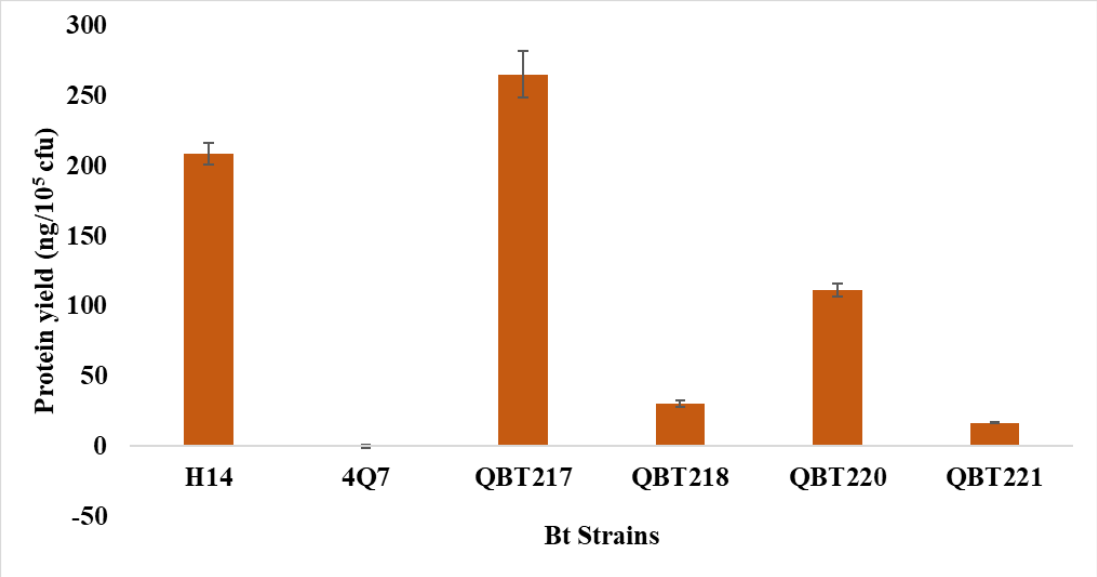

Figure 37. Comparing the protein yield per spore (ng/10^5 cfu) of Qatari *Bti* strains with references in glucose based medium

[*Bti* H14 is the positive control; *Bti* 4Q7 is the negative control; Qatari *Bti* strains: *QBT*217, *QBT*218, *QBT*220, *QBT*221; error bars are standard error values plotted for positive and negative values]

B. Protein yield in Soyameal starch based medium

Estimation of protein production capacity per

In the alternative media, the protein production per ml was the highest for the reference H14 (**figure 38**). H14 could produce 147.3 µg/ml proteins. For the negative control 4Q7, the protein production was the lowest at 7.9 µg/ml. *QBT*217 strain produced 111.9 µg protein per ml and *QBT*220 produced 101.9 µg protein per ml (**table 25**).

Table 25. Determination and statistical analysis of the protein production capacity in soya meal starch based medium

[Positive control is *Bti* strain H14; Negative control is *Bti* strain 4Q7; standard error for each value mentioned with +/- in brackets]

Strains	**Protein concentration (µg/ml)**			**Average protein concentration (µg/ml)**
	I	II	III	
4Q7	7.775	6.427	9.573	**7.925** +/- 0.91
H14	149.798	143.955	148.000	**147.251** +/- 1.73
***QBT*217**	97.663	127.775	110.247	**111.895** +/- 8.73
***QBT*220**	100.809	110.247	94.517	**101.858** +/- 4.57

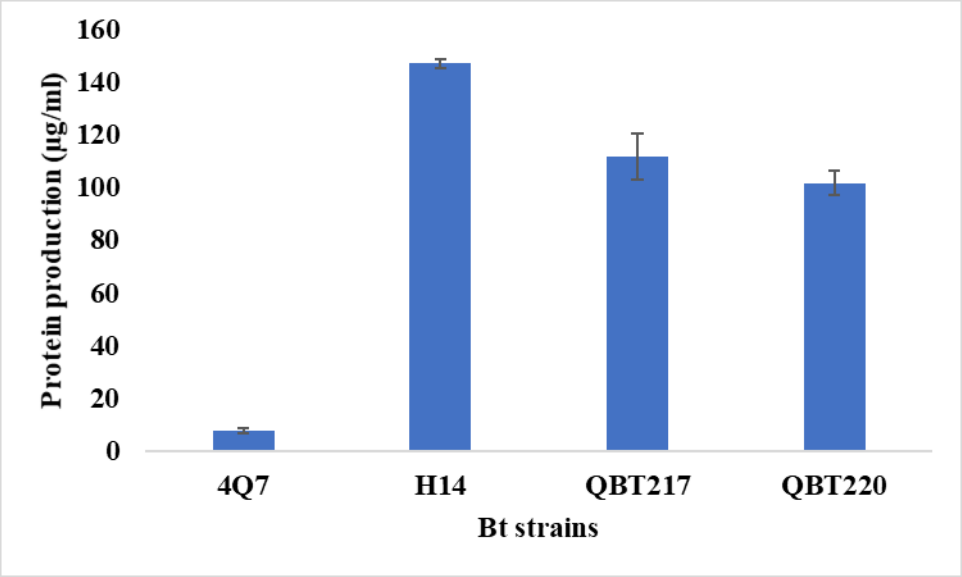

Figure 38. Comparing the protein production capacity (µg/ml) of Qatari *Bti* strains with references in soya meal starch based medium

[*Bti* H14 is the positive control; *Bti* 4Q7 is the negative control; Qatari *Bti* strains: *QBT*217, *QBT*220; error bars are standard error values plotted for positive and negative values]

Estimation of cell biomass of Bti strains

The cell biomass seen for H14 was the highest at 1883.3 x 10^5 cfu/ml (**figure 39**). The second highest cell biomass was seen in the negative control 4Q7 where there were 1476.7 x 10^5 cfu/ml. The *QBT*217 and *QBT*220 had 1370 x 10^5 cfu/ml and 1013.3 x 10^5 cfu/ml, respectively (**table 26**).

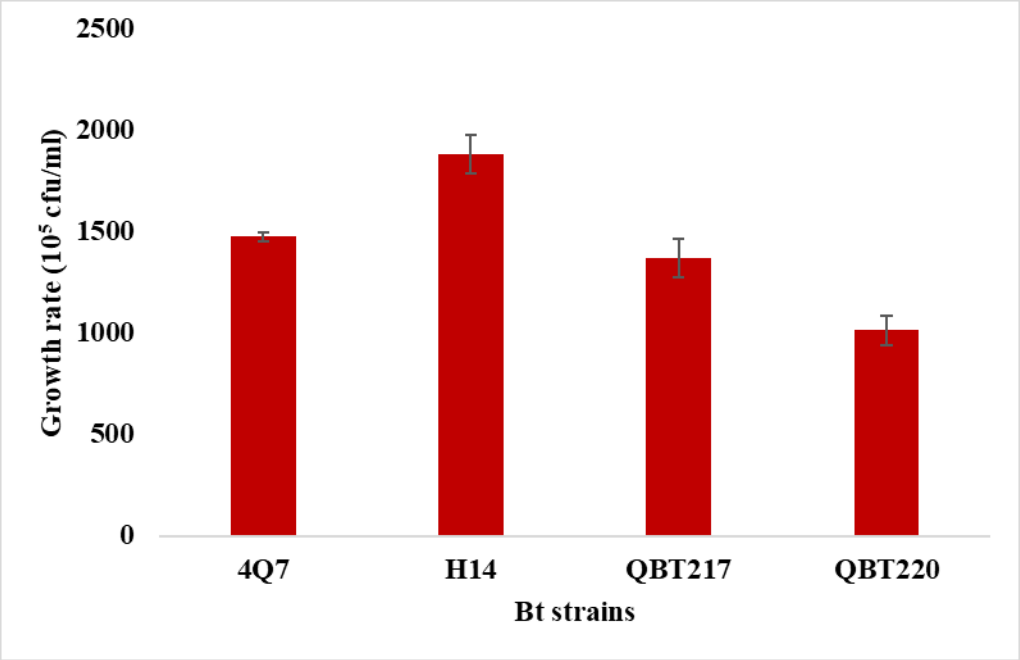

Figure 39. Comparing the cell biomass (10^5 cfu/ml) of Qatari *Bti* strains with references in soya meal starch based medium

[*Bti* H14 is the positive control; *Bti* 4Q7 is the negative control; Qatari *Bti* strains: *QBT*217, *QBT*220; error bars are standard error values plotted for positive and negative values]

Table 26. Determination and statistical analysis of the cell biomass of *Bti* strains in soyameal starch based medium

[Positive control is *Bti* strain H14; Negative control is *Bti* strain 4Q7; standard error for each value mentioned with +/- in brackets]

Strains	**Actual cell biomass (cfu/ml)**			**Average 10^5 cfu/ml**
	I	II	III	
4Q7	1.44 x 10^8	1.52 x 10^8	1.47 x 10^8	**1476.67 +/- 23.33**
H14	2.06 x 10^8	1.74 x 10^8	1.85 x 10^8	**1883.33 +/- 93.86**
***QBT*217**	1.48 x 10^8	1.18 x 10^8	1.45 x 10^8	**1370 +/- 95.39**
***QBT*220**	0.91 x 10^8	0. 97 x 10^8	1.16 x 10^8	**1013.33 +/- 75.35**

Estimation of protein yield of Bti strains per spore

In the alternative media, the protein yield was the highest in *QBT*220 with the yield of 95.59 ng/10^5 cfu (**figure 40**). The reference H14 and *QBT*217 had almost the same yield of 73.13 ng/10^5 cfu and 76.36 ng/10^5 cfu, respectively (**table 27**).

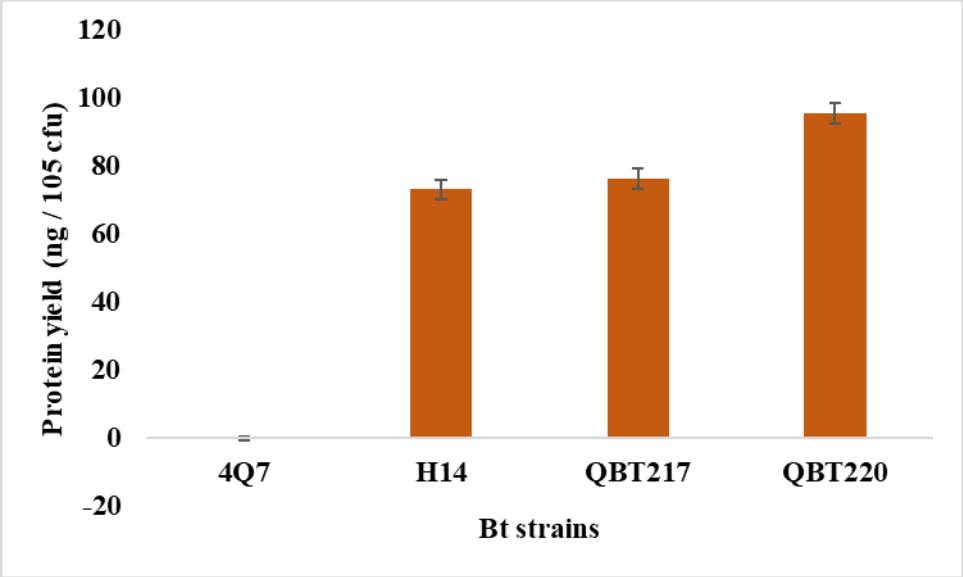

Figure 40. Comparing the protein yield per spore (ng/10^5 cfu) of Qatari *Bti* strains with references in soyameal starch based medium

[*Bti* H14 is the positive control; *Bti* 4Q7 is the negative control; Qatari *Bti* strains: *QBT*217, *QBT*220; error bars are standard error values plotted for positive and negative values]

Table 27. Determination and statistical analysis of protein yield for Qatari *Bti* strains with references in soya meal starch based

[Positive control is *Bti* strain H14; Negative control is *Bti* strain 4Q7; standard error for each value mentioned with +/-]

Strains	**Protein production (µg/ml)**	**Cell biomass (10^5 cfu/ml)**	**Protein yield (ng/10^5 cfu)**	**δ endotoxin content (ng/10^5 cfu)**
4Q7	7.925 +/- 0.91	1476.67 +/- 23.33	5.35	**0.00 +/- 0.53**
H14	147.251 +/- 1.73	1883.33 +/- 93.86	78.48	**73.13 +/- 2.99**
***QBT*217**	111.895 +/- 8.73	1370 +/- 95.39	81.71	**76.36 +/- 3.02**
***QBT*220**	101.858 +/- 4.57	1013.33 +/- 75.35	100.94	**95.59 +/- 2.95**

4.5.3. Discussion

The main goal of this study was to evaluate the four highly insecticidal *Bti* strains (Chapter 4.2) based on their ability to produce δ- endotoxins (quantitative analysis). The strains tested for their protein production per cell were *QBT*217, *QBT*218, *QBT*220 and *QBT*221. The study included two sections: testing the four strains in glucose-based medium and testing the important strains in alternative medium. All the strains were first grown in a glucose-based media that is known to enhance the sporulation and δ-endotoxin production in *Bt* (Ghribi *et al*., 2005). First the concentration of protein produced per ml was calculated and it was found that the reference H14 had produced the highest protein quantity per ml (figure 35). This was followed by *QBT*217, *QBT*218, *QBT*220 and *QBT*221. On the contrary, when the cell biomass of these strains were compared, the trend seen was very different. The reference H14, *QBT*217 and *QBT*220 had a much lower cell biomass compared to *QBT*218, *QBT*221 and 4Q7 (figure 36).

The cultures for all the strains began with approximately equal number of cells. So, after culturing for same time, the huge differences in their cell counts show the differences in their cell biomass. It is even more interesting to note that the protein produced per ml do not correspond to the cell biomass. This means that the higher amount of protein produced could be due to either of the two reasons: the higher number of cells or the higher capacity to produce - δ- endotoxins. When the protein yield per cell was calculated, it was seen that local strain *QBT*217 has the highest protein yield per cell followed by the reference H14 and then the local strain *QBT*220. The protein yield per cell for other two local strains (*QBT*218 and *QBT*221) were much lower (figure 37). *QBT*217 and *QBT*220 were hence chosen as candidate strains.

In the second part of this study, the protein yields were evaluated for the strains *QBT*217, *QBT*220 and the references H14 and 4Q7 in soyameal-starch-based medium. This alternative medium enhanced the protein production for all strains. Like glucose-based media, in the alternative media also the protein production per ml was the highest in case of the reference H14 followed by *QBT*217 and then *QBT*220 (figure 38). H14 had the highest cell biomass followed by *QBT*217 and then *QBT*220 (figure 39). When the protein yield was calculated, it was seen that the *QBT*220 had the highest yield followed by *QBT*217 and then H14 (figure 40). This was an interesting result as the local strains had much higher yield of δ- endotoxins than the reference in the presence of complex sources of carbon and nitrogen.

4.5.4. Conclusion

From the *Bt* collection, four *Bti* strains were chosen for the evaluation of their δ-endotoxin production capacity based on their high insecticidal properties against *Aedes aegypti* larvae. *QBT*217, *QBT*218, *QBT*220 and *QBT*221 showed different protein yields. Among them, *QBT*217 and *QBT*220 had high protein yield per cell compared to the *Bti* strain H14 available today in the market for bio-insecticide production. They

were hence considered as possible candidates for bio-insecticide production. An alternative medium was adopted to check the ability of the strains to grow and produce - δ- endotoxins in the presence of cheap yet complex sources of carbon and nitrogen. *QBT*220 was found to be the best candidate closely followed by *QBT*217 for local bio-insecticide production.

CHAPTER 4.6 RESULTS: INVESTIGATION OF QATARI *BT* STRAINS PLASMID STABILITY AND EFFECT ON δ-ENDOTOXIN PRODUCTION

4.6.1. Introduction

Bt δ-endotoxins, the important components of the crystals formed during sporulation, are encoded by *cry* genes carried by one or more high molecular weight extrachromosomal plasmids. When a *Bt* strain is isolated from the environment and chosen for industrial bio-insecticide production, one of the main concerns is the stability of these plasmids and *cry* genes to ensure the highest δ-endotoxin yields. *Bti* carries its δ-endotoxins coding genes on a mega plasmid called pBtoxis. In this study, Qatari *Bti* strains were screened for the *Bti* δ-endotoxin coding genes by PCR amplifications. The Qatari *Bti* strains showed the presence of all δ-endotoxins genes except the two genes *cry10A* and *cyt1C*. The first part of this study is about the analysis of the study o the stability of pBtoxis plasmid of Qatari *Bti* to monitor any segregational and structural instability that could lead to a loss of or change in the regions carrying the genes. The second objective of this study deals with choosing the best insecticidal strains among the Qatari *Bti* and submit them to plasmid curing conditions to select clones that have high structural stability, retaining the essential δ- endotoxin genes. The clones obtained after plasmid curing, were analysed based on their colony characteristics, the presence of *cry4* δ- endotoxin genes and the presence or absence of insecticidal parasporal crystals. The clones, thus selected, were then tested for their δ- endotoxin yield per spore. The strategy was to compare the δ- endotoxin yields of the clones to that of the original strains and select the clones that show highest yield per spore. The *Bt* clones that show the highest yields would be considered as good candidates for bio-pesticide production, not only based on the yield for production, but also in terms of long-term stability of the genes encoding the δ-endotoxins.

4.6.2. Results

A. Exploration of pBtoxis plasmid stability in Qatari *Bti* strains

The 19 *Bti* strains isolated from Qatar showed the presence of all δ-endotoxin coding genes except *cry10* and *cyt1C* (Chapter 4.3). When we analysed the positions of δ-endotoxin genes on the pBtoxis, it was identified that these two genes are located next to each other between positions 30 kb to 50 kb from the origin; in the order *cry4B* – *cry10A* – *cyt1C*. Mapping was done along this region with primers designed to amplify overlapping fragments (**figure** 41). The figure depicts the amplifications that gave the expected PCR products in green colour and the ones that did not work in red colour.

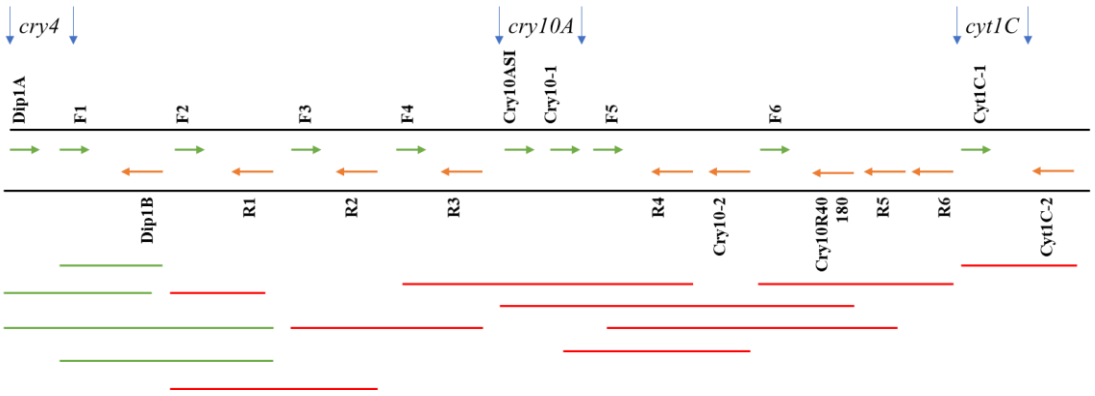

Figure 41. Mapping of part of pBtoxis plasmid in Qatari *Bti*

[The region between 30 kb and 50 kb from the origin of the pBtoxis plasmid is depicted by the black lines; forward primers designed depicted by green arrows; reverse primers designed depicted by the orange arrows; names of the primers listed in black; positions of genes depicted by the respective names and blue arrows; green lines: PCR amplifications that gave the expected product; red lines: PCR amplifications that did not give any amplifications.]

B. Evaluation of the effect of plasmid loss on δ- endotoxin production

Induction of Bti plasmid curing

Plasmid curing was conducted for two Qatari *Bti* strains *QBT*217 and *QBT*220. After seven overnight culturing at 42^0C, each culture was diluted till 10^{-5}. The diluted culture was spread on a LB plate to obtain isolated colonies, which were analysed after overnight incubation. For *QBT*220, 75 clones were isolated and transferred to fresh plate, each. For *QBT*217, 43 clones were isolated and transferred to a fresh plate, each. Among the *QBT*220 clones, only one type of colony was observed: white (**figure** 42b). On the other hand, for *QBT*217, two types of colonies were observed: 26% white and 74% yellow (**figure** 42a).

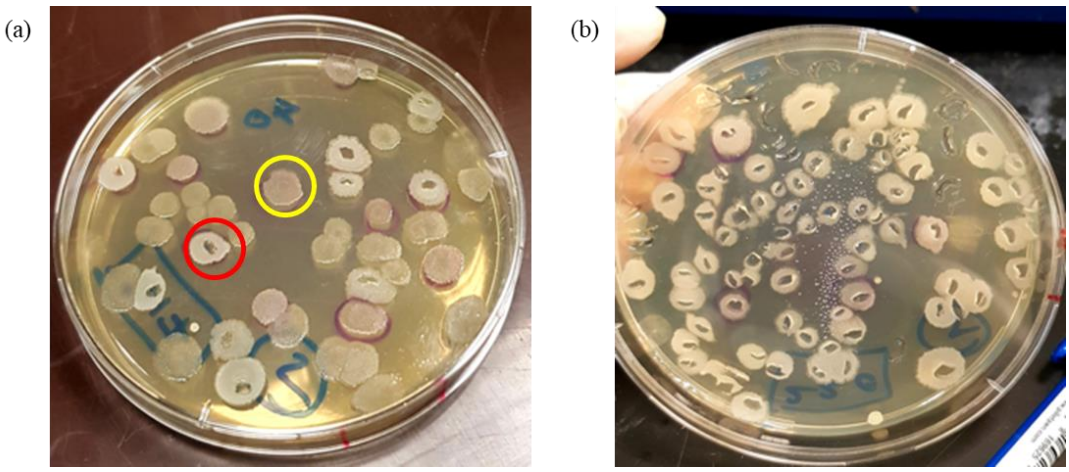

Figure 42. Morphology of colonies among the clones obtained after plasmid curing [(a): LB Agar for *QBT*217 showing two types of colonies; (b): LB Agar for *QBT*220 showing the single type of colonies; yellow circle: the yellow colored colonies obtained for clones of *QBT*217; red circle: the white colored colonies obtained for clones of *QBT*217]

Loss of genetic material among the obtained new clones

The white colony colour is a new characteristic, as the original *QBT*220 and *QBT*217 have yellow colony colour. It can be concluded that the culturing of these 2 strains at high temperature might have affected their genetic material giving this white colony colour to *QBT*220 and *QBT*217. In fact, these strains have many extrachromosomal high molecular plasmids among which the pBtoxis plasmid carrying the - δ- endotoxin coding genes.

Investigation of the presence of pBtoxis plasmid in the Bt heat treated strains

The interest in these clones resides in the possible importance of the clones that might have lost part of their plamids while managing to retain p*Bt*oxis and the insecticidal δ-endotoxin genes. To check whether the new clones retain the p*Bt*oxis plasmid, their total DNA was isolated and used to amplify *cry4* gene (figure 43, figure 44). For *QBT*220, out of 75 clones, only two clones gave the expected amplification: 220KN65 and 220KN66 (**figure 46**). In case of *QBT*217, only three clones out of the white colonies gave the expected PCR amplification: 217KN33, 217KN35 and 217KN39 (**figure 45**). Among the yellow colony clones of *QBT*217, 75% of the clones gave the expected amplification and the other 25% did not (**figure 45**).

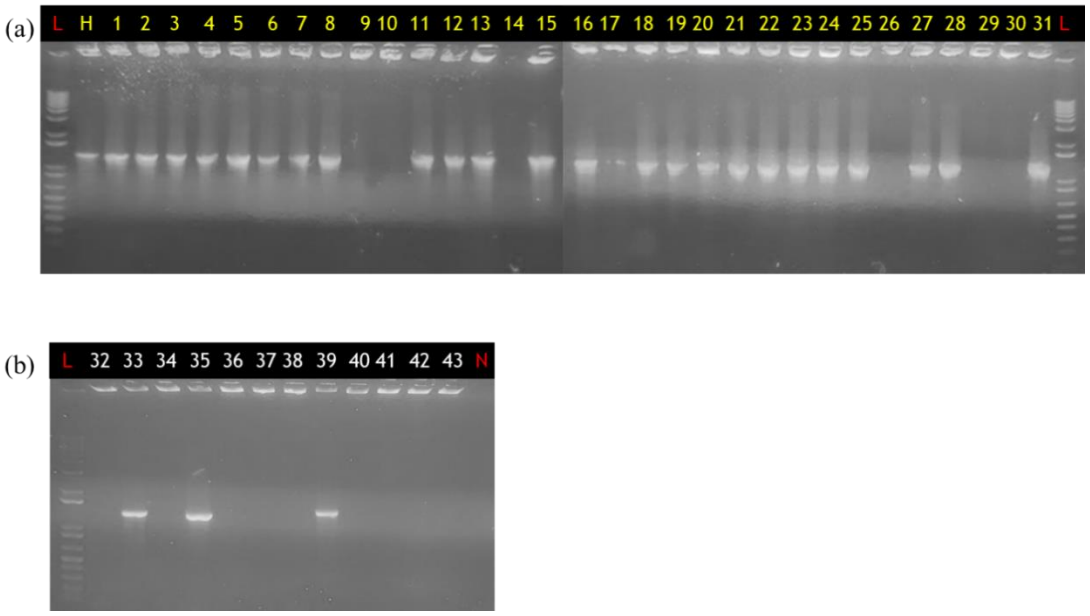

Figure 43. Exploration of the presence of *cry4* gene in the *QBT*217 mutants by PCR [(a): PCR products obtained for clones that show yellow colonies; (b) PCR products obtained for clones that show white colonies; H: positive control *Bti* H14; L: 1 kb plus linear DNA ladder; N: negative control with no DNA template added for PCR; Lane 1 to Lane 43: PCR products using DNA template from clones 217KN1 to 217KN43]

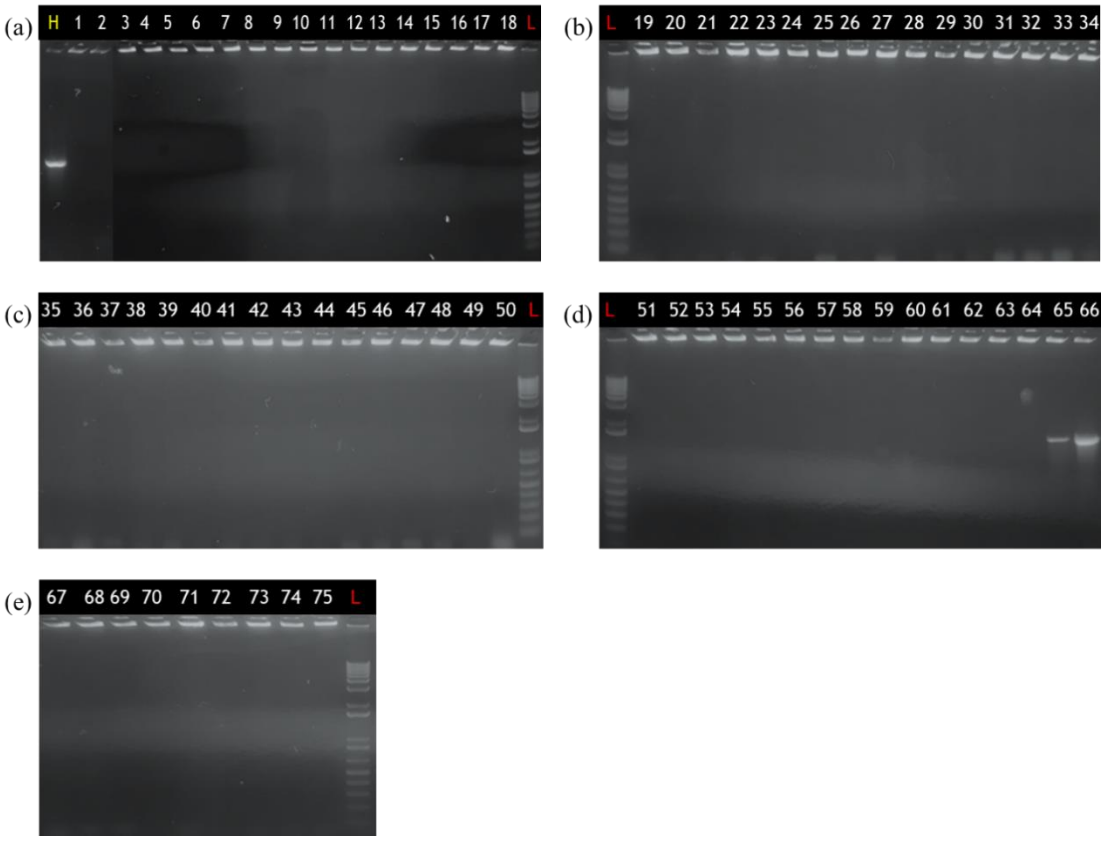

Figure 44. Agarose gel showing the PCR amplifications for cry4 gene in clones of *QBT*220

[H: PCR positive control *Bti* H14; L: 1 kb plus linear DNA ladder; Lane 1 to Lane 75: PCR products using DNA template from clones 220KN1 to 220KN75]

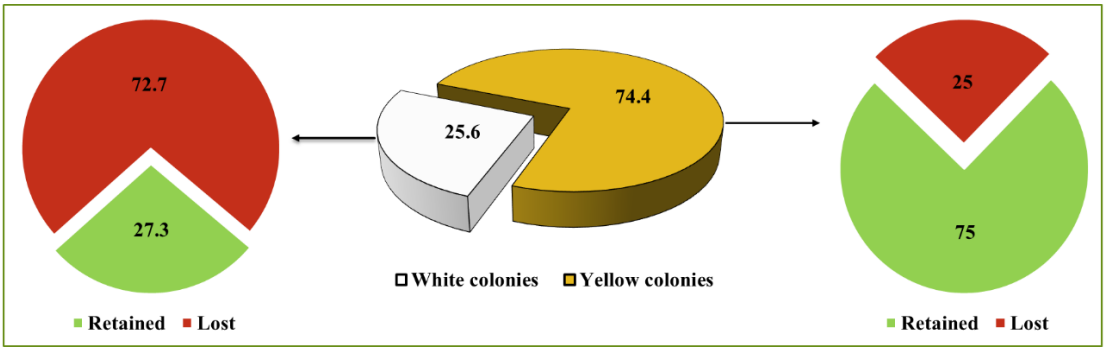

Figure 45. Distribution of *QBT*217clones : colony characteristics & presence of *cry4* gene

[middle disc depicts the distribution of clones obtained based on their colony color; pie-chart on right depicts the distribution of clones forming yellow colonies based on the presence of *cry4* gene; pie-chart on left depicts the distribution of clones forming white colonies based on the presence of *cry4* gene; Lost: No amplification obtained for *cry4* specific primers; Retained: expected PCR product obtained for *cry4* specific primers]

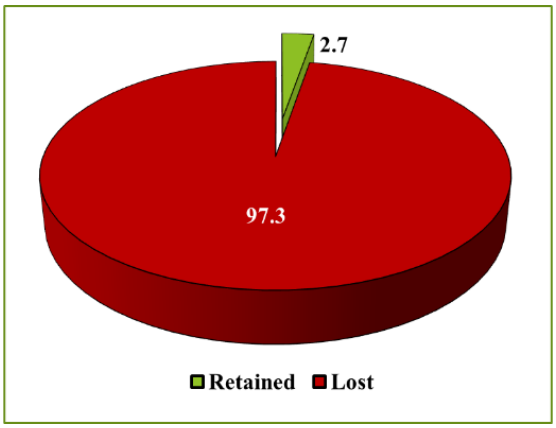

Figure 46. Distribution of clones o*Bt*ained for *QBT*217: colony characteristics & presence of *cry4* gene

[Lost: No amplification obtained for *cry4* specific primers; Retained: expected PCR product obtained for *cry4* specific primers; only white colonies obtained for clones from *QBT*220]

Effect of the loss of the genetic material on important functions.

Among the clones forming white colonies, the ones that showed the presence of *cry4* gene are the clones that have undergone genomic instability while retaining the δ-endotoxin coding genes. We proposed to explore the effect of this genomic instability on δ-endotoxin production in these obtained clones. The clones forming white colonies and lost the *cry4* gene were used as negative control for δ- endotoxin production. The clones forming yellow colonies and have retained the *cry4* gene are the true positive control apart from the original strain itself. After culturing the three groups of interest in Glucose based sporulation media, it was found that the clones forming white colonies with *cry4* gene form normal spores and crystals; like the original strain. While the clones forming white colonies and lost *cry4* gene showed the presence of normal spores but lacked crystals. The third group of clones, forming yellow colonies and having *cry4* gene failed to sporulate even after five days of incubation in sporulation media. Hence, three clones of *QBT*217 and two clones of *QBT*220 were chosen for δ- endotoxin production: 217KN33, 217KN35, 217KN39 (figure 47), 220KN65 and 220KN66 (figure 48). For negative control, 217KN42 and 220KN9 were chosen. Positive controls were the original strains of *QBT*217 and *QBT*220.

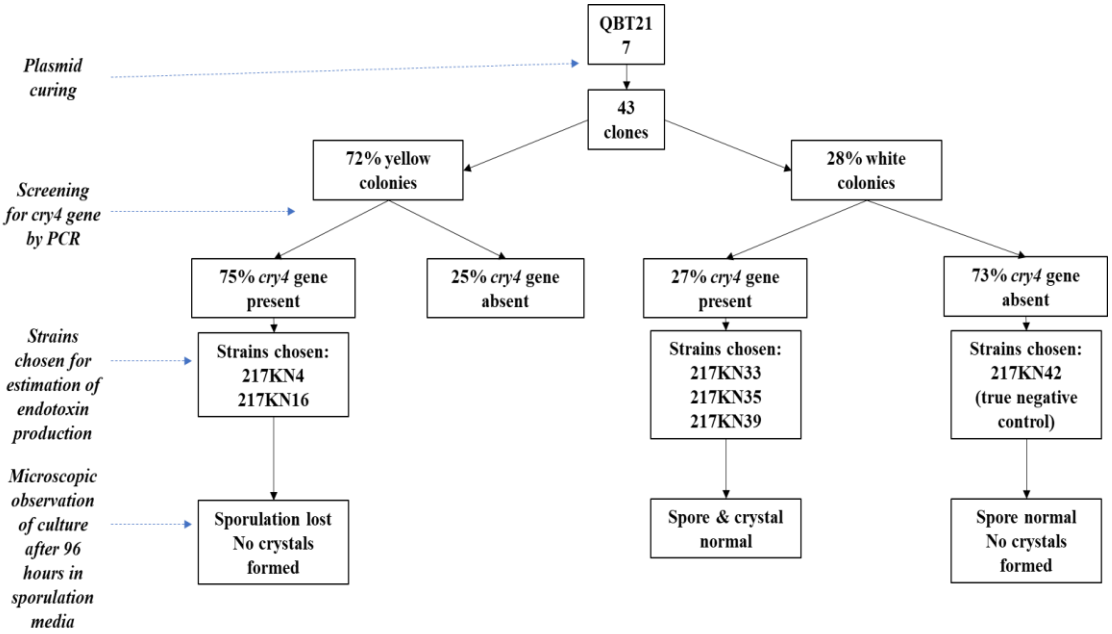

Figure 47. Characteristics of *QBT*217 clones chosen for estimation of δ- endotoxin yield

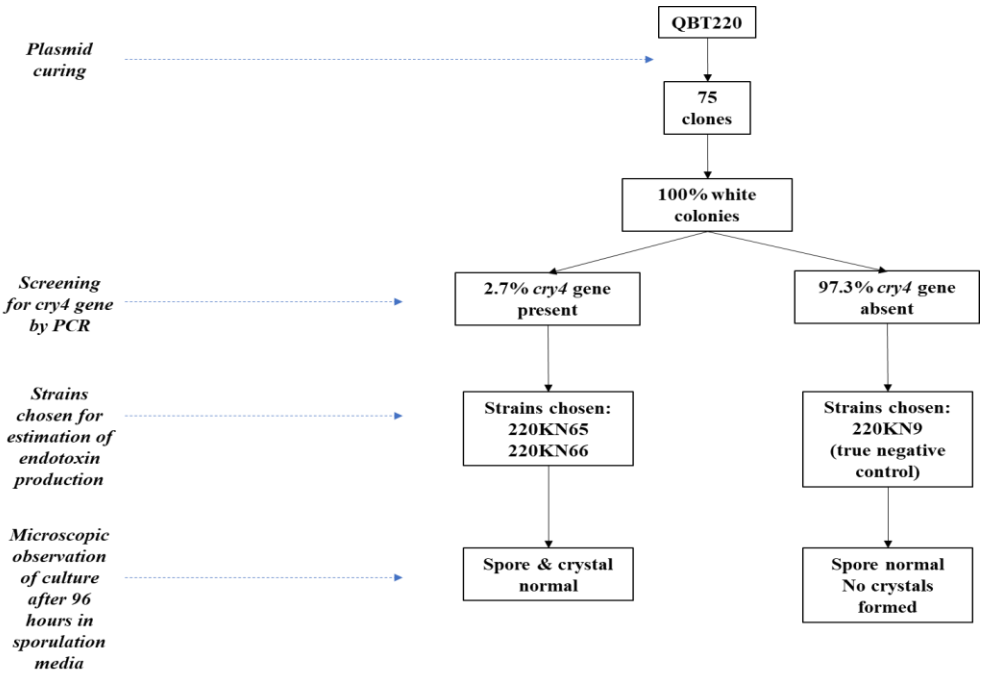

Figure 48. Characteristics of *QBT*220 clones chosen for estimation of δ- endotoxin yield production

Estimation of the δ- endotoxin yields of new clones

δ- endotoxin yield of the new clones was estimated by the same methodology used in Chapter 4.5. The colonies forming white colonies and retaining the *cyt4* gene were selected for the δ- endotoxin production studies. As negative control, the acrystalliferous clones forming white colonies and lacking the *cry4* gene was used. As positive control the original strains *QBT*217 and *QBT*220 were used.

A. Comparison of cell biomass:

For *QBT*217 and *QBT*220, it was observed that the new clones had much higher cell biomass than the original strains. Among the new clones, the acrystalliferous strains (in red, **table 28**) had higher cell biomass then the other strains (in green, **table 28**) with normal spores and crystals.

Table 28. Determination and statistical analysis of cell biomass of plasmid cured clones [217KN33, 217KN35, 217KN39, 220KN65 and 220KN66: the clones with normal spore and crystal; 217KN42 and 220KN9: acrystalliferous clones used as negative control; QBT217 and QBT220: original strains used as positive control]

Strains	**Growth (cfu/ml) x 10^6**			**Average growth (cfu/ml) x 10^6**
	I	II	III	
217KN33	32	31	34	**32.33** (+/- 0.88)
217KN35	98	100	98	**98.67** (+/- 0.67)
217KN39	49	55	50	**51.33** (+/- 1.86)
217KN42	220	215	229	**221.33** (+/- 4.1)
***QBT*217**	8	10	12	**10** (+/-1.16)
220KN9	130	137	141	**136** (+/- 3.22)
220KN65	40	43	43	**42** (+/- 1)
220KN66	36	34	38	**36** (+/- 1.16)
***QBT*220**	15	21	17	**17.67** (+/- 1.77)

B. Comparison of total protein production:

Like the cell biomass, the amount of δ- endotoxin produced by the strains was the lowest among the original strains. Among the new clones, the acrystalliferous strains (in red, table 29) produced the lowest amount of proteins, compared to the other clones

(in green, table 29) with normal spores and crystals.

Table 29. Determination and statistical analysis of crystal protein production of plasmid cured clones

[217KN33, 217KN35, 217KN39, 220KN65 and 220KN66: the clones with normal spore and crystal; 217KN42 and 220KN9: acrystalliferous clones used as negative control; QBT217 and QBT220: original strains used as positive control]

Strains	**Protein production (ng/ml)**			**Average protein production (ng/ml)**
	I	II	III	
217KN33	10.08×10^4	10.53×10^4	10.13×10^4	**10.25** (+/- 0.14) x 10^4
217KN35	19.07×10^4	18.94×10^4	17.27×10^4	**18.43** (+/- 0.58) x 10^4
217KN39	10.58×10^4	9.77×10^4	10.26×10^4	**10.2** (+/- 0.24) x 10^4
217KN42	5.32×10^4	5.09×10^4	4.78×10^4	**5.06** (+/- 0.16) x 10^4
***QBT*217**	2.77×10^4	2.89×10^4	2.38×10^4	**2.67** (+/- 0.15) x 10^4
220KN9	2.67×10^4	2.62×10^4	2.44×10^4	**2.58** (+/- 0.07) x 10^4
220KN65	7.65×10^4	8.06×10^4	7.7×10^4	**7.8** (+/- 0.13) x 10^4
220KN66	8.37×10^4	8.42×10^4	8.15×10^4	**8.31** (+/- 0.08) x 10^4
***QBT*220**	2.1×10^4	2.26×10^4	1.82×10^4	**2.06** (+/- 0.13) x 10^4

C. Comparison of δ- endotoxin yield:

The determination of δ- endotoxin yield was calculated by subtracting the protein yield of the negative control clones (in red, **table 30**) from each of the studied clones (in green, **table 30**). The yield of total protein per cell was calculated by dividing the values of total protein production by the growth obtained each millilitre. Among the clones for *QBT*217, 217KN33 showed a higher δ-endotoxin yield per spore compared to the original strain. The other two clones 217KN35 and 217KN39 showed a lower δ-endotoxin yield per spore compared to the original strain *QBT*217. In case of clones for *QBT*220, both the clones showed much higher δ-endotoxin yield per spore compared to that of the original strain *QBT*220.

Table 30. Determination and statistical analysis of δ- endotoxin yield of plasmid cured clones

[217KN33, 217KN35, 217KN39, 220KN65 and 220KN66: the clones with normal spore and crystal; 217KN42 and 220KN9: acrystalliferous clones used as negative control; QBT217 and QBT220: original strains used as positive control]

Strains	**Protein production (ng/ml) x** 10^4	**Growth (cfu/ml)** x 10^6	**Protein yield (ng/10^5cfu)**	**δ- endotoxin yield (ng/10^5cfu)**
217KN33	**10.25** (+/- 0.14)	**32.33** (+/- 0.88)	**317.14** (+/- 4.47)	**294.27** (+/- 4.79)
217KN35	**18.43** (+/- 0.58)	**98.67** (+/- 0.67)	**186.73** (+/- 5.30)	**163.86** (+/- 5.62)
217KN39	**10.2** (+/- 0.24)	**51.33** (+/- 1.86)	**198.98** (+/- 3.71)	**176.11** (+/- 4.03)
217KN42	**5.06** (+/- 0.16)	**221.33** (+/- 4.1)	**22.87** (+/- 0.32)	**0**
***QBT*217**	**2.67** (+/- 0.15)	**10** (+/-1.16)	**271.5** (+/- 16.82)	**248.63** (+/- 17.14)
220KN9	**2.58** (+/- 0.07)	**136** (+/- 3.22)	**18.94** (+/- 0.10)	**0**
220KN65	**7.8** (+/- 0.13)	**42** (+/- 1)	**185.84** (+/- 3.67)	**166.90** (+/- 3.77)
220KN66	**8.31** (+/- 0.08)	**36** (+/- 1.16)	**231.26** (+/- 5.27)	**212.32** (+/- 5.37)

Strains	Protein production (ng/ml) x 10^4	Growth (cfu/ml) x 10^6	Protein yield (ng/10^5cfu)	δ- endotoxin yield (ng/10^5cfu)
***QBT*220**	**2.06** (+/- 0.13)	**17.67** (+/- 1.77)	**117.27** (+/- 4.9)	**98.33** (+/- 5)

D. Evaluation of the effect of plasmid curing on δ-endotoxin production

Among the clones of *QBT*217, clones 217KN35 and 217KN39 had significantly lower δ- endotoxin yield compared to the original strains *QBT*217. They showed a decrease of about 29% to 34% in their δ- endotoxin yield. The clone 217KN33 showed an increase in its δ-endotoxin yield but, but statistically not significant as shown by the error value (table 31). In the case *QBT*220, both the clones 220KN65 and 220KN66 showed a significant increase in their δ- endotoxin yield. 220KN65 showed an increase of about 68% and 220KN66 showed an increase of about 116%. As indicated by their error values, they are statistically significant (table 31).

Table 31. Estimation of the increase/decrease of the δ-endotoxin production in the *Bti* cured clones

[Changes depicted as positive percentage indicates an increase and in negative indicates a decrease in the δ- endotoxin yield of clones per spore compared to the original strains; underlined values for the clones of *QBT*220 show statistically significant increase in their δ- endotoxin yield]

Strains	**Change in** δ- endotoxin **yield (%)**
217KN33	+18.36 (+/- 20.27)
217KN35	-34.1 (+/- 13.67)
217KN39	-29.14 (+/- 13.07)
220KN65	**+69.74 (+/- 17.68)**
220KN66	**+115.93 (+/- 32.94)**

4.6.3. Discussion

The plasmid instability was analysed among the Qatari *Bti* strains from the collection in two different sections. The first study involved identifying the reason why Qatari *Bti* strains failed to give expected PCR amplifications with primers specific for two *Bti* gene: *cry10A* and *cyt1C*. The second study involved choosing the highly insecticidal *Bti* strains and obtain clones from them by plasmid curing process and analyse the effect on their δ-endotoxin yield per spore.

In the first study, it was found that all 19 Qatari *Bti* strains lost part or all of each of the

two genes, *cry10A* and *cyt1C*. The absence of the amplification could be explained by: a change in the gene sequence, or a plasmid structural instability and gene loss. When analysing the positions of the two genes, it was found that they are next to each other on the mega plasmid pBtoxis of *Bti*. On further analysis it was found that they are present next to *cry4* gene between the positions 30 kb and 50 kb from the origin. Primers were designed to map this region starting from *cry4* gene that was found to be present in Qatari *Bti*. Mapping revealed that the region of pBtoxis with *cry4* gene was intact, but the region from about 38 kb from the origin is lost. This structural instability is a signature of Qatari *Bti* strains as they all give the same results. This instability is not just associated with the lost genes, but the entire region of pBtoxis after *cry4* gene.

In the second study of plasmid stability, the two strains *QBT*217 and *QBT*220 were chosen because of their high insecticidal property compared to the reference *Bti* and other Qatari *Bti*. They were both individually used for plasmid curing experiment over a seven-day period. After seven days, *QBT*217 gave 43 clones and *QBT*220 gave 75 clones. *QBT*217 clones showed two different types of colony colours: yellow colour like the original strain of *QBT*217 and white colour. *QBT*220 had all white coloured colonies. The white coloured clones were of interest as the new characteristic means loss of part of genome. For the interest of this study, it was essential to choose clones that have lost a part of the genome but retained essential δ- endotoxin genes. Hence the DNA from clones obtained were used as template to amplify part of the *cry4* gene. On checking the PCR products on agarose gels, it was found that some clones had lost the *cry4* gene. In the case of *QBT*217 clones three of the clones forming white colonies retained the *cry4* gene and other had lost it. The three that retained the *cry4* gene were the candidate clones for δ-endotoxin production experiments. The others that lost *cry4* gene were considered as negative control. Among the *QBT*217 clones forming yellow

colonies, the ones that retained the *cry4* gene were to be used as positive control along with the original strain for δ-endotoxin production experiments. It was found that they did not sporulate even after 5 days. They had lost some part of their sporulation machinery but retained the δ-endotoxin genes. Hence it was decided to use only original strains as positive control. In case of *QBT*220, only one type of colonies was obtained: white. Among them, only two clones retained the *cry4* gene. These two were the candidate strains for the δ-endotoxin production experiments. The ones that had lost the *cry4* gene were the negative control and the original strain was the positive control. Before using the clones of both strains that have lost *cry4* gene as negative control, it was essential to check the presence of crystals. Absence of Cry4 protein should eliminate the crystal formation. On microscopic observation, it was found that the *cry4*$^{-}$ strains were acrystalliferous. They formed spores during sporulation with no parasporal crystals.

δ- endotoxin yield per spore was estimated for each of the candidate clones of *QBT*217 and *QBT*220 along with the negative control clones of *QBT*217 and *QBT*220 (figure 47 & figure 48), and the original strains as positive control. It was found that the cell biomass of the clones were much higher than the original strains. This could be explained by the fact that loss of part of genome usually increases the cell division rate. When the δ- endotoxin yield per spore was estimated, it was found that both the clones of *QBT*220 had gained a significant δ- endotoxin yield per cell. They showed an increase in the yield by 69% to 115% compared to the original strain *QBT*220. In case of clones of *QBT*217, one clone showed an increase in δ-endotoxin production and the other two clones had significantly lost the δ- endotoxin yield per spore compared to *QBT*217.

4.6.4. Conclusion

The plasmid stability of the Qatari *Bti* strains was explored. Among the Qatari *Bti*, it was found that they were subjected to pBtoxis plasmid structural instability and consequently lost two of the δ- endotoxin genes: *cyt1C* and *Cry10A*. This plasmid structural instability is a common signature among the *Bti* strains isolated from Qatar as they all have lost that region of their pBtoxis.

The two *Bti* strains *QBT*217 and *QBT*220, having the highest insecticidal activity, were chosen for heat induced plasmid curing segregational instability experiments. The clones were characterized based on their colony characteristics, presence of *cry4* gene and microscopic observation of sporulated culture. The clones were studied for their δ-endotoxin yield per spore. Two clones of *QBT*220 were identified that have about 69% to 115% increase in their δ- endotoxin yield per spore compared to the original strain *QBT*220. These clones have the most stable pBtoxis plasmid as they retained the essential genes even after plasmid curing and, in the process, lost other non-essential parts of their genome. Loss of part of genome helped them to increase the δ-endotoxin production capacity.

CHAPTER 5: CONCLUSION AND PERSPECTIVES

The aim of this PhD research project was to characterize the 441 Qatari *Bacillus thuringiensis* (*Bt*) strains, isolated by our research group from Qatar, that produce spherical crystals and identify candidate strains for insecticidal activity against Dipteran insects like mosquitoes. This study focused on finding a sustainable solution to two of the huge health hazards faced by humans today: the diseases caused by the pathogen carrying vectors like mosquitoes and the health issues caused by the toxic chemical insecticides used to control these vectors. An efficient biological control of the disease vectors would solve both health hazards. *Bacillus thuringiensis* (*Bt*) has been recognized as the best biological control for this purpose and there is a need to screen many unexplored ecologies for *Bt* strains that are efficient enough to replace the chemical insecticides in the market.

In this study, a collection of 441 *Bt* strains producing spherical crystals were characterized and classified based on crystal morphology, plasmid patterns, protein patterns of crystal δ-endotoxins and corresponding *cry* and *cyt* genes (Chapter 4.1). Crystal morphology divided the collection into five classes. One class of 19 *Bti* strains that resembled the reference strain H14 and four other morphologies that were Non-*Bti* like. The plasmid patterns divided the collection into six groups. One group of 19 *Bti* strains that resembled that of H14 and five groups of Non-*Bti* strains. Based on protein patterns, the collection was divided into 15 groups; one *Bti* pattern and 14 Non-*Bti* patterns. As the protein patterns gave the maximum number of classes among the collection, the true groups were made based on this category. The collection was broadly divided into two groups: 19 *Bti* strains and 422 Non *Bti* strains. The Non-*Bti* strains were further divided into 14 classes based on their protein patterns. A true representative was chosen for each class. All the other studies were performed with 19

Bti strains, 14 Non *Bti* representatives, positive control *Bti* H14 and negative control *Bti* 4Q7.

In another part of the study, the insecticidal properties of the strains were investigated against Dipteran insect larvae of *Culex pipiens* and *Aedes aegypti* (Chapter 4.2). The qualitative studies showed that the *Bti* strains were all insecticidal, while the Non-*Bti* representatives were not insecticidal against *Culex pipiens* 3rd instar larvae. Quantitative insecticidal bioassays with 3rd instar larvae of *Aedes aegypti Bora Bora* showed that four *Bti* strains *QBT*217, *QBT*218, *QBT*220 and *QBT*221 had higher efficiency than the reference strain H14. By statistical analysis, it was found that although all four had similar insecticidal fiducial range for their LC_{50} values, *QBT*220 was the most insecticidal among them.

To understand the insecticidal activity, *Bt* strains were screened for the presence of Dipteran specific delta endotoxin proteins (Chapter 4.3). The first strategy was screening by amplifying part / whole of the endotoxin coding genes. This worked well for *Bti* strains. It was found that the Qatari *Bti* strains carried all the insecticidal endotoxin genes except *cry10A* and *cyt1C*. As all the Qatari *Bti* strains showed the absence of these two genes, further analyses were performed to explore this phenomenon. It was found that there is a structural instability in the region of pBtoxis plasmid between the positions 30 kb and 50 kb from the origin (Chapter 4.6). Plasmid mapping showed that the entire regions from 38 kb to 50 kb was missing in case of Qatari *Bti*. Among the Non-*Bti* strains, *QBT*674 was found to carry the gene for Cry2 δ-endotoxin. This is the first report of a spherical crystal forming *Bt* strain that carries *cry2* gene without the presence of cuboidal crystals. The second strategy used for screening for endotoxins was protein profiling using LC/MS for Non-*Bti* representative strains. It was found that a Non *Bti* strain *QBT*555 expresses *Bti* δ-endotoxins like

Cry10, Cry11 and Cyt1A. The absence of Cry4 protein in this *Bt* strain explains why its crystal proteins were ineffective against Dipteran insect larvae despite having the other endotoxins.

The *Bt* strains were further characterized based on their haemolytic and cytolytic properties (Chapter 4.4). As expected, the *Bti* strains have high haemolytic activities, but the Non-*Bti* strains showed varying degrees of haemolytic activities. Normally, the *Bt* strains with no haemolytic and no insecticidal activities are the ones with cytolytic activities. But, this study found *Bti* strains having insecticidal and haemolytic activities, to also possess high cytolytic activities. *QBT*229 was the most effective against lung cancer epithelial cells. The anti-cancer parasporin protein coding genes were absent in the Qatari *Bt* strains. The other protein Cyt1A responsible for cytolytic activities was further explored at the molecular and biochemical levels. It was found that Cyt1A protein of highly cytolytic *QBT*229 had five consecutive amino acid replacements in its β sheet. These amino acid replacements make the β sheet highly positive and polar. The β sheet is involved in the direct binding of the Cyt1A protein to the lipid bipolar membrane and recruiting Cry11 to the membrane. The highly positive and polar β sheet enhances both the functions of Cyt1A; thereby increasing its total cytolytic property.

Bti strains were selected for δ-endotoxin production experiments based on two conditions: high insecticidal activity and low cytolytic activity. The four strains *QBT*217, *QBT*218, *QBT*220 and *QBT*221 were tested for the δ-endotoxin (crystal/spore) production capacity. It was found that *QBT*217 and *QBT*220 had the highest δ-endotoxin yield per spore, compared to that of H14 and other Qatari *Bti* (Chapter 4.5). The two efficient strains were also tested in an alternative sporulation medium called Soya meal-starch based media. In the presence of complex sources of carbon and nitrogen, *QBT*220 showed the highest δ-endotoxin production compared to

the reference H14 and *QBT*217.

The candidate strains for production of bioinsecticides against Dipteran insects were chosen as *QBT*217 and *QBT*220. In the scale-up process and industrial level production, there is always a chance of losing the insecticidal endotoxin coding genes due to plasmid instability. Hence, the plasmid curing technique was conducted with *QBT*217 and *QBT*220 to isolate clones with highest plasmid stability (Chapter 4.6). In the process, the effect of plasmid curing on δ-endotoxin production was evaluated. The positive effect on δ-endotoxin production means choosing clones with higher δ-endotoxin production properties. Two important mutant clones of *QBT*220 were isolated, 220KN65 and 220KN66, with an increase in their δ-endotoxin yield per spore of about 69% and 115%, respectively.

In summary, from our Qatari *Bt* collection, the *Bti* strains *QBT*220 was recognized as the most efficient *Bt* strain to produce bio-insecticides against Dipteran insects. Two of its clones obtained by plasmid curing showed an increase of up to 115% in the δ-endotoxin yield per spore, even higher than the original strain *QBT*220. Novel *Bt* strains were recognized among the collection. *QBT*674 is aspherical crystal producing strains with *cry2* gene but does not produce cuboidal crystal. *QBT*555 is a Non *Bti* strain with molecular profile very different from *Bti* strains, but expresses *Bti* proteins like Cry11, Cry10, Cyt1A and p*Bt*152. *QBT*229 is an insecticidal and haemolytic *Bt* strain that also showed high cytolytic activity. Novel proteins were identified, like Cyt1A protein of the *Bti* strain *QBT*229 that had five amino acid replacements in its β sheet that enhanced its cytolytic activity.

Perspectives:

The candidate strains *QBT*220 and its isolated mutants in this project, should be used for scale-up experiments and optimize the growth conditions for production at fermenter level. *QBT*229 should be studied further for its anticancer property using more cancer and normal cell lines; adherent and suspension. *QBT*674 strains will have to be studied at molecular level to understand the reason why it fails to produce the cuboidal crystal despite the presence of *cry2* structural gene and the regulatory ORF2. Induced plasmid cured mutants are excellent candidates to be explored further for the enhancement of the production and / or activity of *Bt* toxins other than δ-endotoxins. The plasmid cured clones that have lost the sporulation machinery and retained the delta endotoxin genes are also interesting as these cells could be used as good model for developing encapsulated crystals without spores. The protein profiling of the Non *Bti* strains can be continued for the screening of any other proteins that are produced by the *Bt* strains during vegetative or sporulation stage as the procedure has been standardized in this PhD project. The thorough characterization and true classification of these 441 *Bt* strains producing spherical crystals, have been performed in this PhD project and hence it will be easy to screen this very precious gene bank for any interesting and high value molecule and activity in the future.

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APPENDIX

APPENDIX A: PUBLISHED ARTICLE 1

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Diversity of *Bacillus thuringiensis* strains from Qatar as shown by crystal morphology, δ-endotoxins and *cry* gene content

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Diversity of *Bacillus thuringiensis* Strains From Qatar as Shown by Crystal Morphology, δ-Endotoxins and *Cry* Gene Content

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Bacillus thuringiensis (Bt) based insecticidal formulations have been recognized as one of the most successful, environmentally safe and sustainable method of controlling insect pests. Research teams worldwide are in search of Bt diversity giving more choices of bio-insecticides and alternatives to address insect resistance. In fact, there are many unexplored ecologies that could harbor novel Bt strains. This study is the first initiative to explore Bt strain diversity in Qatar. A collection of 700 Bt isolates was constructed. Scanning electron microscopy of Bt crystals showed different crystal forms, with a high abundance of spherical crystals compared to the bipyramidal ones. Among the spherical crystals, four different morphologies were observed. The δ-endotoxin content of parasporal crystals from each Bt isolate revealed that there are 16 different protein profiles among the isolates of the collection. On the other hand, plasmid pattern analysis showed seven different plasmid profiles. Their insecticidal activity was predicted by exploring the δ-endotoxin coding genes and conducting qualitative insecticidal bioassays. 19 smooth spherical crystal producing isolates have been identified that could be possible candidates for endotoxin production targeting Dipteran insects. Another group of 259 isolates producing bipyramidal and cuboidal crystals could target Lepidopteran and Coleopteran insects. The remaining 422 isolates have novel profiles. In conclusion, Qatari soil ecology provides a good collection and diversity of Bt isolates. In addition to strains harboring genes encoding common endotoxins, the majority are different and very promising for the search of novel insecticidal endotoxins.

Keywords: ***Bacillus thuringiensis*, crystals, δ-endotoxins profiling, *cry* genes, plasmids**

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INTRODUCTION

Bacillus thuringiensis (Bt) is an entomopathogenic, rod shaped, Gram positive, spore-forming and aerobic bacterium found usually in soil, grain dusts, dead insects and water (Lambert and Peferoen, 1992). During the sporulation stage, they produce parasporal insecticidal protein crystals or δ-endotoxins (Jouzani et al., 2008a,b). Bt is considered the most successful bioinsecticidal alternative available to man today, owing to its toxicity toward a broad range of insect pests such as Dipteran, Lepidopteran and Coleopteran (Federici et al., 2006; Lacey et al., 2015). Based on their serotype and phylogenetic features, Bt is classified into subspecies; which are further classified into serotypes and

strains (Seifinejad et al., 2008). Some of the well-studied subspecies include Bt *israelensis* and Bt *kurstaki* that are currently being used as a source for endotoxin protein production at a commercial level (Dambach et al., 2014; Elleuch et al., 2015; Jeong et al., 2017; Zhang et al., 2017). Bt *israelensis* (Bti) produces a combination of endotoxins (Cry) and hemolytic proteins (Cyt) during sporulation stage and crystallize them in a spherical form. On the other hand, Bt *kurstaki* (Btk) produces a combination of other endotoxins (Cry) and crystallize them into bipyramidal form and cuboidal form (Adang et al., 2014). These endotoxins are target specific, for example, the spherical crystals of Bti are toxic to Dipteran insects while the bipyramidal and cuboidal crystals of Btk are toxic to Lepidopteran and Coleopteran insects (Jain et al., 2017). The parasporal crystal form is an indication of its Cry proteins content and is hence used as the first criterion of classification of Bt isolates (López-Meza and Ibarra, 1996; Ben-Dov et al., 1997; Mahalakshmi et al., 2012). As per the database of known endotoxins maintained by Crickmore et al. (2016), there are currently 74 known families of *cry* genes having 770 different *cry* genes and three *cyt* families having 38 *cyt* genes (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/ accessed on 8th October 2017). Apart from Cry and Cyt proteins, Bt also produces other insecticidal proteins called Vip (vegetative insecticidal proteins); which as the name suggests, are expressed during the vegetative stage (Abdelkefi-Mesrati et al., 2011; Abdelmalek et al., 2016) and other useful proteins like Bacteriocins (Jung et al., 2008; Kamouneh et al., 2011). The understanding of the Bt isolates with specific insecticidal genes, becomes even more complex, when one considers that these genes are mostly expressed on plasmids that can be transferred among each other; completely or partially (Rolle et al., 2005). Hence, every ecology might have Bt isolates with unique combinations of insecticidal genes. Research teams worldwide are constantly screening different ecologies to find such novel Bt isolates (Campanini et al., 2012; Soares-da-Silva et al., 2015; El-Kersh et al., 2016).

Knowing that Bt screening programs have never been conducted before in Qatar, this is the first study in the country where 700 Bt isolates were collected from Qatari soil in order to study the diversity and characteristics of Qatari Bt strains. For such big collections of isolates the first criterion of categorization is the crystal shape of the isolates. Ultimately our aim was to study the endotoxins produced by the isolates.

Techniques commonly used, to group and choose a true representative, include plasmid pattern comparisons, PCR (polymerase chain reactions) amplifications of known insecticidal genes, protein analysis, Pulsed-Field Gel Electrophoresis (PFGE), and Ribotyping. (Saadaoui et al., 2010; Sellami et al., 2013; Elleuch et al., 2015). Recent advances in the technologies have seen the use of next generation sequencing and "omics" studies like Genomics, Transcriptomics and Proteomics. (Dong et al., 2016). Although all the techniques have their advantages and limitations, there is always a need to use the right order of these techniques in order to avoid missing out on unique isolates; especially when it comes to big collections of isolates.

Abbreviations: Bt, *Bacillus thuringiensis*; Bti, *Bacillus thuringiensis israelensis*; Btk, *Bacillus thuringiensis kurstaki*; PCR, Polymerase chain reactions; PFGE, pulsed field gel electrophoresis; Plas, plasmid pattern; Prot, protein pattern; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Collection, Isolation, and Preservation of Qatari Bt Samples

Seven hundred Bt isolates were collected from soil samples in Qatar. Spore-forming isolates were obtained using the acetate selection method from Travers et al. (1987) with slight modifications. One gram of each soil sample was suspended in 10 ml of Luria Bertini (LB) broth, buffered with 250 mM Sodium Acetate (pH 6.8). The mixture was then incubated in a shaker incubator at 30°C for 4 h. After incubation, the samples were heated at 80°C for 15 min. From each of these samples, 100 µl was spread on T3 agar plates and incubated for 72 h at 30°C. Each pure isolate was subjected to microscopic observation to confirm the presence of spores and crystals. Each parasporal crystal forming Bt isolate was named QBT followed by its serial number. Bt isolates were then grown on T3 sporulation media (Travers et al., 1987) for 96 h and the spores-crystal mixture was stored in 30% glycerol at −80°C.

Identification of Crystal Morphology

Each sample was grown on T3 agar plates and incubated at 30°C for 96 h. After sporulation, the spore crystal mixture was checked under light microscope to identify the shape of the crystals and group the collection based on their crystal shapes: Bipyramidal, Cuboidal and Spherical. The crystals were then studied in detail under FEI Nova NanoSem 450 Scanning Electron Microscope (SEM), USA to identify intrinsic details and differences between crystals of different isolates. *Bacillus thuringiensis israelensis* (Bti) and *Bacillus thuringiensis kurstaki* (Btk) were used as reference strains.

Ribotyping Based on 16s rRNA Gene

The plasmid DNA of of Bt isolates representing the 4 classes forming different spherical crystals was used as template for amplification of 16s rRNA gene using the primer sets Rib73 (5′-AGAGTTTGATCCTGGCTCAG-3′) and Rib74 (5′-AGGAGGTGATCCAGCCGCA-3′). The amplification was carried out using polymerase chain reaction (PCR) in Applied Biosystems 96 wells Veriti Thermal Cycler from Thermo Fisher, USA. The amplified products were run on a 1.2% agarose gel. Expected band (1.5 kb) was gel purified using QIAquick gel extraction kit from Qiagen by following the instruction manual. The purified product was sequenced by Sangers sequencing using 3500 Series Genetic Analyzer by Thermo Fisher Scientific, USA. The 16s ribosomal DNA sequences were submitted to NCBI (National Center for Biotechnology Information) database. The sequences obtained were compared to published sequences on NCBI database.

Purification of Crystal Proteins

After complete sporulation, T3 culture with spore crystal mixture of each isolate was centrifuged and the pellet was washed thrice with 1 N NaCl. The pellet was then washed 3x with distilled water. The spore-crystal pellet was re-suspended in 50 mM NaOH and incubated at room temperature for 1 h to solubilize the crystal proteins.

Studying Protein Patterns by SDS-PAGE

The purified crystal proteins were combined with 2x boiling buffer (containing 0.1% ß-mercaptoethanol, 1% SDS, 0.025% Bromophenol blue and 10% glycerol) in the ratio 2:1, respectively. The samples were then boiled for 5 min along with a GelPilot broad range protein marker. Samples were loaded on a SDS-PAGE gel with a 10% separating gel and 3% stacking gel. The electrophoresis was run at 100 V for 2 h. The gels were stained with a staining solution containing 0.025% Coomassie Brilliant Blue R250. Destaining was performed overnight with a solution containing Ethanol, Glacial Acetic acid and Water in the ratio 5:7:88, respectively.

Isolation of Plasmid DNA and Plasmid Profiling

Total plasmid DNA was isolated by alkaline lysis method combined with lysozyme treatment and purified using alcohol precipitation as per Sambrook et al. (1989) with slight modifications. The plasmid profiles were obtained by running on 1% agarose gels with 0.5 µg/ml Ethidium bromide. The gels were loaded with 40 µl of each sample, and run at 10 V overnight in 1% TAE tank buffer. The plasmid patterns of the Bti and Btk were used as references.

Investigation of Hemolytic Activity

Blood Nutrient agar plates were prepared by adding 5 ml fresh sheep's blood to the 100 ml autoclaved Nutrient agar medium. The plates were divided into grids and each isolate was inoculated onto the Blood agar plates using sterile toothpicks. The plates were then incubated at 30°C overnight. The zone of clearance around the colony of each isolate were checked and the results were interpreted accordingly.

PCR Amplifications of Insecticidal Genes

Plasmid DNA was used as template for the PCR amplification of genes encoding Cry and Cyt proteins that are insecticidal to Dipteran, Lepidopteran and Coleopteran insects (**Table 1**). The PCR amplifications were carried out as per Jaoua et al. (1996) using Applied Biosystems 96 wells Veriti Thermal Cycler from Thermo Fisher. Two groups of primers were used for amplifications. First group consisted of Dipteran specific genes including *cry4A/4B, cry11, cry10, cyt1A, cyt1C, cyt2A, p19,* and *p20. Second* group consisted of Lepidopteran and Coleopteran specific genes including *cry1A, cry1IA, cry1B, cry1D, vip3a,* and *cry2.*

Protein Preparation for Insecticidal Bioassay

A single colony from 24 h incubated LB agar plates was re-suspended in 50 ml LB broth in 250 ml flask and incubated at 30°C in a shaker incubator for 24 h. The O.D of the culture was checked after 24 h and 50 ml T3 broth was inoculated with this pre-culture such that the starting O.D of all the isolates were 0.1. The T3 broth cultures were incubated at 30°C in shaker incubator for 96 h, ensuring complete sporulation. The broth was centrifuged at 10000 rpm for 10 min and the pellet was re-suspended in 10 ml distilled water. The spore crystal mixture was then diluted 5 times and was used as testing solution for the bioassay.

Qualitative Bioassay of Insecticidal Activity

Third and fourth instar larvae of *Culex pipiens complex* were used for bioassay. For each test, five larvae were transferred to the test solution. And incubated overnight at room temperature. All the 19 Bti like isolates and the representatives of other classes with spherical crystals were tested for insecticidal activity. The number of larvae that survived was calculated for each Bt isolate δ-endotoxin sample.

RESULTS

Characterization of Bt Isolates Based on Crystal Morphology and Phylogeny

The observation of crystal forms of the 700 isolates carried out by light microscope allowed the collection to be classified into two main classes.:441 isolates producing spherical crystals and 259 isolates producing bipyramidal and cuboidal crystals. The crystals were observed by SEM in order to evidence any further differences among the same crystal forms of each class. The results showed that the bipyramidal and cuboidal crystals resembled the standard crystals of the reference Bt *kurstaki* HD1. However, the spherical crystals showed four different shapes when studied at higher magnifications of SEM. In fact, among the collection of 441 spherical crystal producing isolates four groups were identified: smooth spherical (like the reference Bti), spherical with undulated surface, spherical but deflated balloon shape, spherical with one concave side and pointy edged shape (**Figure 1**).

The plasmid DNA of Bt isolates representing the 4 classes forming different spherical crystals was used for the amplification of the 16s ribosomal DNA using the primers Rib73 and Rib74. The PCR products obtained were purified from the gel using the QIAquick gel extraction kit, following the manual instructions. The purified PCR products were then sequenced by Sangers sequencing. The sequences were submitted to NCBI GeneBank for 16s rRNA and the accession numbers for the same were obtained as MG995012, MG995013, MG995014, MG995015. The *in-silico* analyses of these sequences showed that they had very high (up to 99%) similarity to the published sequences of Bt 16s rRNA genes in the NCBI database. This confirms that the isolates that were identified and characterized in this collection definitely belong to the Bt family.

TABLE 1 | Primers used in this study for the exploration of genes encoding endotoxin, accessory proteins and Cyt.

Gene	Primers	Sequences	Reference
cry1A	Lep1A	5′ CCGGTGCTGGATTTGTGTTA 3′	Carozzi et al., 1991
	Lep1B	5′ AATCCCGTATTGTACCAGCG 3′	
cry1B	Cry1B1	5′ CTTCATCACGATGGAGTAA 3′	Cerron et al., 1994
	Cry1B2	5′ CATAATTTGGTCGTTCTGTT 3′	
cry1D	Cry1D1	5′ CTGCAGCAAGCTATCCAA 3′	Cerron et al., 1994
	Cry1D2	5′ ATTTGAATTGTCAAGGCCTG 3′	
cry1IA	Cry5A	5′ ATGAAACTAAAGAATCAAGA 3′	Masson et al., 1998
	Cry5B	5′ ACCTGTGCTATACCATTTCA 3′	
cry2	Cry2-1	5′ GTTATTCTTAATGCAGATGAATGGG 3′	Ben-Dov et al., 1997
	Cry2-2	5′ CGGATAAAATAATCTGGGAAATAG 3′	
vip3a	Vip1	5′ ATGAACAAGAATAATACTA 3′	Abdelkefi-Mesrati et al., 2005
	Vip3	5′ TTACTTAATAGAGACATCGT 3′	
cry4A/4B	Dip2A	5′ GGTGCTTCCTATTCTTTGGC 3′	Carozzi et al., 1991
	Dip1B	5′ ATGGCTTGTTTCGCTACATC 3′	
cry10	Cry10-1	5′ ATATGAAATATTCAATGCTC 3′	Porcar et al., 1999
	Cry10-2	5′ ATAAATTCAAGTGCCAAGTA 3′	
cry11	Cry11-1	5′ TTAGAAGATACGCCAGATCAAGC 3′	Bravo et al., 1998
	Cry11-2	5′ CATTTGTACTTGAAGTTGTAATCCC 3′	
cyt1A	Cyt1A1	5′ GTTGTAAGCTTATGGAAAAT 3′	Zghal et al., 2008
	Cyt1A2	5′ TTAGAAGCTTCCATTAATA 3′	
cyt1C	Cyt1C1	5′ CAAAATCTACGGGAGCAAGG 3′	Designed for this study
	Cyt1C2	5′ GGAAGGATCCCTTTGACTTTT 3′	
cyt2A	Cyt2A1	5′ AATACATTTCAAGGAGCTA 3′	Guerchicoff et al., 1997
	Cyt2A2	5′ TTTCATTTTAACTTCATATC 3′	
p19	p19-1	5′ GCAGGAGGAACATCACCATT 3′	Designed for this study
	p19-2	5′ GGATTTGCTGAGCAGGTCAT 3′	
p20	p20-1	5′ TGACGAGGAAACAGAGTATACGA 3′	Designed for this study
	p20-2	5′ TGAAAGGTTAAACGTTCCGATT 3′	

δ-Endotoxin Profiling Among Bt Isolates

The protein patterns of the isolates producing bipyramidal and cuboidal crystals were similar to that of the reference subspecies *kurstaki*. They have one protein of about 130 kD, two proteins of about 65 kD and a protein of about 40 kD. However, the spherical crystals harbor different protein patterns when compared to the reference subspecies *israelensis*. In fact, 15 different protein patterns (**Figure 2**) were evidenced. One protein pattern matched that of the reference Bt *israelensis* H14. These profiles showed mainly the presence of one protein of 130 kD, two proteins of about 65 kD, three proteins of about 45 kD and two proteins of about 27 kD. But 14 other types of protein patterns were observed among the collection. The main proteins as per their sizes are listed in **Table 2**. Hence, as per the protein patterns, 16 groups (Prot 1 to Prot 16) were evidenced in the collection: 256 Btk like isolates with one protein pattern (Type 11), 19 Bti like isolates with one protein pattern (Type 1) and 14 protein patterns shown by 422 spherical crystals producing isolates (bifurcations shown in **Table 2**).

Distribution of Bt Collection Based on Plasmid Patterns

The investigation of plasmid patterns of the Bt isolates showed that all bipyramidal and cuboidal crystal producing isolates had the same plasmid pattern; similar to that of the reference Bt *kurstaki* HD1. Additionally, the 19 Bti like spherical crystal producing isolates have the same plasmid pattern as that of the Bt *israelensis* H14. However, among the other 422 spherical crystal producing isolates, only 5 types of plasmid patterns were observed. Contrary to the 16 groups of protein patterns in our collection, we have only 7 types (Plas 1 to Plas 7) of plasmid patterns: one Btk like, one Bti like and 5 Non-Bti like (**Figure 3**). These findings showed that the 700 isolates harbor a total of seven plasmid patterns and 16 different protein patterns (**Table 2**).

Hemolytic Activity Among Qatari Bt Isolates

The 19 Bti like isolates with smooth spherical crystals, all showed positive hemolysis around their colonies after overnight incubation. The others showed different degrees of hemolysis. Among the crystal forms, the isolates with spherical deflated balloon shaped crystals did not show any hemolytic activity. On the other hand, the isolates with spherical undulated surface crystals showed different hemolytic activities. Some had no activity while some had good or slight activities. The isolates with spherical concave crystals and spherical pointy edged crystals showed good hemolytic activity (**Figure 4**, **Table 2**). The isolates

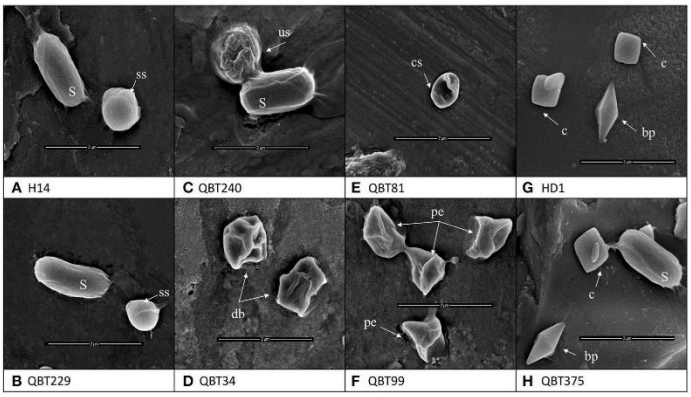

FIGURE 1 | Scanning electron microscopy images of the different types of crystal morphologies and the spores (S) produced by Bt strains of the collection. **(A)** Reference strain *Bacillus thuringiensis israelensis* H14 with smooth spherical [ss] crystal **(B)** Qatari Bti like isolate QBT229 with smooth spherical [ss] crystal **(C)** Spherical crystal with undulated surface [us] QBT240 **(D)** Spherical but deflated balloon [db] shape QBT34 **(E)** Spherical crystals with concave surface [cs] QBT81 **(F)** spherical crystals with pointy edges [pe] QBT99 **(G)** reference strain *Bacillus thuringiensis kurstaki* HD1 with bipyramidal [bp] and cuboidal [c] crystals **(H)** Qatari Btk like isolate QBT375 with bipyramidal [bp] and cuboidal [c] crystals.

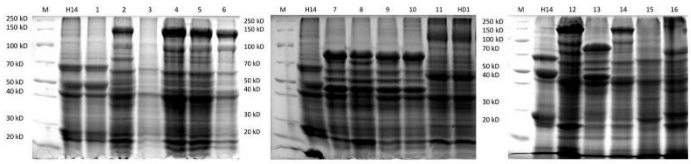

FIGURE 2 | SDS-PAGE gels showing the 16 different protein patterns among the Bt strains collection (1–16). Lanes marked as M is broad range protein marker; H14 is the reference strain *Bacillus thuringiensis israelensis* and HD1 is the reference strain *Bacillus thuringiensis kurstaki*; 1, QBT229; 2, QBT3; 3, QBT6; 4, QBT34; 5, QBT39; 6, QBT212; 7, QBT240; 8, QBT320; 9, QBT418; 10, QBT555; 11, QBT375; 12, QBT41; 13, QBT43; 14, QBT56; 15, QBT81; 16, QBT99.

with bipyramidal crystals showed hemolytic activity among them like the reference Bt *kurstaki*.

Insecticidal Activity via Investigation of δ-Endotoxin Genes and Bioassay

The 19 isolates that resembled the profile of the reference Bt *israelensis* H14 gave the expected PCR product for amplifications with primers designed for exploring genes encoding Dipteran specific endotoxins; except for *cry10* and *cyt1C* genes. Hence, these isolates were tested for their insecticidal activity against *Culex pipiens complex*. The 259 isolates that resembled the profile of the reference Bt *kurstaki* HD1gave all the expected PCR products with primers designed for identifying the presence of genes encoding Lepidopteran and Coleopteran specific endotoxins. The rest of the isolates with spherical crystals did not give expected PCR products with the primers that we tested.

As the 19 Bti like isolates that showed the possible absence of the two important Dipteran specific endotoxin coding genes, the insecticidal bioassay was conducted to check the effect, if any, on the activity. First group consisted of 19 Bti like isolates which were able to kill all the larvae as expected. The second group consisted of 14 representatives that had different protein patterns; they did not show insecticidal activity (**Table 2**).

DISCUSSION

Since its first isolation in 1902 by Ishiwata, Bt has been isolated from various ecologies and has been widely studied. In spite of this worldwide exploration, the research on this bacterium is still incomplete (Melo et al., 2016) because of the many ecologies that still remain unexplored and the fact that Bt based insecticides have not been able to completely replace the harmful chemical insecticides in the market. In this study, one such ecology was

TABLE 2 | Qatari Bt strains collection summarized into 16 classes with the help of true representatives selected based on crystals morphology, proteomic, and genomic characteristics; QBT229 is the representative of Qatari Bti like isolates, QBT376 is the representative of Qatari Btk like isolates, others represent different spherical crystal morphologies observed with various protein and plasmid pattern.

Family representative	Crystal shape	Protein pattern	Protein sizes in classes (kD)	Plasmid patterns	No. of isolates	Haemolytic activity	Insecticidal activity	Bt *cry, cyt* and accessory genes present
H14	Smooth spherical	–		–		Yes	Yes	*cry4A, cry4B, cry11, cyt1A, cyt2A, cry10, cyt1C, p19, p20*
QBT229		Prot 1	130, 65, 45, 27	Plas 1	19	Yes	Yes	*cry4A, cry4B, cry11, cyt1A, cyt2A, p19, p20*
HD1	Bipyramidal and Cuboidal	–		–		Yes	–	*cry1A, cry1IA, cry1B, cry1D, vip3a, cry2*
QBT376		Prot 11	130, 65, 40	Plas 7	259	Yes	–	*cry1A, cry1IA, cry1B, cry1D, vip3a, cry2*
QBT6	Spherical undulated surface	Prot 3	100, 65, 40	Plas 2	7	Yes	No	–
QBT43		Prot 13	85, 65, 55, 45, 30, 27, 22	Plas 3	33	Slight	No	–
QBT212		Prot 6	130, 90, 60, 40, 22	Plas 4	10	No	No	–
QBT240		Prot 7	100, 80, 60, 45, 40,	Plas 4	48	Slight	No	–
QBT320		Prot 8	80, 60, 45, 40, 35	Plas 4	16	Slight	No	–
QBT418		Prot 9	150, 80, 60, 45, 40, 27	Plas 4	9	Slight	No	–
QBT555		Prot 10	180, 150, 80, 60, 45, 40, 27	Plas 4	203	No	No	–
QBT3	Spherical deflated balloon	Prot 2	130, 90, 75, 65, 50, 40, 25	Plas 6	4	No	No	–
QBT34		Prot 4	180, 140, 90, 80, 60, 45, 25	Plas 6	19	No	No	–
QBT39		Prot 5	140, 90, 80, 60, 45, 25	Plas 6	12	No	No	–
QBT41		Prot 12	130, 90, 75, 55, 40, 27, 22	Plas 6	29	No	No	–
QBT56		Prot 14	130, 100, 65, 45, 27, 22	Plas 6	28	No	No	–
QBT81	Spherical concave surface	Prot 15	65, 45, 35, 27	Plas 6	*2*	Yes	No	–
QBT99	Spherical pointy edged	Prot 16	230, 150, 85, 70, 40, 27, 25	Plas 5	*2*	Yes	No	–
				Total	700			

explored for the first time: Qatari soil. Among the bacterial isolates from soil, 700 isolates were identified as Bt as they all produced parasporal crystals during sporulation. The first level of grouping was done by light microscopy, where 259 isolates were found to produce bipyramidal and cuboidal crystals while the majority (441 isolates) produced spherical crystals. This was contrary to the usual Bt screenings of environmental samples, where the majority produces bipyramidal crystals (Meadows et al., 1992; Bernhard et al., 1997). As Qatar started local agricultural activities very recently, the unavailability of crops and associated insect pests could be a reason for the relatively lower number of Lepidopteran specific Bt strains producing bipyramidal and cuboidal crystals. In this work, Scanning Electron Microscopy was used to not only confirm the crystal shape, but also to further magnify and differentiate between the spherical crystal shapes. Although different types of spherical crystals have been reported before (Noguera and Ibarra, 2010), it has been rarely used as a tool for classifying big Bt collections (Djenane et al., 2017). From SEM images, it was found that among the spherical crystals, there are four kinds of spherical shapes in the collection. This shows the wide diversity among the Qatari Bt strains. The identity of the

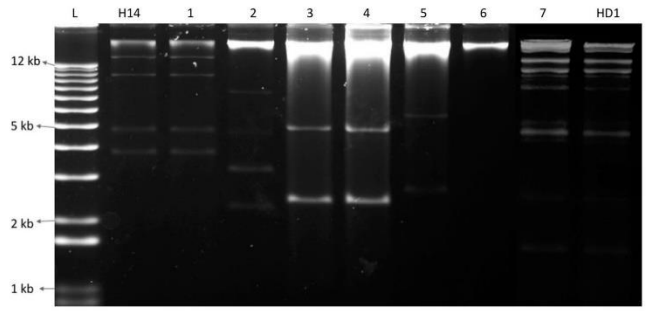

FIGURE 3 | Electrophoresis gel showing seven different plasmid patterns observed among the Bt collection (1–7). L represents a 1 kb plus ladder; H14 is the reference strain Bacillus thuringiensis *israelensis*, HD1 is the reference strain Bacillus thuringiensis *kurstaki*; 1, QBT229; 2, QBT6; 3, QBT43; 4, QBT212; 5, QBT99; 6, QBT3; 7, QBT375.

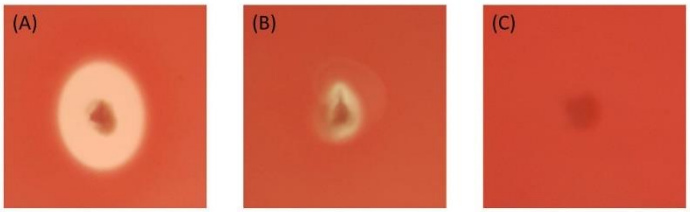

FIGURE 4 | Different types of the hemolytic activities shown by Bt isolates. **(A)** Positive hemolytic activity QBT229 **(B)** Slight hemolytic activity QBT43 **(C)** Negative hemolytic activity QBT3.

isolates as Bt strains were confirmed by 16s ribosomal DNA amplification, sequencing and comparing with the published strains from NCBI database. All the isolates belong to the Bt family. When the proteomic content of the parasporal crystals were studied by SDS-PAGE, it was found that isolates with the same crystal shape also had differences among their protein content. In fact, while there are just six types of crystal shapes in the collection, there are 14 types of protein patterns. As the proteins of the parasporal crystals are often encoded by the genes on plasmids, an equal number of plasmid patterns were expected. But, contrary to the published work and theories regarding Bt parasporal crystals (Aptosoglou et al., 1997; Reyes-Ramírez and Ibarra, 2008; Fagundes et al., 2011), only seven plasmid patterns were found among the isolates of the collection. This discrepancy among the protein and plasmid patterns lead to two conclusions. First, the use of plasmid pattern as a tool to identify the diversity among the isolates of an ecology has limitations. In this study, if the plasmid patterns were used as a primary tool of grouping the collection, the actual diversity of the collection wouldn't have been evidenced. Second, as the interest lies in the crystal proteins that have the insecticidal activity, the protein pattern should be ideally used to classify the collection into groups. This will help one understand the real diversity and select true representatives for molecular and genomic studies.

The hemolytic activity of the strains representatives (Bti like and Btk like isolates) was tested and the collection showed varying degrees of hemolytic activities as expected. Among them, the interesting group was the isolates with spherical crystals having undulated surface. The representatives of this group, had varying hemolytic activities. This single group has isolates with good hemolytic activities, slight hemolytic activity and no hemolytic activity. This shows for the first time, the polymorphism among the isolates with same crystal forms.

PCR amplifications were carried out with primers designed to explore *cry* and *cyt* genes, in order to predict their insecticidal activity. Among the Bt collection, 19 isolates resemble *Bt israelensis* H14 in crystal morphology, plasmid pattern and protein pattern. PCR amplifications showed that they carry the Dipteran specific insecticidal protein coding genes like *cry4A/4B*, *cry11*, *p19*, *p20*, *cyt1A*, and *cyt2*. These 19 isolates among

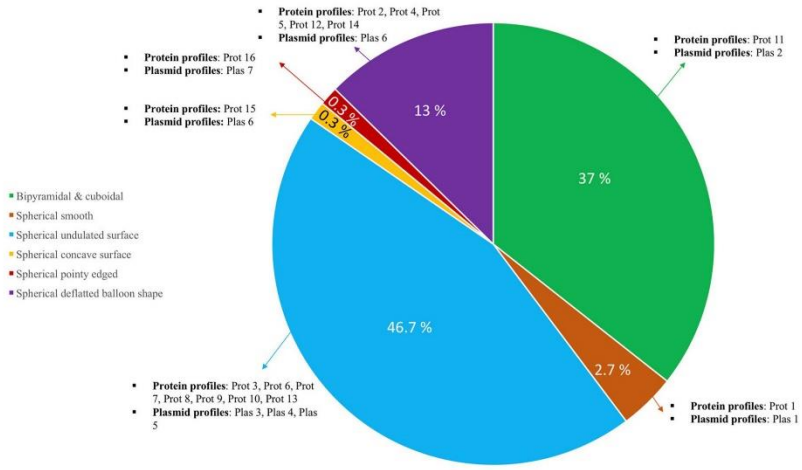

FIGURE 5 | Pie chart showing the diversity among 700 Qatari Bt isolates. Main distribution in the pie is based on crystal forms and each section has been further classified based on the types of protein and plasmid profiles observed among them.

our collection would be the candidates to be tested for their insecticidal activities against Dipteran insects. All these Qatari Bti like isolates failed to give the PCR amplifications for primers designed for *cry10* and *cyt1C* gene. Knowing that these two genes are located next to each other on pBtoxis plasmid of Bti (Berry et al., 2002), it was proposed that there is some kind of structural instability among the Qatari Bti like isolates in this plasmid region. Hence, it was necessary to check the insecticidal activity against Dipteran insects. As shown in **Table 2**, these Qatari Bti like isolates were able to kill all five larvae of *Culex pipiens*, like reference strain *Bt israelensis* H14. The insecticidal activity still persists among these 19 isolates, in spite of the possible absence of *cry10* and *cyt1C* genes. The exploration of the concerned region of the plasmid will be carried out in further genomic studies to understand the structural instability.

The isolates producing bipyramidal and cuboidal crystals carry all the Lepidopteran and Coleopteran specific insecticidal protein coding genes that were tested by PCR. They harbor *cry1A, cry1IA, cry1B, cry1D, vip3a,* and *cry2*. These 259 isolates producing bipyramidal and cuboidal crystals harboring the gene encoding the essential δ-endotoxins are the candidates for bio-larvicidal production against Lepidopteran and Coleopteran insects. Other 422 isolates gave no amplifications for all the primer sets that were tested so far. This leads to the conclusion that the 422 isolates either have new δ-endotoxin genes and/or different forms of the known endotoxin genes, which could not be detected by PCR. They are very promising in the search of novel endotoxins. These isolates will be studied further on genomic as well as proteomic levels to identify the types of delta-endotin genes harbored by them.

In conclusion, in the first study of Bt screenings in Qatar, the 700 isolates were characterized and grouped into 16 classes (**Figure 5**, **Table 2**). Qatar's microbial community offers a good diversity of Bt isolates that could be potential candidates for local production of bio-larvicides against many insect families. The parasporal crystal morphology and protein content of the crystals were given more importance when classifying them. This study shows the advantages of using proteomic techniques over genomic techniques when screening and studying big collections of Bt. We believe that from each group, the representatives that we chose will truly embody its respective group and make it easier to run genomic and proteomic studies accurately with them in the future.

AUTHOR CONTRIBUTIONS

KN, RA-T, DA-T, FA-Y, TA, and SJ made considerable contribution to the design of the work, procurement of the data and planning the work. KN and SJ made the analysis and interpretation of data, wrote the final version of this manuscript, are accountable for all of the work in ensuring the accuracy and reliability of the work.

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APPENDIX B: PUBLISHED ARTICLE 2

Type of article: Original article

Title:
The Replacement of five Consecutive Amino Acids in the Cyt1A Protein of *Bacillus thuringiensis* Enhances its Cytotoxic Activity against Lung Epithelial Cancer Cells

Journal: Toxins

Impact factor: 3.3

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toxins

MDPI

Article

The Replacement of five Consecutive Amino Acids in the Cyt1A Protein of *Bacillus thuringiensis* Enhances its Cytotoxic Activity against Lung Epithelial Cancer Cells

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Abstract: Cyt1A protein is a cytolytic protein encoded by the *cyt* gene of *Bacillus thuringiensis* subsp. *israelensis* (Bti) as part of the parasporal crystal proteins produced during the sporulation. Cyt1A protein is unique compared to the other endotoxins present in these parasporal crystals. Unlike δ-endotoxins, Cyt1A protein does not require receptors to bind to the target cell and activate the toxicity. It has the ability to affect a broad range of cell types and organisms, due to this characteristic. Cyt1A has been recognized to not only target the insect cells directly, but also recruit other endotoxins by acting as receptors. Due to these mode of actions, Cyt1A has been studied for its cytolytic activity against human cancer cell lines, although not extensively. In this study, we report a novel Cyt1A protein produced by a Bti strain QBT229 isolated from Qatar. When tested for its cytotoxicity against lung cancer cells, this local strain showed considerably higher activity compared to that of the reference Bti and other strains tested. The possible reasons for such enhanced activity were explored at the gene and protein levels. It was evidenced that five consecutive amino acid replacements in the β8 sheet of the Cyt1A protein enhanced the cytotoxicity against the lung epithelial cancer cells. Such novel Cyt1A protein with high cytotoxicity against lung cancer cells has been characterized and reported through this study.

Keywords: *Bacillus thuringiensis* subsp. *israelensis*; Novel Cyt1A; cytotoxicity; lung epithelial cancer cell line; protein modelling

Key Contribution: A novel Cyt1A protein has been identified among the Bt collection from Qatar. This reported new Cyt1A protein has an original β8 sheet, with five amino acid replacements, that enhances its cytotoxic activity against lung cancer cell lines.

1. Introduction

Bacillus thuringiensis (Bt) is a Gram-positive, spore forming, aerobic soil bacterium found in different environments [1]. It belongs to the six-member group of *Bacillus cereus*, including others like *Bacillus anthracis*, *Bacillus cereus*, *Bacillus weihenstephanensis*, *Bacillus pseudomycoides*, and *Bacillus mycoides*. The factor differentiating Bt from the others is that it forms parasporal protein crystals during the sporulation stage [2]. The parasporal crystals are made up of many insecticidal proteins called δ-endotoxins including Cry toxins and cytolytic proteins called Cyt toxins [3]. It has

been demonstrated that the Cry toxins are specifically insecticidal to larvae of many insect orders including Lepidoptera, Diptera, Coleoptera, Hymenoptera, Isoptera, etc., [4,5]. The specificity of the Cry toxins towards each order of insects is based on its mode of action, where the Cry toxins require specific binding receptors in the insect midgut to bind. The Cyt proteins, on the other hand, do not require receptors and can initiate the lysis of insect gut epithelial cells without specific binding [6]. Bt Cry toxins are very specific to their host families and, hence, have been recognized as the most efficient and sustainable biological alternative to the harmful chemical insecticides [7].

Apart from the insecticidal activity, Bt is also known for its antimicrobial, antifungal, and anti-cancer properties [8–11]. The anticancer properties of Bt is being widely studied today as it has high cytotoxic activity towards cancer cell lines without affecting the normal cells at the same levels of treatment. The anticancer property of Bt is attributed to two factors: Parasporins and Cytolytic proteins (Cyt) [12]. Parasporins are Cry like proteins with less that 25% amino acid sequence homology with the known Cry toxins. Like Cry toxins, Parasporins also act specifically to their target cancer cell lines as they require specific binding receptors on the cell lines for their activity [13–15]. They have now been classified into six groups: Parasporin 1 to Parasporin 6 [16–18].

Bt has three *cyt* gene families comprising of 38 *cyt* genes (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). Cyt proteins affect a wider range of target cells and organisms as they do not require any specific receptors [19]. They are known to have much lower insecticidal or cytotoxic activity than Cry or Parasporins [20]. They are also very useful for their ability to synergize other proteins, like Cry4 and Cry11 [21,22]. Therefore, through their synergistic action, Cyt proteins enhance the Bt insecticidal and cytotoxic activities.

In the recent years, the lack of efficient treatment strategies and the ever-increasing rates of cancers, have pushed the researchers to study the anticancer property of Bt intensively. In this study, Bt isolates collected locally from Qatari soil were explored for their cytotoxic activity. *Bacillus thuringiensis* subsp. *israelensis* (Bti) H14 and *Bacillus thuringiensis* subsp. *kurstaki* HD1 were used as reference strains. The isolates used in this study were characterized as Bti-like, based on their proteomic and genomic studies [23]. In spite of the similarity between the Bti and Qatari Bti-like isolates, the cytotoxic activity observed among the latter were considerably higher against a lung cancer cell line. After eliminating the possible presence of *parasporin* genes, it was found that the amino acid sequence of the Cyt protein from Qatari Bti strain QBT229 differed considerably from that of the reference H14 along the β8 sheet of the Cyt protein. The β8 sheet is involved in two processes: binding to the lipid bilayer of the target cell membrane and act as a receptor for Cry11 protein. This is a first study where a novel Cyt1A protein has been reported with amino acid replacements in the β8 sheet which enhances the cytotoxicity of the Bt crystal proteins against lung cancer cells.

2. Results

2.1. Determination of Cytotoxicity of Qatari Bt Strain Proteins against Lung Cancer Cells

The standard MTT assay for quantifying the cytotoxic activity of the Bt proteins was performed for this study with some modifications. After 72 h of incubation with solubilized and activated Bt strain proteins, the cells were treated with MTT reagent for three hours. This allowed the reagent to be converted to formazan by the live cells. The quantity of the formazan formed in each well corresponds to the number of live cells in each well. Hence, the untreated well represented 100% viability. The formazan was quantified by solubilizing in dimethyl sulfoxide (DMSO) and checking the absorbance by the purple dye formed during the process. The three Qatari Bti strains QBT213, QBT220 and QBT229 showed varying degrees of cytotoxic activity after 72 h incubation (Figure 1). However, QBT229 showed the highest cytotoxicity. In fact, while the reference strain Bt subsp. *israelensis* H14 and other local Bt subsp. *israelensis* strains QBT213 and QBT220 showed cell viability of more than 60% even at the highest concentration of solubilized Bt proteins tested

(0.5 µg/mL), the Bt subsp. *israelensis* QBT229 could kill more than 50% of cancer cells even at the lowest concentration of solubilized Bt proteins tested (0.15 µg/mL).

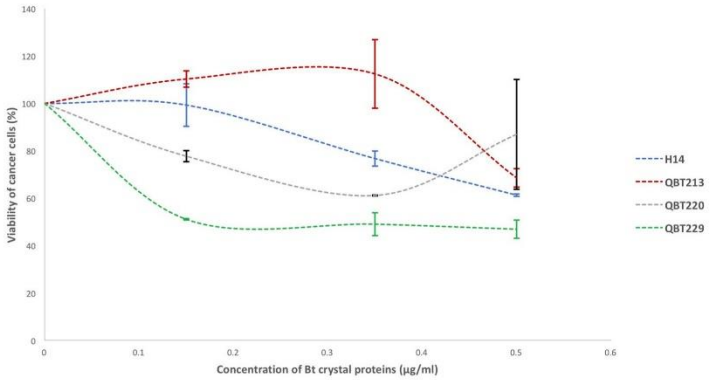

Figure 1. Cytotoxic effects of local Bt subsp. *israelensis* strains against lung epithelial cancer cells (NCI-H1975). Calculated viability of cells plotted against the concentration of endotoxin proteins (treatment).

2.2. Investigation of Genes Encoding Endotoxins, Parasporin, and Cyt Proteins

To understand the difference in cytotoxic activity between the QBT strains and the reference strain Bti H14, PCR screenings were performed to identify the types of genes they might be carrying. For these screenings, primer sets were designed or adopted from the published articles to detect the presence of the genes of interest (Table 1). Among the endotoxin coding genes carried by reference H14, the Qatari Bti-like isolates showed the presence of *cry4A/4B*, *cry11*, *p19*, and *p20*, except *cry10* (Figure 2). The PCR amplification with the cry10 primer sets produced a PCR product of about 2 kb, instead of the expected 1.1 kb given by the reference (Figure 2D). This PCR product was purified from the gel and sequenced by Sanger sequencing and the sequence had no resemblance to any known endotoxin genes. It was concluded that the three Qatari Bt subsp. *israelensis* strains lack the gene *cry10*.

Among the Cyt family of proteins, Qatari isolates showed the presence of *cyt1A* and *cyt2A* genes, but gave no PCR product with primer sets designed for *cyt1C* gene (Figure 2). Among the parasporin genes, neither the reference (as expected) nor the Qatari isolates gave any PCR products for any of the primer sets designed for parasporin genes (data not shown).

Table 1. The list of amino acid replacements seen in Qatari Bt subsp. *israelensis* QBT229 Cyt1A protein compared to the reference Bti H14.

Amino Acid Positions	Bt subsp. *israelensis* H14	Qatari Bt subsp. *israelensis* QBT229
225	Lysine (+) (Charged)	Asparagine (+) (Polar)
226	Phenylalanine (Hydrophobic)	Leucine (Hydrophobic)
227	Alanine (Hydrophobic)	Histidine (+) (Polar)
228	Glutamine (Polar)	Asparagine (+) (Polar)
229	Proline (Hydrophobic)	Histidine (+) (Polar)

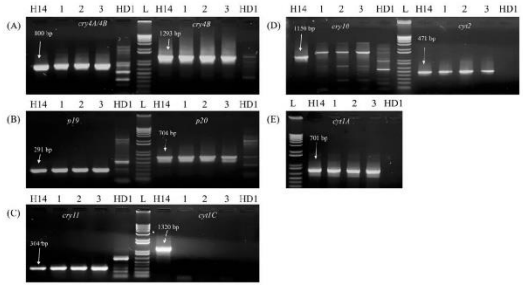

Figure 2. Electrophoresis of the amplified PCR products obtained for each δ-endotoxin gene being explored, Lanes L represent 1 kb plus ladder (100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 650 bp, 850 bp, 1 kb, 1.65 kb, 2 kb, 5 kb, and 12 kb), Lanes 1, 2, and 3 represent QBT213, QBT220, and QBT229 respectively, Lanes H14 and HD1 represent references Bt subsp. *israelensis* and Bt subsp. *kurstaki* respectively: each panel represents PCR amplification results for (**A**) 800 bp of *cry4A/4B* and 1293 bp of *cry4B*; (**B**) 291 bp of *p19* & 704 bp of *p20*; (**C**) 304 bp of *cry11* & 1320 bp of *cyt1C*; (**D**) 1150 bp of *cry10* & 471 bp of *cyt2*; and (**E**) 701 bp of *cyt1A*..

2.3. Investigation of the cyt1A Gene of Qatari Bt subsp. israelensis Strain QBT229

QBT229 showed the highest cytotoxic activity among the Qatari isolates (Figure 1). It showed a LC50 of about 0.15 µg/mL as opposed to the reference H14 that showed 60% viability even at highest concentration tested (0.5 µg/mL). Hence, the *cyt1A* gene sequence of QBT229 (accession number: MG708177) was compared with that of *cyt1A* gene of the reference Bt subsp. *israelensis* H14. The comparison between the sequences revealed at least six nucleotide differences among the sequences.

2.4. Translation and Amino Acid Sequence Alignment to Study the Cyt1A Protein

The DNA sequence of *cyt1A* gene from QBT229 (accession number: MG708177) was translated into amino acid sequence and aligned with published cyt1A protein sequence using in silico tools. The alignment of Cyt1A amino acid sequences of QBT229 and that of reference H14 revealed six amino acid replacements (Figure 3). At position 225, Lysine has been replaced by Asparagine, at position 226 Phenylalanine has been replaced by Leucine, at position 227 Alanine has been replaced by Histidine, at position 228 Glutamine has been replaced by Asparagine and at position 229 Proline has been replaced by another Histidine.

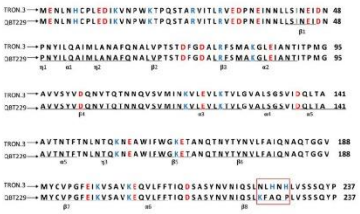

Figure 3. Amino acid alignment of Cyt1A protein sequence from QBT229 and the template TRON.3 representing the Cyt1A sequence and model for Cyt1A protein from *Bacillus thuringiensis* subsp. *israelensis*; red box represents the region of β8 sheet with amino acid replacements; amino acids highlighted in red have negative charges and the amino acids highlighted in blue have the positive charge; amino acids included in secondary structures like β sheets, α helix, and η are marked in the template.

2.5. Chemical Differences Due to Amino Acid Replacements by Protein Modelling

When the region of the protein structure with amino acid replacements (position 225 to 229) were checked, no structural changes were observed in the region (Figure 4A,B). The region was further studied based on possible chemical changes, in order to understand the effect of these changes on the cytotoxicity. In this region, QBT229 has four polar and positively charged amino acids (2× Asn, 2× His) and one hydrophobic amino acid (Leu) that have replaced three hydrophobic amino acids (Phe, Ala, Pro), one polar amino acid (Gln) and one positively-charged amino acid (Lys) (Table 2). Cyt1A protein consists of eight β sheets and six α helices. The region consisting of amino acids from position 225 to 229 forms part of β8 sheet (Figure 3). All these changes in QBT229 have made this region comparatively positively charged and polar (Figure 4C,D, Table 2).

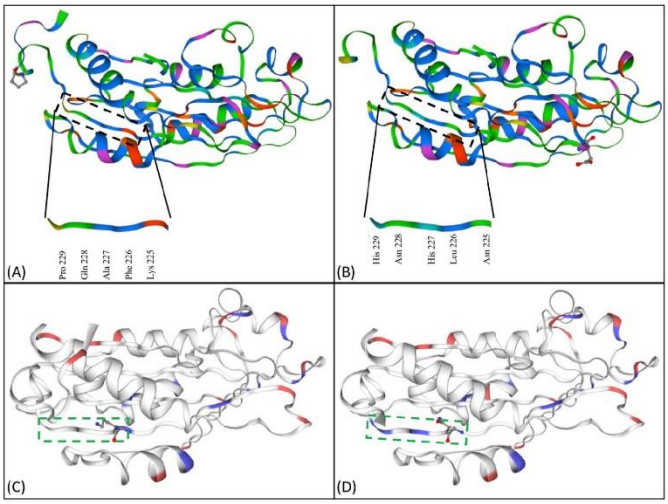

Figure 4. Protein modeling and structural and chemical homology comparisons between Cyt proteins. The ClustalX protein model of the Cyt protein expressed by (**A**) Bt subsp. *israelensis* H14 and (**B**) Qatari Bt subsp. *israelensis* QBT229, showing the amino acid replacements and chemical differences in the region; the positive (blue) and negative (red) charges owing to the amino acids in the Cyt protein of (**C**) Bt subsp. *israelensis* H14 and (**D**) Qatari Bt subsp. *israelensis* QBT229.

3. Discussion

The parasporal crystal proteins of Qatari Bt subsp. *israelensis* strains were tested against lung epithelial cancer cells to quantify their cytotoxic effects by MTT assay. It was found that the cytotoxic activities exhibited by Qatari Bt subsp. *israelensis* strains were much higher than the Bti strain H14. From previous research, it is known that the cytotoxicity by Bt is attributed to either the anticancer proteins called Parasporins or the cytolytic proteins called Cyt [12]. PCR with whole DNA extract from all the Qatari Bti-like isolates and the reference failed to amplify any of the *parasporin* genes. This was expected as the insecticidal activity due to endotoxins and the anticancer property due to parasporins are known to be mutually exclusive among the Bt strains [24,25]. However, the lack of *parasporin* genes warranted further investigation of the *cyt1A* gene. It was found that Qatari Bti like isolates lacked two of the genes that the Bti carried: *cry10* and *cyt1C*. But, they all gave the expected amplification for the PCRs carried out with primers detecting other genes such as *cry4A/cry4B*, *cry11*, *cyt1A*, *cyt2B*,

p19, and *p20*. Among these genes, *cyt1A* gene encodes Cyt1A protein that is known to specifically act on cancer cells [26,27]. As the reference and Bti like isolates showed the presence of *cyt1A* gene, the cytotoxicity could be attributed to its encoded Cyt protein.

To explore the difference in cytotoxic activity among the reference and local isolates, the Cyt protein of one local isolate QBT229 with the highest cytotoxicity was compared with that of the reference. The full *cyt1A* gene was amplified by PCR and the amplified product was gel purified for sequencing by the Sanger's method. The *cyt1A* gene nucleotide sequence (accession number: MG708177) showed considerable differences when compared to the published sequence of *cyt1A* gene from Bti H14 strain. The sequence was translated to amino acid sequence and five consecutive amino acid replacements (position 225 to 229) were detected towards the C terminal of the protein. The sequence was fed to the SWISS-MODEL to render the 3D model of the consequent protein to see if there were any structural differences. When compared, no conformational changes were detected between the Cyt1A structure of reference and that of QBT229 (Figure 4A,B). When different filters for chemical changes in the region were applied to the software, it was found that the studied region of QBT229 gained positive charges and has become more polar than that of the reference (Table 1). Among these five replacements, four amino acids are polar with a positive charge.

It is known that these amino acids form a part of the β8 sheet of the Cyt1A protein (Figure 3). The β sheets of Cyt1A protein are involved in membrane pore formation, in order to activate the cytotoxic effect against the target cell. A change in these β sheets would in turn affect the cytotoxicity of the Cyt1A protein. Additionally, the β8 sheet is known to be involved in synergizing other endotoxins like Cry11. Cry11 is one of the insecticidal endotoxins carried by the mosquitocidal Bt strains, that require specific binding receptors on the membrane of the target cell to act as a toxin. Cry11 does not affect the human cell lines, except when Cyt1A protein is present along with it. Cyt1A binds to the lipid bilayer of the cell membrane and acts as a receptor for the Cry11 protein. Specifically, the three amino acids at the positions K198, E204, and K225 of the Cyt1A protein interact with the amino acids at the positions S259 and E266 of the Cry11 protein [28]. Thus, the interaction takes place between the charged amino acids of Cyt1A (K198, E204, and K225), and one polar (S259) and one charged amino acid (E266) of the Cry11 protein.

In case of the Cyt1A protein of QBT229, it was found that the K225 (Lysine) of β8 sheet has been replaced by N225 (asparagine). In this case, the interactions between Cyt1A and Cry11 protein of QBT229 take place between two charged (K198, E204) and one polar (N225) amino acid of Cyt1A and one charged (E266) and one polar amino acid (S259) of Cry11. Hence, it was postulated that the interactions were enhanced because of the presence of polar molecules on both sites.

Additionally, the overall β8 sheet has been changed chemically due to the five amino acid replacements (Figure 4). The amino acids K225, F226, A227, Q228, and P229 of the Cyt1A has been replaced by N225, L226, H227, N228, and H229 in the case of QBT229 Cyt1A protein. The positively-charged polar amino acids in the position 225, 227, 228, and 229 increases the ability of these amino acids to interact with the polar lipids. These interactions are enhanced because of the availability of more amino acids in this region that could be involved in hydrogen bonding, which is the characteristic of polar amino acids.

In other words, the amino acid replacements seen in Cyt1A protein of QBT229 would most probably increase the affinity between the β8 sheet and the lipid polar heads, increasing the binding affinity between the Cyt1A protein and the membrane, thereby enhancing its cytotoxicity. The specific amino acids replacement at position 225 enhances its ability to recruit the Cry11 protein to the membrane, thereby contributing to the cytotoxicity.

4. Conclusions

The cytotoxic activity of three Qatari Bt subsp. *israelensis* strains was quantified and compared to that of the reference Bt subsp. *israelensis* H14 against lung cancer epithelial cells NCI-H1975. It was observed that the cytotoxic activity among these Qatari Bt strains were higher compared to the reference strain H14. The presence of anticancer proteins called Parasporins was eliminated. Hence, it was postulated that the cytotoxic activity shown against the NCI-H1975 cell lines could be caused by the

Cyt1A protein. The full *cyt1A* gene sequence of the Qatari Bt subsp. *israelensis* strains showing the highest cytotoxic activity (QBT229), showed several differences when compared to the *cyt1A* gene sequence of the reference H14. To understand the possible significances of the nucleotide replacements, the amino acid sequences were studied and it was found that there are five amino acid replacements. These replacements did not bring about any structural differences but, chemically, the region has changed. The Cyt1A protein of QBT229 had four positively-charged polar amino acids instead of one polar and one positively-charged amino acid in the Cyt1A protein of reference Bti. The polarity and the charge of the region with amino acid replacements were enhanced. As this particular region is involved in membrane binding via lipid polar heads, it was concluded that the polar amino acids of β8 sheet of QBT229 has a better binding ability compared to the reference Cyt1A. In addition, the amino acid replacement at position 225 enhances its ability to act as receptor to the Cry11 protein. In our study, it was shown for the first time a novel Cyt1A protein which has amino acid replacements that enhance its cytotoxic activity against lung cancer cells without losing its structural homology.

5. Materials and Methods

5.1. Bt Isolates and Culture Conditions

Four *Bacillus thuringiensis* strains were used in the present study; H14 (Bti reference strain) and three local Bt subsp. *israelensis* isolates from Qatar environment. The Bt isolates were grown on nutrient-rich, low sodium Luria Bertini (LB) media at 30 °C. The pure cultures were stored in the form of spore-crystal mixture on T3 sporulation media [29]. The T3 agar plates inoculated with the Bt isolates were incubated at 30 °C for 96 h for complete sporulation. For all the experiments conducted, the spore-crystal mixtures were inoculated and incubated on LB agar plates overnight at 30 °C to obtain fresh cultures.

5.2. Parasporal Crystal Protein Purification and Solubilisation

During sporulation, Bt produces spores and the associated parasporal protein crystals are released into the sporulation broth. For the cytotoxicity assays, it is necessary to separate these crystals from the spores before activation. The separation was done by solubilizing the crystals at high pH maintained by 50 mM Na_2CO_3 for one hour at room temperature [30]. Once the proteins are solubilized, they were separated from the spores by centrifugation at 10,000 rpm for 10 min at 40 °C. The high pH of the solution was then adjusted to around 7, as the high pH affects the activity of proteases used for the next step of activation. The supernatant containing all the crystal proteins were then activated by trypsin at a final concentration of 15 µg/mL for 1 h at 37 °C. This activation is an essential step to convert the pro-toxins from the crystals to toxins. For the quantitative assay, the proteins were quantified using the spectrophotometer and extrapolating the values on the standard curve of optical densities (O.D) of bovine serum albumin by Bradford's method. The quantified proteins were used as treatment to be added according to the different concentrations decided for the assay: 0.15 µg/mL, 0.35 µg/mL, and 0.5 µg/mL. Each test was triplicated to obtain statistically relevant results.

5.3. Cancer Cell Line and Culture Conditions

The cell line chosen was the adherent lung cancer epithelial cells from ATCC called NCIH1975 [H1975, H1975] (ATCC® CRL5908™). The cells were maintained in ATCC-formulated RPMI-1640 medium added with 10% fetal bovine serum (FBS), 100 µg/mL Streptomycin and 100 IU/mL penicillin. The cells were grown and maintained in humidified 5% CO_2 incubator at 37 °C.

5.4. Quantitative Cytotoxic Bioassay

The cytotoxicity was determined by the cell proliferation assay called MTT assay [31]. The activity was quantified by comparing the percentage of viable lung cancer epithelial cells remaining after the treatment in the treated wells and untreated wells of cells. Each of the five concentrations were repeated thrice. Each well of the 96-well microtiter plates were seeded with 5000 cells and incubated

overnight for attachment. Then the media from each well was replaced with fresh media containing fixed concentrations of proteins for treatment. The treatments were carried out for 72 h. After 72 h, 100 µL of MTT reagent was added to each well and incubated for three hours at 37 °C. 50 µL of DMSO was added to each well and absorbance was calculated using the plate reader (Tecan, Switzerland). The cytotoxicity was calculated by comparing the cell viability observed in the treated wells to that of the untreated wells based on their respective absorbance.

5.5. Isolation of Plasmid DNA

The total plasmid DNA was isolated from fresh bacterial cultures by alkaline lysis method and purified by alcohol precipitation [32]. The cells were lysed by high pH maintained by NaOH and lysozyme digestion at 37 °C. The cell debris and proteins were separated from the nucleic acids by phenol: chloroform: isoamyl alcohol (25:24:1) precipitation. The nucleic acids were purified by ethanol precipitation overnight at −20 °C. The RNA molecules were digested by RNase enzyme (4 µg/mL) at 37 °C for one hour to obtain plasmid DNA.

5.6. Exploration of Endotoxin and Parasporin Encoding Genes

The primers were designed or adopted from published articles for the δ-endotoxin genes, *parasporin* genes and cytolytic (*cyt*) genes (Table 2). Polymerase chain reactions (PCR) were conducted with these primer sets for each plasmid sample. The reactions were carried out with Qiagen Taq PCR Master Mix Kit (Germany). The cycles for the amplifications included one denaturation step for 5 min at 95 °C followed by 35 cycles consisting of 1 min denaturation at 95 °C, 1 min annealing at the appropriate temperature and 1 min 30 s polymerization at 72 °C. The final extension was set at 72 °C for 7 min. A gel of 1.2% agarose stained with ethidium bromide was used for the visualization of the PCR products by gel electrophoresis.

Table 2. List of primers used in this study to explore the Bt subsp. *israelensis* genes.

Sr. No	Genes	Primer Pairs	Sequences	Amplicon Size	References
1	*cry4A*, *cry4B*	Dip1A Dip1B	5′ CAAGCCGCAAATCTTGTGGA 3′ 5′ ATGGCTTGTTTCGCTACATC 3′	800 bp	[33]
2	*cry4B*	Dip2A Dip2B	5′ GGTGCTTCCTATTCTTTGG 3′ 5′ TGACCAGGTCCCTTGATTAC 3′	1293 bp	[33]
3	*cyt1A*	Cyt1A1 Cyt1A2	5′ GTTGTAAGCTTATGGAAAAT 3′ 5′ TTAGAAGCTTCCATTAATA 3′	701 bp	[34]
4	*cyt2*	Cyt2-1 Cyt2-2	5′ AATACATTTCAAGGAGCTA 3′ 5′ TTTCATTTTAACTTCATATC 3′	471 bp	[35]
5	*cry11*	Cry11-1 Cry11-2	5′ TTAGAAGATACGCCAGATCAAGC 3′ 5′ CATTTGTACTTGAAGTTGTAATCCC 3′	304 bp	[36]
6	*cry10*	Cry10-1 Cry10-2	5′ ATATGAAATATTCAATGCTC 3′ 5′ ATAAATTCAAGTGCCAAGTA 3′	614 bp	[37]
7	*cyt1C*	Cyt1C1 Cyt1C2	5′ CAAAATCTACGGGAGCAAGG 3′ 5′ GGAAGGATCCCTTTGACTTTT 3′	1320 bp	[23]
8	*p19*	P19-1 P19-2	5′ GCAGGAGGAACATCACCATT 3′ 5′ GGATTTGCTGAGCAGGTCAT 3′	291 bp	[23]
9	*p20*	P20-1 P20-2	5′ TGACGAGGAAACAGAGTATACGA 3′ 5′ TGAAAGGTTAAACGTTCCGATT 3′	704 bp	[23]
10	*parasporin1*	PS1-94F1 PS1-94R4	5′ AGCACCTAATGATGATAGAGGAA 3′ 5′ CCCAGATTCAAATAATAACCAAGA 3′	511 bp	[16]
11	*parasporin2*	PS2-F PS2-R	5′ GATGGTATTGCATTAAATAATGAAAC 3′ 5′ TTCTCCACCAATTTCAAAGACT 3′	306 bp	[16]
12	*parasporin3*	PS3-F PS3-R	5′ ATACAAGATGTGAGGAAATGATGA 3′ 5′ GTATGGCTCAGCTCAATTTGA 3′	526 bp	[16]
13	*parasporin4*	PS4-F PS4-R	5′ ACTAGTCAGCCTATAATCAGAACGA 3′ 5′ ACTATTCCAGTACCAGTGTAACC 3′	377 bp	[16]
14	*parasporin5*	PS5-F PS5-R	5′ TCAACGCCACAATTAACAAATA 3′ 5′ TCCCTTGTATAGTTGCCTTTGT 3′	397 bp	[16]
15	*parasporin6*	PS6-F PS6-R	5′ TGTTTACTATGTGAAAGGTGGAGA 3′ 5′ CAATAGTGGTTCCTATTGGACC 3′	446 bp	[16]

5.7. Gel Purification and DNA Sequencing of PCR Products

Forty microliters of the PCR product to be sequenced was loaded on a 1% agarose gel and the gel electrophoresis was carried out for one hour at 50 V. The DNA band to be sequenced was cut from the gel by visualizing the same on a gel dock with UV light. The DNA was purified from the gel using QIAquick gel extraction kit from Qiagen (Hilden, Germany), as per the manual instructions. The purified product was then sequenced by Sanger sequencing.

5.8. Translation, Alignment and Comparison of Amino Acid Sequences

The DNA sequence obtained from sequencer was translated into amino acid sequence in silico using the translate tool of ExPasy available on Swiss Institute of Bioinformatics research portal. The sequence was aligned to the published sequence of the *cyt* gene using the pBLAST tool on the National Centre for Biotechnology Information (NCBI) website.

5.9. In Silico Structural Homology Comparison of Cyt Proteins

To understand the structural and/or chemical differences among the functional Cyt protein structures of QBT229 and the reference Bt subsp. *israelensis* H14, their respective amino acid sequences were used to find the in silico structure of the proteins by SWISS-MODEL software [38]. As a reference for the protein modelling, the 3D structure of the Cyt1A1 protein (TRON.3) from *Bacillus thuringiensis* subsp. *israelensis* was used. Cohen et al. [39] had modelled the structure of Cyt1A1 protein by X-ray crystallography with a resolution of 2.19 Å. The structures of the two Cyt proteins were compared for any conformational changes due to amino acid replacements. The structures were also compared chemically by applying the different filters of the software including charged regions, hydrophobic regions, polar regions, etc.

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Author Contributions: S.J. is supervisor of the student K.N; S.J. and K.N. conceived and designed the experiments; K.N. and A.I. performed the experiments; K.N., S.J., A.I., R.M., and R.A.-T. analyzed the data; S.J., A.I., and R.M. contributed with reagents and materials; K.N. and S.J. designed and drafted the paper; and S.J, A.I., and R.A.-T. reviewed the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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APPENDIX C: Positions of primers designed for Plasmid mapping of p*Bt*oxis

CAAGCCGCAAATCTTGTGGAATGTATTTCTGAAGAATTATATCCAAAAGAAAAAA
>>>>>>>(Dip1A)>>>>>>
TGCTGTTATTAGATGAAGTTAAAAATGCGAAACAACTTAGTCAATCTCGAAATGT
ACTTCAAAACGGGGATTTTGAATCGGCTACGCTTGGTTGGACAACAAGTGATAAT
ATCACAATTCAAGAAGATGATCCTATTTTTAAAGGGCATTACCTTCATATGTCTG
GGGCGAGAGACATTGATGGTACGATATTTCCGACCTATATATTCCAAAAAATTGA
TGAATCAAAATTAAAACCGTATACACGTTACCTAGTAAGGGGATTTGTAGGAAGT
AGTAAAGATGTAGAACTAGTGGTTTCACGCTATGGGGAAGAAATTGATGCCATC
ATGAATGTTCCAGCTGATTTAAACTATCTGTATCCTTCTACCTTTGATTGTGAAGG
GTCTAATCGTTGTGAGACGTCCGCTGTGCCGGCTAACATTGGGAACACTTCTGAT
ATGTTGTATTCATGCCAATATGATACAGGGAAAAAGCATGTCGTATGTCAGGATT
CCCATCAATTTAGTTTCACTATTGATACAGGGGCATTAGATACAAATGAAAATAT
AGGGGTTTGGGTCATGTTTAAAATATCTTCTCCAGATGGATACGCATCATTAGAT
AATTTAGAAGTAATTGAAGAAGGGCCAATAGATGGGGAAGCACTGTCACGCGTG
>>>>>>>>(F1)>>>>>>>>
AAACACATGGAGAAGAAATGGAACGATCAAATGGAAGCAAAACGTTCGGAAAC
ACAACAAGCATATGATGTAGCGAAACAAGCCATTGATGCTTTATTCACAAATGTA
<<<<<<<(Dip1B)<<<<<<
CAAGATGAGGCTTTACAGTTTGATACGACACTCGCTCAAATTCAGTACGCTGAGT
ATTTGGTACAATCGATTCCATATGTGTACAATGATTGGTTGTCAGATGTTCCAGGT
ATGAATTATGATATCTATGTAGAGTTGGATGCACGAGTGGCACAAGCGCGTTATT
TGTATGATACAAGAAATATTATTAAAAATGGTGATTTTACACAAGGGGTAATGGG
GTGGCATGTAACTGGAAATGCAGACGTACAACAAATAGATGGTGTTTCTGTATTG
GTTCTATCTAATTGGAGTGCTGGCGTATCTCAAAATGTCCATCTCCAACATAATCA
TGGGTATGTCTTACGTGTTATTGCCAAAAAAGAAGGACCTGGAAATGGGTATGTC
ACGCTTATGGATTGTGAGGAGAATCAAGAAAAATTGACGTTTACGTCTTGT

GAAGAAGGATATATTACGAAGACAGTAGATGTATTCCCAGATACAGATCGTGTA
CGAATTGAGATAGGCGAAACCGAAGGTTCGTTTTATATCGAAAGCATTGAATTAA
TTTGCATGAACGAGTGATTAATAAAAAATAACTAAAGCTTTAAAAACCATGGAG
AAAGTTTTCTCCATGGTTTTTAATTTCTGCATTTATTAATTCTGGTACAAAAAATA
TATAGAAAACATAAAAAATAGATATCTAGAGGACATAAAATTTATACAAATATC
AATTTCATTAGTATAGAACGTTTATCCAATAATAATTATCACCATTTAAACTATCC
AAACTACAATTGTGATCCAAGTTATGATGATTAATAGATGAACTGATTTAGGATT
TGAAAATAGATTTTCATCAAACAAAATGAGGGAAAGAATATGCAGGGAAATCAA
>>>>>>>>>(F2)>>>>>
CAATCGATTTCAAATCTTAATACTTCGGGATTATTTCGGGGTACAGCCGAGATAC
GTGTAAGAATAGACCGTATCCTTACCTTTATTTCTATATTGGATTTATTGAGCTTA
TAAATATTGTTTCTGTGGTTTCGTCTATTTTTATTAAGTAAATTGTCTATTATGGGT
TAACCCTAATCATTCATTAGTTACTGAAAACATTTTATTTCATTTGTGTGATTAGG
ATGAGTGATAATAGATTCAAAAACAAAAAAGCTGGATTAATGCCAGCTTTTTTTA
CAATTATAATTACCCATAAATATAAAATATTATTTTTGTGTTTCTCTTTCACCTAC
AACCTAAGCTAATAATAAAATAGAGCTAGTTACTAACAATGCTATAAAAACTCCC
ATTAAACATTTCTCCTTTTTACAAAAAATATTTTACCATATTATACCGCATCAGGA
<<<<<<
CGGATCAAATTGTAATAACAAGGAGCATTTATGTTGCAAATGTCACAGAAAAAT
<<(R1)<<<<<<<<
ATTTTTTGGAAAAGAATATATAAAAATGACCTATTTTTACACGTATCTCGGTTGTA
>>>>>>
CCCCATACACAGAAACAGACATAAAACAGACATAAAACACACCTTCCTTTCCTAT
>>(F3)>>>>>>>>

GATTTTACATAAAAAATAGCGTGTTTTTTCATTTTAAGAAAATTTAATTTTGTTAG
CGTAATAGCGATATATGTGCTTTTTAAATAAATTATTAAAATCTTAATGCTTTAAG
TAGAAAATTATATAAAACATAGAAAAATTGAGCTATCAAGGCACCTTGCCATAA
ACTTAAGTTAGAAGAAAGATAAGGGTGTTTTTTAAGATACATATGTAATATACAG
AGATTCGTTCCACTGGCTGAAAGAACGATTATTAGGCCTCTTTGAAGGTAGTCTC
GGAGCTTTGCTTTTGAAAGAGTGAAAATTGTGCCCAGTAGAAAGCAACGATTTGG
TTCTCGTTATCGAACTAAAGTCATAGGACTTATTGAGTTTATTTACCGTGAGGAA
ATAGAGAAACTGAGTGGTACCGCAATAATCACGCCTCAGGAATCAAGCGTATAA
TGCGTATTTGATTGTGGAGGTGAGATTTTTTATTGTTTAAAGTAAGAATAATATTT
TAAAAAGATCATAGAAATTCAAGGCAAAACAGGAGAGAATGATTAAGTTATTTA
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CTAAGAAAAACCGTGCAGGATGATATCAATGGTTCTGTTGCAAAGTTTGAAAAA
AATTATAAATGGGTGGATAAATAAGGGGAATAACTTATGCAGTGGCCATTGATTG
TTGTAATTCATTGTACACTGAAAAGGCAAGATTTGTTTGGAAATTTCGCCTCTGTT
TATATATAGCCTGAATCGTTTCGATTCCTTTTATGGTACGAGAAGCATGACGAGG
AGTTTGAAATCCAGCTGATTTCACAAACCGACGTTTGATATGTCGATGATCTTGTT
<<<<<<<<<(R2)<<<<<<<<<
CAATAAGATTATTACGATATTTAATGGTACAGTGATTGGTATGTACATAATACTC
AGCTTTCTGTAATTTTTTGAATGCACAAAGTAAAGCTGGGGCCTTGTCTGTCGTAA
>>>>>>(F4)>>>>>
GAACCGATGGTTCTCCAAAATGTTTGACCAATCTTTTCATAAAGGCATAAGCAGC
TTGATGGATTACGTGTTTTTCAAACTTGAAAGTCCAGTGTATGTCCATCACTATCA
ATCGCGCGGTATAAATAACACCATTCTCCTTTGACTTTGATATACGTCTCATCTAA
ATGCCAAGTATGGAGTGCCGATTTGTTTTTTTTCTTCCATATTCGATAGATTAGCT
GGCCATATTCATGAACCCAACGCATGATCGTTGTGGGATGATCTGACACACCACG
TTCCTGAAAAATCTCAGATACATCACGATAGCTTAAAGAAAAACGACAGTAATA
GCCAATGGCTACTAAAATAATGTCTTTCTTGAACTGTTTTCCTTTAAAATATCTCA
TGTAGCATTCTCCTCAGCACATTTTCCCTACAGTCTCCTTTTTCGTGGAATGGGCA
<<<<<<<<(R3)<<<<<<<<
AAAACATCCTGCTACCGAAACGCTGGATTGTGGAACAAACTTTTTCTTGGTTAGA
AAACTACCGCAGACTACGGAAGAACTGTGAGTAAACACTTGAAAATAGTAGACA
GAGTTGCTTATTGGCATCTGTGGTGATTTTATTAAAAAGATTCTAGATAGGTTCTA
AGGGTGAAGTAGGGAAATGGATACCTTTGTGCAAACAGGAGGGGTTTATGAACC
GCTGTGGTCTGGAGTTAGAACGTGGATGGATGAGAAGGGCTGGTATTATGAAGT
GAGACAAACAATGTGAACAGGATATCTTCCATCCAAGATGTCCTGTTTTTCTGTA
CTCTCTACTATGGTTGGACTCAAAACGCTTATAGAAGAATTTGAGGATGTTGAAG
GGGGATAGGAGGTGATTTGATATATTTTTGGGAAATGTAAAGGTTGATTGTCGGA
AAATGATGAGGTTATTTGTAGAAAAGATGTAACAGGAATACATATAGAAAATTA
CGAATACTTTAAAATGCATAAGACATATTGAAAAAAGAATGATCAATCACTACAT
AGGAAGGAATATCTATAGGATTTGCGAAAATGATAAATTATGTACAGATAGGTTC
TTGTTAAGTCATATGAATTAAAAAATGCTTTAAAATAATCTTTGTTGCAACAGAA
AAGAGTTGTGTCTAATTTGAGTATGGGAGGAATAGATATGAATCCATATCAAAAT
>>(Cry10ASI)>
AAGAATGAATATGAAATATTCAATGCTCCATCCAATGGTTTTAGCAAGTCTAATA
>>(CRY10-1)>>>> >>>>>
ACTATTCTAGATATCCATTAGCAAATAAGCCAAATCAACCACTGAAAAACACGA
>>>>(F5)>> >>>>>>>>>>>
ATTACAAAGATTGGCTCAATGTGTGTCAAGATAATCAACAATATGGCAATAATGC
GGGGAATTTTGCTAGTTCTGAAACTATTGTTGGAGTTAGTGCAGGTATTATTGTA
GTAGGAACTATGTTAGGAGCTTTTGCTGCCCCTGTCTTAGCTGCAGGTATAATATC
TTTTGGGACTTTGTTGCCGATCTTTTGGCAAGGATCTGACCCTGCAAATGTTTGGC
<<<<<<<<<(R4)<<<<<<<<<
AGGATTTGTTAAACATCGGAGGAAGGCCTATACAAGAAATAGATAAAAACATAA
TTAATGTACTAACTTCTATCGTAACACCTATAAAAAATCAACTTGATAAATATCA
AGAATTTTTCGATAAATGGGAGCCAGCACGTACACACGCTAATGCTAAAGCAGT

ACATGATCTCTTTACTACCTTAGAACCTATAATAGATAAAGATTTAGATATGTTA
AAAAATAATGCTAGCTATCGAATACCAACACTCCCTGCATATGCACAAATAGCTA
CTTGGCACTTGAATTTATTAAAACATGCTGCTACCTATTACAATATATGGCTGCAA
<<<<<<(CRY10-2)<<<<<
AATCAAGGTATAAATCCAAGTACTTTCAATTCATCTAATTACTATCAGGGCTATTT
AAAACGTAAAATACAAGAATATACTGACTATTGTATACAAACGTACAATGCAGG
ACTAACTATGATTAGAACTAATACTAACGCAACATGGAATATGTATAATACTTAC
CGTTTAGAAATGACTCTAACTGTGTTAGATCTTATTGCTATTTTTCCAAATTATGA
CCCAGAAAAATATCCAATAGGAGTTAAATCTGAACTTATCAGAGAAGTTTATACG
AATGTTAATTCAGATACATTTAGAACCATAACAGAACTAGAAAATGGATTAACTA
GAAATCCTACATTATTTACTTGGATAAACCAAGGGCGTTTTTACACAAGAAATTC
>>>>>>>>(F6)>>>>>>>>
TCGAGACATTCTTGATCCTTATGATATTTTTTCTTTTACAGGTAACCAGATGGCCT
<<<<(Cry10R40180)<<<
TTACACATACTAATGATGATCGCAACATAATCTGGGGAGCGGTTCATGGAAATAT
TATTTCTCAAGACACATCCAAAGTATTTCCTTTTTATAGAAACAAACCTATTGATA
AGGTCGAAATTGTCAGACATAGAGAGTACTCAGATATAATATATGAAATGATATT
TTTTTCGAATAGCAGTGAAGTATTTCGATATTCATCCAATTCAACAATAGAAAAT
AATTATAAAAGAACTGATTCTTATATGATTCCAAAACAAACATGGAAAAATAAA
GAATATGGTCATACTCTATCGTATATAAAAACTGATAATTATATATTTTCAGTAGT
TAGAGAAAGAAGAAGAGTTGCATTTAGTTGGACACATACTAGTGTTGATTTCCAA
AATACAATAGATTTAGATAACATCACCCAAATCCACGCTCTAAAAGCTTTGAAGG
TAAGTTCTGATTCGAAAATTGTGAAAGGTCCTGGTCACACAGGTGGAGACTTGGT
<<<<<<<<(R5)<<<<<<<<
AATTCTTAAAGATAGTATGGATTTTAGAGTTAGATTTTTAAAAAATGTTTCTCGAC
AATATCAAGTACGTATTCGTTATGCTACTAATGCTCCAAAGACAACAGTATTCTT
AACCGGAATAGATACTATAAGTGTGGAGCTCCCTAGTACCACTTCCCGCCAAAAC
CCAAATGCTACAGATTTAACATATGCAGATTTTGGATATGTAACATTTCCAAGAA
CAGTTCCAAATAAAACATTTGAAGGAGAAGACACTTTATTAATGACCTTATATGG
TACACCAAATCATTCATATAATATATATATTGACAAAATCGAATTTATTCCAATC
ACTCAATCTGTATTAGATTATACAGAGAAGCAAAATATAGAAAAAACACAGAAA
ATAGTGAATGATTTATTTGTTAATTAAAACAAAGTTCTTACTAAAATAGATAGTA
TGGCTGTTAAAAAGTAAGCGAGAAAGGTCGTGAACCCTATGTTTACAAGTGGTGC
GAAAAATAGGTTAAAGCTAGAAACGACAGATTATGAAATAGATCAAGTGGCTAA
TGCTATAGAATGTATGTCAGATGAACAATATTCAAAAGAAAAACTGATGTTATGG
GATCAAGTAAAACATGCAAAATACCTTAGTCAGTCTCGAAATTTGCTTCAAAATG
GTGATTTTGAAGATGTATTTCATGGATGGACTACAAGTGATCATATGTACATTCA
GTCGGATAATTCTACTTTTAAAGGAAATTATCTGAATATATCTGGGGCGCGAGAC
ATATACTTAACGATATTTCCAACATACATTTACCAAAAAATTGATGAATCAAAAT
TAAAACCGTATACACGTTACCTAGTAAGGGGATTTGTAGGAAGTAGTAAAGATGT
AGAACTAGTGGTTTCACGCTATGGAAAAGAAATAGATACAGTCATGAATGTACC
<<<<<<<<(R6)<<<<<<<<
ATTTGATATCCCGTATGTATCTTCTAGGCCTGTTTGTAATGAATTATATGATGGTG
AACAACAACCGTATCCAAATGGGAATGTAGGATATTATAATCCAATGTCAGCTTT
TACGCCTTCTTACACATCTGATGCTCGTCAGTGTATGCCAGGGAAAAAACAGATA
GTCTGTCAAGATTCTCATCAGTTTAAGTTCCATATTGATACAGGTGAAGTAGATT
ATAATACAAATATAGGGATTTGGGTCATGTTTAAAATATCTTCCCCAGATGGATA
CGCATTATTAGATAATTTAGAAGTAATTGAAGAAGGGCCAATAGATGGGGAAGC
ACTGTCACGCGTGAAACACATGGAGAAGAAATGGAACGATCAAATGGAAGCAAA
ACGTTCG

APPENDIX D: LC/MS data o*Bt*ained for *Bti* H14 and *QBT*555 from Spectrum Mill software

Data for *Bti* H14

numSpectra	numPeptsUnique	percentCoverage	accession_number	entry_name
42	25	55.5	P21256	Pesticidal crystal protein Cry11Aa
14	10	56.3	A0A141DWM3	Cry11 (Fragment)
28	18	55.3	A0A160LGP4	ATP synthase subunit beta
17	14	35	A0A160LF20	Succinate dehydrogenase
22	16	26	A0A160LCR0	Aconitate hydratase
19	15	30.2	A0A160L5S6	Elongation factor G
15	15	38	A0A160LG56	Acyl-CoA dehydrogenase
19	16	18.1	P05519	Pesticidal crystal protein Cry4Ba
19	16	16.8	Q1RN84	Delta-endotoxin
6	6	10	Q8KNV1	Pesticidial crystal protein
21	14	50.3	A0A160L545	Elongation factor Tu
18	12	31	A0A160L637	60 kDa chaperonin
17	13	32.6	A0A160LGW0	ATP synthase subunit alpha
15	13	37.2	A0A160LF66	Isocitrate dehydrogenase [NADP]
12	10	45.7	A0A160L7C7	Ornithine aminotransferase
45	10	74.2	P0A382	Type-1Aa cytolytic delta-endotoxin
13	11	21.7	P09662	Pesticidal crystal protein Cry10Aa
15	9	32.3	A0A160LCH5	Betaine aldehyde dehydrogenase
13	11	23.7	A0A160LF44	Chaperone protein DnaK
10	8	32.9	A0A160LDN9	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
12	9	46.8	A0A160LF	L-lactate dehydrogenase

			U6	
12	9	34.9	A0A160LD53	Protein RecA
12	9	16.5	A0A160LG25	3-hydroxyacyl-CoA dehydrogenase
10	8	44.7	A0A160LH75	ATP synthase gamma chain
9	9	12.4	A0A160L7N6	Peptidase M6
11	10	34.9	A0A160LDB5	Succinate--CoA ligase [ADP-forming] subunit beta
10	9	25.9	A0A161J2P3	Aminopeptidase
9	7	25.3	A0A160LA39	2-methylcitrate dehydratase
10	8	16.4	A0A160LCN8	Peptide ABC transporter substrate-binding protein
10	7	42.2	A9UF62	Camelysin
9	8	25.2	Q8KNP4	Hemagglutinin-related protein
9	8	19.3	A0A160L5W3	Formate acetyltransferase
10	7	19	Q8KNU9	Possible two-domain toxin
8	8	32.6	A0A161J275	30S ribosomal protein S2
9	7	36.7	Q8KNU0	Uncharacterized protein
9	7	46.7	A0A160LFU4	Universal stress protein
8	7	16.6	A0A160LB62	Methylmalonate semialdehyde dehydrogenase [acylating]
8	7	18.8	A0A160L6A3	Alkyl hydroperoxide reductase subunit F
8	6	27.7	A0A160LF61	Glyceraldehyde-3-phosphate dehydrogenase
6	6	31.7	A0A160LA15	3-hydroxyisobutyrate dehydrogenase
7	6	26.8	A0A169DC63	Sporulation protein
8	7	18.6	A0A160L629	1-pyrroline-5-carboxylate dehydrogenase
6	6	11.1	A0A160LDH2	Translation initiation factor IF-2
9	7	32.4	A0A160L6R5	30S ribosomal protein S3
7	6	20.1	A0A160LGT1	3-hydroxybutyryl-CoA dehydrogenase

6	6	23.9	A0A160L6U0	Iron-sulfur cluster carrier protein
6	6	16.9	A0A169DRV7	Fe-S cluster assembly protein SufB
6	6	17	A0A160L5P5	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B
7	5	21.1	A0A160LA65	Ethanol-active dehydrogenase/acetaldehyde-active reductase
7	6	17	A0A160L5V8	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]
7	6	10.4	A0A160LFC4	Acetyl-CoA synthetase
7	6	10.1	A0A160L5Z2	Protein prkA
7	6	7.8	A0A160LEW9	Pyruvate carboxylase
5	5	24.6	A0A160LD49	4-hydroxy-tetrahydrodipicolinate synthase
8	5	35.7	Q45723	Type-2Ba cytolytic delta-endotoxin
4	3	24.3	O30895	Cytolytic toxin homolog (Fragment)
8	5	19.2	A0A160LBA3	Peptide ABC transporter substrate-binding protein
8	6	37	A0A161J2H1	30S ribosomal protein S4
7	6	27.6	A0A169D7Y5	Cell division protein FtsN
6	6	34.6	A0A160L5N5	50S ribosomal protein L5
5	5	22.1	A0A160LD69	Aspartate-semialdehyde dehydrogenase
5	5	18	A0A160LFR7	Citrate synthase
5	5	17.4	A0A160LEZ9	ATP-dependent Clp protease ATP-binding subunit ClpX
6	5	23.6	A0A160LFC2	Electron transfer flavoprotein subunit alpha
5	5	18.5	A0A169D9G9	30S ribosomal protein S1
7	6	20.2	A0A169DGE4	Phosphoserine aminotransferase

5	5	18.7	A0A160LGB3	Enolase
6	4	35.5	A0A160LGF0	Ribosome hibernation promoting factor
7	5	29.3	A0A160LAK9	Uncharacterized protein
5	4	42.2	A0A160L5N2	Alkyl hydroperoxide reductase
6	4	45.3	A0A160L7X7	Putative phosphoesterase ATN07_06430
6	6	29.5	A0A160LG10	Fe-S cluster assembly ATPase SufC
5	5	31.6	A0A160LGA1	Succinate dehydrogenase
7	6	13.9	A0A160L9M5	Lysine 2,3-aminomutase
5	5	14.8	A0A160LDC0	Glycosyl hydrolase
6	5	14.6	A0A160LHA4	Fe-S cluster assembly protein SufD
5	4	21.6	A0A169D9J8	Histidinol-phosphate aminotransferase
5	5	21.3	A0A160LFK3	Aminomethyltransferase
4	4	21.2	A0A160L5T6	Glyoxal reductase
5	5	10.9	A0A160L9L1	Glutamate dehydrogenase
5	5	33.8	A0A169DT47	ATP synthase subunit delta
5	5	26.5	A0A160L535	50S ribosomal protein L1
5	4	18.4	A0A1L2Z056	Uncharacterized protein
6	4	16.5	A0A160L709	3'-5' exoribonuclease YhaM
5	5	9.1	A0A169DI69	Peptide ABC transporter substrate-binding protein
4	4	15.9	A0A160L622	Alanine dehydrogenase
5	5	15.3	A0A160LE75	2-oxoisovalerate dehydrogenase
4	4	41.7	A0A160LG72	Uncharacterized protein
7	4	77.9	A0A160LEF8	Stage V sporulation protein S
5	4	17.7	A0A161JQF5	Cell division protein FtsZ

5	4	31.8	Q7AL75	20 kDa accessory protein
6	4	26.4	A0A160LGD8	ATP-dependent Clp protease proteolytic subunit
4	4	23.5	A0A160LGY3	Fructose-bisphosphate aldolase
4	4	10.5	A0A160LBR2	Hydrolase
4	4	5.7	A0A169DSL5	Protein translocase subunit SecA
4	4	12.9	A0A169DSF9	Phosphoglycerate kinase
3	3	14.8	A0A160L8C8	Ketol-acid reductoisomerase (NADP(+))
4	4	11	A0A161J6W7	Dihydrolipoyl dehydrogenase
5	4	38.3	A0A161JQU0	ATP synthase epsilon chain
5	4	33.3	A0A160L6Q7	30S ribosomal protein S7
5	4	11.8	A0A160LF48	Asparagine--tRNA ligase
6	4	15.8	A0A160LDC5	GTP-sensing transcriptional pleiotropic repressor CodY
4	3	11.9	A0A160LG78	Pyridine nucleotide-disulfide oxidoreductase
4	4	22.5	A0A161J6E1	Putative pyruvate, phosphate dikinase regulatory protein
4	4	12.9	A0A160L977	Beta-ketoacyl-ACP reductase
4	4	29.1	A0A160LGN9	ATP synthase subunit b
4	4	15.5	A0A169D7F4	Peptide ABC transporter ATP-binding protein
4	4	35.6	A0A169D8J9	SSEB protein
4	3	27.5	A0A160LEJ9	Superoxide dismutase
4	4	17.9	A0A160LF01	Rod shape-determining protein MreB
4	3	14.3	A0A169DBU4	Quinone oxidoreductase
4	4	6.3	A0A160L7N5	Bacillolysin

5	4	16.6	A0A160LDJ0	Succinate--CoA ligase [ADP-forming] subunit alpha
4	4	9.2	A0A160LE46	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
3	3	14.9	A0A160LHH7	UTP--glucose-1-phosphate uridylyltransferase
3	3	13.6	A0A161J7A4	Nucleotide-binding protein ATN07_26615
4	4	6.9	A0A160LG26	Aspartate--tRNA(Asp/Asn) ligase
4	4	12.8	A0A160LHC3	Acetyl-CoA acetyltransferase
3	3	11.7	A0A169DC37	Penicillin-binding protein
4	3	12.1	A0A160LH82	Serine hydroxymethyltransferase
3	3	10.4	A0A160L701	4-hydroxyphenylpyruvate dioxygenase
3	3	5	A0A160L5X5	Glutamate synthase
4	3	17.9	A0A160LGB2	Glyceraldehyde-3-phosphate dehydrogenase
4	3	31.6	A0A160L7Q4	Putrescine importer
4	3	11.3	A0A160L4Z9	Ribose-phosphate pyrophosphokinase
3	3	14.3	A0A160LE62	Oxidoreductase
4	4	13.3	A0A160L6R9	Enoyl-CoA hydratase
4	3	10.8	A0A169DF93	Probable malate:quinone oxidoreductase
3	2	10.3	A0A160LCY3	Glutamine synthetase
3	3	21	A0A160L5U1	30S ribosomal protein S5
3	3	32.4	A0A169DFA6	Antibiotic biosynthesis monooxygenase
3	3	22	A0A160L5Q6	50S ribosomal protein L13
3	3	15.5	A0A161IQ44	2-methylisocitrate lyase
3	3	28.7	A0A160LGX6	Spore coat protein GerQ

3	3	16.4	A0A160L835	Glycerol-3-phosphate dehydrogenase [NAD(P)+]
3	2	9.6	A0A160L870	Poly(R)-hydroxyalkanoic acid synthase
3	3	22	A0A160LDH9	Uridylate kinase
4	3	7.5	A0A160L5R2	ATP-dependent Clp protease ATP-binding subunit ClpC
4	3	7.9	A0A160LAE4	Uncharacterized protein
4	3	19.3	A0A160L5T3	50S ribosomal protein L4
3	3	6.4	A0A160LCX7	Anaerobic ribonucleoside-triphosphate reductase
2	2	10.9	A0A160LH29	UDP-glucose 4-epimerase
3	3	18.1	A0A160L844	Polyhydroxyalkanoate biosynthesis repressor PhaR
3	3	20	A0A160LGQ3	Uracil phosphoribosyltransferase
3	3	26	A0A160L5M7	50S ribosomal protein L23
3	3	15	A0A169D159	50S ribosomal protein L10
3	3	14.7	A0A160L547	50S ribosomal protein L3
3	2	24.4	A0A169DBF2	DNA starvation/stationary phase protection protein
2	2	7.2	A9P7G0	Glucanase
3	3	9.4	A0A169D0L3	Inosine-5'-monophosphate dehydrogenase
3	3	9.4	A0A160LAI8	Tellurite resistance protein
4	3	5.5	A0A160LA54	AMP-dependent synthetase
4	3	24.8	A0A160L5R5	50S ribosomal protein L11
3	3	10.7	A0A160LE05	Pyruvate dehydrogenase (Acetyl-transferring) E1 component subunit alpha
3	3	5.7	A0A160LFH8	Leucine--tRNA ligase

3	2	4	A0A160LE32	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase
3	2	11.6	A0A160LDG3	Dipicolinate synthase subunit A
3	3	15.3	A0A161J6A8	Acetyltransferase
4	3	16.5	A0A160LKA0	Uncharacterized protein
2	2	40	A0A160LA37	DNA-binding protein
2	2	12.1	A0A160LGV7	Putative heme-dependent peroxidase ATN07_27860
3	2	19.4	A0A160L8F1	Uncharacterized protein
2	2	23.3	A0A160L573	50S ribosomal protein L17
2	2	8.9	A0A160LE64	Probable butyrate kinase
3	3	11.7	A0A160L901	Enoyl-[acyl-carrier-protein] reductase [NADH]
3	2	5	A0A160L941	Peptide ABC transporter substrate-binding protein
3	3	8.4	A0A160LC26	3-phosphoglycerate dehydrogenase
3	2	11.1	A0A1L2YZW6	L-threonine dehydratase catabolic TdcB
2	2	15.7	A0A160LHA6	Methyltransferase
2	2	13.7	A0A160LAC1	Lipase
2	2	15.8	A0A160L8L6	Menaquinol-cytochrome C reductase
3	3	11.9	A0A160LDI7	Cell division protein FtsA
3	2	18	A0A160L556	50S ribosomal protein L14
3	3	9.4	A0A169D3F1	Uncharacterized protein
2	2	13.1	A0A160L920	Aspartate--ammonia ligase
2	2	3.6	A0A169D7D1	Chaperone protein ClpB
3	2	13.8	A0A160LD44	Spore coat protein
2	2	8.5	A0A160LGP9	Peptidase M4

2	2	8.8	A0A160LDI5	Polyphenol oxidase
2	2	5.1	A0A160LB36	ABC transporter ATP-binding protein
2	2	53	A0A169DHV2	Cold-shock protein
2	2	18.4	A0A160L538	50S ribosomal protein L7/L12
2	2	15.7	A0A161IQ33	Acetyltransferase
2	2	7.4	A0A160L744	Stage V sporulation protein R
2	2	25.5	A0A160L8J0	DNA-binding protein
2	2	16.3	A0A169DJ64	2-oxoacid ferredoxin oxidoreductase
2	2	9.6	A0A160L7F9	Peptide ABC transporter ATP-binding protein
2	2	8.1	A0A160LDA7	Elongation factor Ts
3	2	11.5	A0A160LGS1	Fructose-1,6-bisphosphatase
2	2	19.8	A0A160LA06	Uncharacterized protein
2	2	9.6	A0A160LAX5	Peptidase P60
2	2	6.4	A0A160L7B5	Adenylyltransferase
2	2	11.3	A0A160L609	Fumarylacetoacetase
2	2	5.6	A0A160LCB1	Histidine kinase
2	2	6	A0A160LGL8	L-lactate dehydrogenase
2	2	11.5	A0A160LH59	Single-stranded DNA-binding protein
2	2	7.3	A0A169DRJ8	Glucose-6-phosphate isomerase
2	2	5.4	A0A161J604	Zn-dependent hydrolase
3	2	10.8	A0A160LF68	Enoyl-CoA hydratase
2	2	10.1	A0A160LFG1	Site-determining protein
2	2	9.5	A0A160LD52	Uncharacterized protein
2	2	21.3	A0A169D609	Uncharacterized protein

2	2	8.6	A0A161IPI5	Glutamine ABC transporter substrate-binding protein
2	2	10.8	A0A160L6A0	Chemical-damaging agent resistance protein C
2	2	8.1	A0A161J5J8	Aspartate phosphatase
2	2	6.5	A0A160L8L5	SAM-dependent methyltransferase
2	2	7.8	A0A169D367	Ornithine carbamoyltransferase
2	2	17.8	A0A160L6T2	30S ribosomal protein S11
2	2	4.7	A0A160LDH7	Uncharacterized protein
2	2	10.8	A0A160L843	Nucleoside diphosphate kinase
2	2	13.1	A0A160LDW2	Protein Ri*Bt*
2	2	8.3	A0A160LFH2	Bifunctional protein FolD
2	2	11.3	A0A160L591	30S ribosomal protein S8
2	2	23.7	A0A160LDX6	Uncharacterized protein
2	2	16	A0A169D855	DNA recombinase
2	2	8.3	A0A160L6M9	Probable transcriptional regulatory protein ATN07_02915
2	2	9.4	A0A160L5P1	50S ribosomal protein L6
2	2	11.7	A0A160LF42	Adenine phosphoribosyltransferase
2	2	6	A0A160L9S6	Alkaline serine protease
2	2	12.4	A0A160L8C5	Methylthioribulose-1-phosphate dehydratase
1	1	14.6	A0A160LB18	PadR family transcriptional regulator
2	2	4.5	A0A160LG46	Phosphoenolpyruvate carboxykinase (ATP)
2	2	6.8	A0A169D1A0	50S ribosomal protein L2
2	2	8	A0A160LEJ4	RNA polymerase sigma factor SigA
1	1	5.5	A0A160L7F1	3-oxoacyl-[acyl-carrier-protein] synthase 2

2	2	3.5	A0A161J750	Acyl--CoA ligase
1	1	6	A0A161J744	Malate dehydrogenase
2	2	12.3	A0A160LDQ6	Uncharacterized protein
1	1	6.8	A0A160LH58	Ribosome-binding ATPase YchF
2	1	24.4	A0A160LD72	DNA-binding protein
1	1	4.9	A0A160LE18	Acetylornithine aminotransferase
1	1	13.2	A0A160LAN3	ESAT-6-like protein
1	1	5.9	A0A160LHP2	Histidine kinase
1	1	7.8	A0A169DPW8	Probable thiol peroxidase
1	1	9.2	A0A160LAZ0	Cytoplasmic protein
1	1	11.8	A0A160LC95	N-acetylmuramoyl-L-alanine amidase
1	1	3.2	A0A160LFX0	NADH dehydrogenase
1	1	19.1	A0A160L4Y9	Transition state regulator Abh
1	1	14.8	A0A160LFJ3	Helix-turn-helix transcriptional regulator
1	1	2.5	A0A160LGQ6	Peptidase M24
1	1	5	A0A160L9P5	Alpha/beta hydrolase
1	1	7	A0A160L7M5	HTH-type transcriptional regulator Hpr
1	1	6.8	A0A161J732	Uncharacterized protein
1	1	9.4	A0A169DI91	CoA-binding protein
1	1	6.2	A0A160LAE7	3-ketoacyl-ACP reductase
1	1	4.9	A0A160LE89	Leucine dehydrogenase
1	1	4.9	A0A161J1S8	Histidine kinase
2	1	5.3	A0A160L8W5	Flagellin
2	1	7.8	A0A160L5M1	30S ribosomal protein S12
1	1	4.4	A0A160L	D-alanine aminotransferase

1	1	10.6	A55 A0A160L5 F4	10 kDa chaperonin
2	1	6.9	A0A160L HH8	Hydroxymethylpyrimidine/phosp homethylpyrimidine kinase
1	1	6.7	A0A160L9 I2	Flavohemoprotein
1	1	8.1	A0A160LE M5	Ribosome-recycling factor
1	1	9.8	A0A160LF 63	Transcriptional repressor NrdR
1	1	5.5	A0A160LE 24	Thymidine phosphorylase
1	1	9.6	A0A160LE R7	4a-hydroxytetrahydrobiopterin dehydratase
1	1	14.7	A0A169D1 82	30S ribosomal protein S10
1	1	14.5	A0A160LE Z3	50S ribosomal protein L27
1	1	5.2	A0A160L D39	2-oxoglutarate ferredoxin oxidoreductase subunit alpha
1	1	6	A0A160LF I3	Potassium transporter
1	1	3.1	A0A169D3 13	Uncharacterized protein
1	1	7.9	A0A160LE Z1	Delta-aminolevulinic acid dehydratase
1	1	1.5	A0A160L7 K6	Flotillin
1	1	2.4	A0A160L8 55	Stage IV sporulation protein A
1	1	9.6	A0A160L DP5	50S ribosomal protein L19
1	1	1.8	A0A160L AA1	Thymidine phosphorylase

Data for *QBT*555

Num Spectra	Num Peps Unique	Percent Coverage	accession_number	entry_name
18	11	28.6	P21256	Pesticidal crystal protein Cry11Aa

7	5	31.7	A0A141DWM3	Cry11 (Fragment)
15	13	40.6	A0A160LDB5	Succinate--CoA ligase [ADP-forming] subunit beta
18	11	17.9	A0A160LCR0	Aconitate hydratase
13	10	21.7	A0A160LF44	Chaperone protein DnaK
13	9	30.5	A0A160LGP4	ATP synthase subunit beta
15	10	30	A0A160LF66	Isocitrate dehydrogenase [NADP]
15	8	30.7	A0A160LCH5	Betaine aldehyde dehydrogenase
11	9	24.5	A0A160LI82	Formate--tetrahydrofolate ligase
13	9	21.9	A0A160L629	1-pyrroline-5-carboxylate dehydrogenase
10	9	20	A0A160LG56	Acyl-CoA dehydrogenase
12	7	33.5	A0A160L7C7	Ornithine aminotransferase
10	9	18.1	A0A160L5W3	Formate acetyltransferase
10	8	26.3	A0A160LA39	2-methylcitrate dehydratase
15	8	28.6	A0A160L545	Elongation factor Tu
12	8	16.4	A0A160L5S6	Elongation factor G
7	6	25.7	A0A160LGB3	Enolase
11	6	55.4	P0A382	Type-1Aa cytolytic delta-endotoxin
7	6	25.4	A0A160LDN9	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
11	8	15.9	A0A160LGW0	ATP synthase subunit alpha
8	7	35.3	A0A160LAK9	Uncharacterized protein
9	6	32.1	A0A160LF61	Glyceraldehyde-3-phosphate dehydrogenase
7	7	24.1	A0A160LD69	Aspartate-semialdehyde dehydrogenase
9	6	18.1	A0A160L6A3	Alkyl hydroperoxide reductase subunit F
8	8	13.7	A0A169DI69	Peptide ABC transporter substrate-binding protein
6	6	23.8	A0A160LF01	Rod shape-determining protein MreB
7	6	19	A0A160LEZ9	ATP-dependent Clp protease ATP-binding subunit ClpX

7	5	19.1	A0A160LA65	Ethanol-active dehydrogenase/acetaldehyde-active reductase
5	5	9	A0A160L5L9	DNA-directed RNA polymerase subunit beta
5	5	9.8	A0A160LCX7	Anaerobic ribonucleoside-triphosphate reductase
2	2	5.3	A0A1L2Z063	Anaerobic ribonucleoside-triphosphate reductase
8	5	13.6	A0A160L637	60 kDa chaperonin
7	5	18.4	A0A169D0L3	Inosine-5'-monophosphate dehydrogenase
5	5	27	A0A160LGN2	Rod shape-determining protein MreB
6	5	18	A0A169D9G9	30S ribosomal protein S1
7	5	15.3	A0A160LE46	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
8	6	55.4	A0A160L843	Nucleoside diphosphate kinase
6	5	12.8	A0A160L8I8	Asparagine synthetase B
6	5	25.1	A0A160LFU6	L-lactate dehydrogenase
6	5	9.7	A0A160LFC4	Acetyl-CoA synthetase
6	5	12.6	A0A169DRV7	Fe-S cluster assembly protein SufB
5	5	24.4	A0A161J275	30S ribosomal protein S2
5	5	14	A0A161J6W7	Dihydrolipoyl dehydrogenase
5	5	22.1	A0A160LE89	Leucine dehydrogenase
5	4	25.3	A0A160LD49	4-hydroxy-tetrahydrodipicolinate synthase
7	5	40.6	A0A160L5N2	Alkyl hydroperoxide reductase
5	4	17.6	A0A160LDA7	Elongation factor Ts
6	5	19.7	A0A161JQF5	Cell division protein FtsZ
6	6	22.8	A0A160L6R5	30S ribosomal protein S3
5	5	25.1	A0A160LFH2	Bifunctional protein FolD
5	5	10.7	A0A160LF20	Succinate dehydrogenase
5	5	14	A0A160LFR7	Citrate synthase
4	4	16.4	A0A160L6L9	8-amino-7-oxononanoate synthase
5	4	21	A0A169D367	Ornithine carbamoyltransferase
4	4	7.7	A0A160LDH2	Translation initiation factor IF-2

6	5	20.8	A0A160L7R7	Uncharacterized protein
5	4	17.2	A0A160LEE4	Probable glycine dehydrogenase (decarboxylating) subunit 1
5	5	21.7	A0A160LE64	Probable butyrate kinase
5	4	14.1	A0A160L4Z9	Ribose-phosphate pyrophosphokinase
5	4	29.3	A0A160L7Q4	Putrescine importer
6	5	8.8	A0A160LG25	3-hydroxyacyl-CoA dehydrogenase
4	4	17.4	A0A160LFK3	Aminomethyltransferase
4	4	21.1	A9UF62	Camelysin
4	4	13.1	A0A169DAH3	Histidine kinase
4	4	8.8	A0A160LB62	Methylmalonate semialdehyde dehydrogenase [acylating]
4	4	15.6	A0A160LF95	Alanine dehydrogenase
5	4	11.5	A0A169D2P2	Glutamyl-tRNA(Gln) amidotransferase subunit A
5	3	22.4	A0A160LGQ3	Uracil phosphoribosyltransferase
4	4	24.7	A0A169D7F4	Peptide ABC transporter ATP-binding protein
4	4	16.9	A0A160LHH7	UTP--glucose-1-phosphate uridylyltransferase
4	4	10	A0A160LG46	Phosphoenolpyruvate carboxykinase (ATP)
4	4	5.2	A0A169DSL5	Protein translocase subunit SecA
4	4	10.3	A0A160L9M5	Lysine 2,3-aminomutase
3	3	41.1	A0A160L8J0	DNA-binding protein
4	3	20.8	A0A160L835	Glycerol-3-phosphate dehydrogenase [NAD(P)+]
4	3	18.2	A0A160L535	50S ribosomal protein L1
4	4	14.6	A0A160L6U0	Iron-sulfur cluster carrier protein
4	3	14.9	A0A160LGB2	Glyceraldehyde-3-phosphate dehydrogenase
5	4	13.5	A0A160LEF7	2-oxoisovalerate dehydrogenase
4	4	13.7	A0A160LGT1	3-hydroxybutyryl-CoA dehydrogenase
3	3	6.4	A0A160L5R2	ATP-dependent Clp protease ATP-binding subunit ClpC
3	3	7.1	A0A160LAY	Asparagine synthetase B

			6	
4	3	13.3	A0A160L977	Beta-ketoacyl-ACP reductase
5	3	16	A0A160LD53	Protein RecA
3	3	21.8	A0A160LGV7	Putative heme-dependent peroxidase ATN07_27860
3	2	13.1	A0A160LE24	Thymidine phosphorylase
4	3	14.4	A0A160LFU4	Universal stress protein
4	3	31.9	A0A169DBF2	DNA starvation/stationary phase protection protein
3	3	6.6	P09662	Pesticidal crystal protein Cry10Aa
4	4	4.2	A0A160L7N6	Peptidase M6
4	3	10.7	A0A160LFC2	Electron transfer flavoprotein subunit alpha
3	3	17	A0A161J2H1	30S ribosomal protein S4
3	3	12.7	A0A160LGT5	CTP synthase
3	3	13.9	A0A169DSF9	Phosphoglycerate kinase
4	3	18.9	A0A160L8F1	Uncharacterized protein
4	3	13.2	A0A160L870	Poly(R)-hydroxyalkanoic acid synthase
4	3	13.7	A0A160LG78	Pyridine nucleotide-disulfide oxidoreductase
3	3	14.9	A0A161IQ44	2-methylisocitrate lyase
3	3	7.4	A0A160LBR2	Hydrolase
5	3	10.6	A0A160L5V8	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]
4	3	19.3	Q45723	Type-2Ba cytolytic delta-endotoxin
4	3	24.3	O30895	Cytolytic toxin homolog (Fragment)
3	3	5.2	A0A160LA54	AMP-dependent synthetase
3	3	12.6	A0A160LGA1	Succinate dehydrogenase
3	3	13	A0A161IQT9	Acetyl-CoA acetyltransferase
3	3	0.9	A0A160LAM7	Non-ribosomal peptide synthetase
3	3	23.5	A0A160LF42	Adenine phosphoribosyltransferase
2	2	11.8	A0A161J744	Malate dehydrogenase
3	3	2.5	A0A160LEW9	Pyruvate carboxylase
2	2	15.8	A0A160LEZ1	Delta-aminolevulinic acid dehydratase
4	2	9.2	A0A160LH82	Serine hydroxymethyltransferase

4	3	17	A0A161J6E1	Putative pyruvate, phosphate dikinase regulatory protein
3	3	8.3	A0A160LCY3	Glutamine synthetase
4	2	10.9	A0A160LE09	Dihydroorotase
2	2	10.2	A0A161J7A4	Nucleotide-binding protein ATN07_26615
3	2	40	A0A160LA37	DNA-binding protein
4	3	28	A0A160LDP5	50S ribosomal protein L19
3	3	7.7	A0A160LAM2	Cytochrome P450
3	2	18.8	A0A160L541	Hypoxanthine phosphoribosyltransferase
3	2	11.7	A0A160LB63	Chorismate mutase
2	2	19.2	A0A160LEJ9	Superoxide dismutase
3	3	3.1	A0A169D171	DNA-directed RNA polymerase subunit beta'
4	2	11.5	A0A160LDC5	GTP-sensing transcriptional pleiotropic repressor CodY
3	2	5.7	A0A169DPJ0	Threonine--tRNA ligase
4	2	23.2	A0A160L7X7	Putative phosphoesterase ATN07_06430
2	2	8.7	A0A160L5Q2	Arginine deiminase
2	2	14	A0A160LGY3	Fructose-bisphosphate aldolase
3	3	12.2	A0A160LG10	Fe-S cluster assembly ATPase SufC
3	2	13.9	A0A160LEC1	3-hydroxybutyrate dehydrogenase
2	2	8.1	A0A160L5E1	Pyridoxal 5'-phosphate synthase subunit PdxS
2	2	10.8	A0A160LG89	Phage shock protein A
3	2	14.3	A0A169D7Y5	Cell division protein FtsN
2	2	10.5	A0A160LFJ6	S-adenosylmethionine synthase
2	2	30.2	A0A160L6T2	30S ribosomal protein S11
2	2	12.2	A0A160LEM3	2-oxoisovalerate dehydrogenase
3	2	5.8	A0A169DF93	Probable malate:quinone oxidoreductase
2	2	8.4	A0A169D9E4	Two-component system response regulator
3	2	8.1	A0A161J5J8	Aspartate phosphatase
3	2	8.6	A0A160LDJ0	Succinate--CoA ligase [ADP-forming] subunit alpha
2	2	6.9	A0A160L8M	Carboxypeptidase 1

			6	
2	2	9.1	A0A161J1I4	Aminotransferase
2	2	16.3	A0A160L5R5	50S ribosomal protein L11
2	2	16.1	A0A160LH59	Single-stranded DNA-binding protein
3	2	18	A0A160L556	50S ribosomal protein L14
3	2	13.8	A0A169D159	50S ribosomal protein L10
3	2	6.9	A0A160LAE4	Uncharacterized protein
2	2	21.5	A0A160LGX6	Spore coat protein GerQ
4	2	25	A0A161JQF0	Uncharacterized protein
2	2	6.5	A0A160LG37	Proline dehydrogenase
2	2	4.8	A0A160L5P5	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B
2	2	13.5	A0A160L7M5	HTH-type transcriptional regulator Hpr
2	2	16.6	A0A160LDH9	Uridylate kinase
2	2	5.3	A0A160LCN8	Peptide ABC transporter substrate-binding protein
2	2	20	A0A160L573	50S ribosomal protein L17
2	2	10	A0A161JQE1	Aspartokinase
2	2	5.2	A0A160LDS5	GTP-binding protein TypA
3	2	13.3	A0A160LF68	Enoyl-CoA hydratase
2	2	18.3	A0A160LF63	Transcriptional repressor NrdR
2	2	5.7	A0A160LEC4	Probable glycine dehydrogenase (decarboxylating) subunit 2
2	2	7.5	A0A160LE50	Ornithine carbamoyltransferase
2	2	11.4	A0A160L547	50S ribosomal protein L3
2	2	7	A0A160LD39	2-oxoglutarate ferredoxin oxidoreductase subunit alpha
3	2	10.3	A0A161J6A8	Acetyltransferase
3	2	6.5	A0A169DNU4	Glutamate-1-semialdehyde 2,1-aminomutase
2	2	12.8	A0A160L6Q7	30S ribosomal protein S7
2	2	5.8	A0A160LE75	2-oxoisovalerate dehydrogenase
2	2	7.1	A0A161J604	Zn-dependent hydrolase
2	2	6.5	A0A160LHA4	Fe-S cluster assembly protein SufD
2	2	0.9	A0A169DD32	Non-ribosomal peptide synthetase

3	2	9.4	A0A160LH75	ATP synthase gamma chain
2	2	7	A0A160LA15	3-hydroxyisobutyrate dehydrogenase
2	2	8	A0A160LEJ4	RNA polymerase sigma factor SigA
2	2	5.2	A0A160LA52	Acyl-CoA dehydrogenase
2	2	5	A0A160L5P9	Lysine--tRNA ligase
3	2	9	Q8KNU0	Uncharacterized protein
3	2	7.7	A0A160L804	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex
2	2	15.9	A0A169D8J9	SSEB protein
2	2	3.6	A0A160LF48	Asparagine--tRNA ligase
2	2	11.7	A0A160L5N5	50S ribosomal protein L5
2	2	4.1	A0A160LDD1	Uncharacterized protein
2	2	4.5	A0A161J2P3	Aminopeptidase
2	2	4.4	A0A161JQ17	Carboxylase
2	2	25.7	A0A160L553	50S ribosomal protein L29
2	2	5.7	A0A169DC63	Sporulation protein
1	1	5.5	A0A160L7F1	3-oxoacyl-[acyl-carrier-protein] synthase 2
2	2	8.9	A0A160LGN9	ATP synthase subunit b
2	2	5.2	A0A169DGE4	Phosphoserine aminotransferase
2	2	10.8	A0A160L6A0	Chemical-damaging agent resistance protein C
2	2	7.3	A0A160LFE5	UDP-N-acetylmuramate--L-alanine ligase
2	1	27.7	A0A169D1E5	Translation initiation factor IF-1
2	1	9.5	A0A161IQ33	Acetyltransferase
2	2	7.2	A0A160LDT4	Ribonuclease J
2	2	1.9	Q1RN84	Delta-endotoxin
1	1	11.1	A0A160L5T3	50S ribosomal protein L4
1	1	3.6	A0A160LAU3	ATP-dependent DNA helicase RecQ
1	1	6.5	A0A160L7Q1	UDP-glucose 4-epimerase
2	1	4.3	A0A160LD99	Transcription termination/antitermination protein NusA
3	2	9.1	A0A169D4J6	Butanediol dehydrogenase
2	2	3.4	A0A160LDI7	Cell division protein FtsA
2	1	3.3	A0A161JQC7	Imidazolonepropionase

1	1	4.4	A0A160L709	3'-5' exoribonuclease YhaM
2	1	6.3	A0A160L5U5	DNA-directed RNA polymerase subunit alpha
1	1	9.3	A0A160LGP5	Uncharacterized protein
2	1	24.4	A0A160LD72	DNA-binding protein
1	1	3.9	A0A169DTB1	Acyl-CoA dehydrogenase
2	1	8.7	A0A160LGI4	Triosephosphate isomerase
1	1	1.8	A0A160LG26	Aspartate--tRNA(Asp/Asn) ligase
1	1	7.2	A0A160L5U1	30S ribosomal protein S5
1	1	13	A0A160LG72	Uncharacterized protein
1	1	10.4	A0A160L5M7	50S ribosomal protein L23
1	1	6.8	A0A160LIC5	Stage 0 sporulation protein A
1	1	3.9	A0A160LFC9	Argininosuccinate synthase
1	1	3.9	A0A160LGT2	Acyl-CoA dehydrogenase
1	1	13.2	A0A160LAN3	ESAT-6-like protein
1	1	8.2	A0A160L8L6	Menaquinol-cytochrome C reductase
1	1	6.1	A0A160LGF0	Ribosome hibernation promoting factor
1	1	1.9	A0A169D7D1	Chaperone protein ClpB
1	1	9.8	A0A160LDQ2	Short-chain dehydrogenase
1	1	5.6	A0A160LBA3	Peptide ABC transporter substrate-binding protein
1	1	5.8	A0A160LE62	Oxidoreductase
2	1	19.1	A0A160L4Y9	Transition state regulator Abh
1	1	3	Q8KNU9	Possible two-domain toxin
1	1	4.7	A0A160L624	Redox-sensing transcriptional repressor Rex
1	1	26	A0A160LFM7	ArsR family transcriptional regulator
1	1	10.3	A0A169D855	DNA recombinase
1	1	7.8	A0A169D9J8	Histidinol-phosphate aminotransferase
1	1	5.9	Q8KNU6	Uncharacterized protein
1	1	2.3	A0A160LGH4	Pyruvate kinase
1	1	6.8	A0A160L591	30S ribosomal protein S8
1	1	2.7	A0A169D0R5	Methionine--tRNA ligase
1	1	2.9	Q8KNP4	Hemagglutinin-related protein
1	1	3.6	A0A160LG02	Lipoyl synthase
1	1	3	A0A160LF90	Lon protease

1	1	1	A0A169D7U8	2-oxoglutarate dehydrogenase E1 component
1	1	1	A0A160LF27	Alanine--tRNA ligase
2	1	6.2	A0A160LGD8	ATP-dependent Clp protease proteolytic subunit
1	1	3.9	A0A160L901	Enoyl-[acyl-carrier-protein] reductase [NADH]
1	1	0.9	A0A1L2Z054	Aldehyde-alcohol dehydrogenase
1	1	2.3	A0A160L5J6	Serine--tRNA ligase
1	1	3	A0A160LC26	3-phosphoglycerate dehydrogenase
1	1	6.1	A0A169D1A0	50S ribosomal protein L2
1	1	3.4	A0A160LF25	Enoyl-CoA hydratase
1	1	3.1	A0A169DM18	Acetyl-CoA carboxylase biotin carboxylase subunit
1	1	1	A0A169DK17	Carbamoyl-phosphate synthase large chain

APPENDIX E: Cytolytic activities of *Bt* strains tested

Table 32. Cytolytic activity results of *Bti* strains against lung cancer epithelial cells

Strains	Concentrations of crystal proteins (µg/ml)						Treatment time
	0	0.15	0.25	0.35	0.5		
H14	100	73.71	70.58	60.00	43.68	Percentage of viable cancer cells (%)	24 hrs
	100	73.51	63.55	57.40	53.66		48 hrs
	100	99.33	91.89	76.73	61.28		72 hrs
*QBT*205	100	94.67	78.09	84.42	70.22		24 hrs
	100	87.11	82.17	78.95	71.33		48 hrs
	100	85.03	74.07	72.46	58.27		72 hrs
*QBT*213	100	87.86	90.35	81.34	60.32		24 hrs
	100	76.56	69.26	74.21	54.94		48 hrs
	100	103.56	109.15	92.86	65.61		72 hrs
*QBT*214	100	85.74	70.45	62.01	53.85		24 hrs
	100	85.71	74.87	67.94	62.62		48 hrs
	100	86.56	82.98	87.91	76.64		72 hrs
*QBT*215	100	87.16	88.42	65.03	44.79		24 hrs
	100	82.44	101.98	88.14	86.90		48 hrs
	100	88.9	92.96	98.31	77.79		72 hrs
*QBT*216	100	93.10	78.59	86.44	68.55		24 hrs
	100	82.05	77.95	73.42	56.73		48 hrs
	100	90.58	72.15	59.81	56.34		72 hrs
*QBT*217	100	80.30	62.60	55.43	41.09		24 hrs
	100	62.95	69.77	64.81	55.89		48 hrs
	100	74.4	66.04	60.88	58.16		72 hrs
*QBT*218	100	73.60	73.71	71.25	55.37		24 hrs
	100	87.88	75.26	77.31	84.09		48 hrs
	100	101.37	75.44	76.27	71.39		72 hrs
*QBT*220	100	79.44	69.90	72.52	42.31		24 hrs
	100	76.15	79.69	66.05	58.24		48 hrs
	100	76.08	73.49	61.01	70.54		72 hrs
*QBT*221	100	102.40	88.68	58.47	65.19		24 hrs
	100	98.47	82.88	66.84	52.34		48 hrs
	100	98.38	91.92	71.98	61.04		72 hrs
*QBT*222	100	115.83	88.29	77.33	84.54		24 hrs
	100	78.45	76.85	59.47	54.49		48 hrs
	100	77.27	82.98	75.04	66.36		72 hrs
*QBT*223	100	98.99	91.03	93.35	77.31		24 hrs
	100	68.95	64.81	61.14	54.94		48 hrs
	100	82.72	80.49	75.74	67.96		72 hrs
*QBT*224	100	87.27	64.89	64.46	72.64		24 hrs
	100	77.59	67.05	60.25	59.60		48 hrs
	100	97.32	83.59	95.35	97.98		72 hrs

*QBT*225	100	83.44	90.57	75.94	52.56		24 hrs
	100	75.04	64.67	61.87	57.34		48 hrs
	100	101.07	74.09	83.73	85.32		72 hrs
*QBT*226	100	95.40	98.54	78.14	68.27		24 hrs
	100	94.72	96.09	74.40	66.76		48 hrs
	100	85.28	79.27	76.64	79.47		72 hrs
*QBT*227	100	106.66	103.80	61.72	60.43		24 hrs
	100	94.03	79.20	66.23	59.05		48 hrs
	100	81.51	73.76	75.38	73.2		72 hrs
*QBT*228	100	96.64	92.15	66.49	61.28		24 hrs
	100	103.33	96.56	83.05	75.40		48 hrs
	100	89.95	83.39	81.21	75.34		72 hrs
*QBT*229	100	52.14	74.33	64.63	66.40		24 hrs
	100	54.55	69.00	70.43	67.71		48 hrs
	100	51.3	47.98	52.44	49.62		72 hrs
*QBT*230	100	72.48	99.74	77.86	61.56		24 hrs
	100	80.79	73.65	76.73	72.95		48 hrs
	100	92.25	54.98	51.43	40.75		72 hrs
*QBT*608	100	93.86	87.03	72.38	68.02		24 hrs
	100	103.66	88.87	58.76	48.76		48 hrs
	100	96.2	87.87	80.02	75.74		72 hrs

Table 33. Cytolytic activity results of Non *Bti* strains against lung cancer epithelial cells

Strains	Concentrations of crystal proteins (µg/ml)						Treatment time
	0	0.15	0.25	0.35	0.5		
*QBT*3	100	95.83	95.98	93.96	99.22	Percentage of viable cancer cells (%)	24 hrs
	100	73.36	72.60	69.71	73.86		48 hrs
	100	85.93	91.99	76.11	72.16		72 hrs
*QBT*6	100	86.27	90.22	87.19	86.44		24 hrs
	100	82.36	101.64	84.99	79.09		48 hrs
	100	97.58	89.56	89.27	78.40		72 hrs
*QBT*34	100	99.15	104.60	94.65	91.01		24 hrs
	100	68.56	84.73	78.12	73.27		48 hrs
	100	84.24	81.53	72.05	72.67		72 hrs
*QBT*39	100	98.58	90.98	94.47	94.92		24 hrs
	100	75.52	81.19	73.98	73.54		48 hrs
	100	77.38	73.04	87.30	70.78		72 hrs
*QBT*41	100	96.67	101.09	96.82	89.40		24 hrs
	100	95.35	87.48	91.95	75.17		48 hrs
	100	80.57	85.71	83.84	78.45		72 hrs
*QBT*43	100	74.75	94.65	65.15	57.46		24 hrs
	100	69.73	63.73	60.74	56.32		48 hrs

	100	92.12	90.57	89.76	82.04		72 hrs
*QBT*56	100	83.73	112.20	92.65	81.05		24 hrs
	100	59.04	79.92	69.17	66.49		48 hrs
	100	84.66	91.98	88.93	100.30		72 hrs
*QBT*81	100	96.20	85.68	97.98	80.03		24 hrs
	100	74.15	71.27	63.13	71.83		48 hrs
	100	92.99	87.77	88.90	89.92		72 hrs
*QBT*99	100	81.00	71.81	69.31	73.60		24 hrs
	100	97.27	80.37	90.85	105.34		48 hrs
	100	80.75	74.68	74.66	76.04		72 hrs
*QBT*212	100	78.24	85.46	85.27	69.41		24 hrs
	100	87.48	73.80	82.92	85.02		48 hrs
	100	88.93	84.73	86.60	92.64		72 hrs
*QBT*240	100	87.02	103.28	92.85	84.00		24 hrs
	100	81.12	75.47	76.71	82.21		48 hrs
	100	82.38	100.30	83.82	79.16		72 hrs
*QBT*320	100	83.12	75.47	101.75	76.17		24 hrs
	100	96.72	98.18	96.87	95.85		48 hrs
	100	72.63	71.84	69.60	63.01		72 hrs
*QBT*418	100	92.72	96.54	62.98	62.44		24 hrs
	100	71.38	78.82	77.41	76.80		48 hrs
	100	99.71	92.53	88.23	105.10		72 hrs
*QBT*555	100	85.39	73.33	62.59	52.00		24 hrs
	100	88.54	85.14	69.69	77.41		48 hrs
	100	74.29	78.37	74.05	70.11		72 hrs