Journal of Neonatal Surgery
ISSN(Online): 2226-0439
Vol. 14, Issue 32s (2025)
https://www.jneonatalsurg.com

OPEN ACCESS

A Complete Examination of Bacillus thuringiensis: Its Properties and Functions of Proteins

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Cite this paper as: Hasna KP, Rasheeda EV, (2025) A Complete Examination of Bacillus thuringiensis: Its Properties and Functions of Proteins..*Journal of Neonatal Surgery*, 14, (32s) 9426-9433.

ABSTRACT

Bacillus thuringiensis is an ubiquitous, gram-positive, aerobic, endospore-forming environment-friendly bacterium. The development of this bacterium brings the most significant advances in crop protection technology. Bacillus thuringiensis produces various types of insecticidal proteins, including Cry, Cyt, Vip (vegetative insecticidal proteins), and Sip (secreted insecticidal proteins), each with distinct modes of action and target specificities. Cry protein is famous in its insecticidal activity against many insect species . Cyt proteins exhibit broad cytotoxic activity against both invertebrate and vertebrate cells (Bravo et al., 2007). Besides this protein Bacillus thuringiensis also produces Vip (vegetative insecticidal proteins) and Sip (secreted insecticidal protein), both of which have insecticidal activity. Vip proteins are divided into four groups Vip1, Vip2, Vip3, and Vip4 (Palma et al., 2014). The organism also exhibits parasporal crystals during the stationary phase of its growth cycle, making it a unique organism from others (Moazamian et al., 2018). These proteinaceous bodies reported several distinctive characteristics, and crystals have unique toxic activities against certain insects and some other invertebrates (Charles et al. 2000) and human cancer cells (Mizuki et al., 2000)

Keywords: *Cry protein, Cyt protein, parasporin, Vip protien , Bombyx Mori.*

1. INTRODUCTION

Bacillus thuringiensis (commonly abbreviated as Bt) has gained enormous attention in both basic and applied microbiology, agriculture, and biotechnology (Ibrahim et al., 2010). Many Bt strains produce proteinaceous crystalline inclusions (also known as crystal proteins or δ-endotoxins) during the sporulation phase, which are poisonous to some insects when consumed. The main emphasis of Bt's function as a biological pesticide is these insecticidal proteins, also known as Cry and Cyt proteins (and occasionally vegetative insecticidal proteins, or Vip) (Palma et al., 2014).

Bt and its toxins are widely utilized in organic farming, transgenic (Bt) crops, and integrated pest management (IPM) due to their low environmental persistence, high selectivity to target pests, and relative safety to non-target organisms. The molecular biology, structural biochemistry, mode of action, evolution of resistance, and environmental behavior of Bt proteins have all been extensively studied in recent decades (Schnepf et al., 1998). This work aims to present a compre.

investigation of *B. thuringiensis*—covering its historical background, various protein and its functions etc. In doing so, we will also review challenges such as insect resistance, protein stability in the environment, and biosafety considerations (Ibrahim et al., 2010).

In the following review, we first lay out the historical background, general characteristics and classification of *B. thuringiensis*, then explore in depth each major class of insecticidal proteins (Cry, Cyt, Vip), their structural and functional features, mode of action against insects, interactions with insect midgut receptors, and factors influencing potency.

2.1 Discovery and Importance of Bacillus thuringiensis

In 1901 Shigetane Ishiwata, a Japanese silkworm pathologist isolated a new bacterium as a pathogen from the sotto disease of the silkworm *Bombyx Mori* and he named the bacterium *Bacillus sotto.* The disease was blamed for the large scale loss of silk worms in Japan and surrounding area (K, 1915). Later in 1915, Ernest Berliner isolated the bacterium from Schlaffsucht disease in flour moth caterpillars, he discovered at a flourmill in the German state of Thuringia and the bacteria was given the apt name Bacillus Thuringiensis by him (Ibrahim et al., 2010). He also identified the organism as gram-positive, spore-forming bacterium in the *Bacillus cereus* group. Later the bacterium used as a biopesticide because of the production of crystalline parasporal inclusion during the sporulation period of the bacterium (Okumura et al., 2008). The sporulation phase contains seven stages. Synthesis of parasporal inclusion begins at the second and third stage, by the end of the fifth stage the crystals reach their maximum size. The spores and crystals were released into the environment at the time of maturation of spores. The attractive property of *Bacillus thuringiensis* is its capacity to produce proteinaceous crystals. These crystals

contain toxic protein against insects, and such toxic protein are called δ-endotoxin. Based on hemolytic activity, δ-endotoxin divided in to Cry and Cyt protein (Aldeewan et al., 2014). Cry proteins are nonhemolytic protein and it exhibits experimentally verifiable toxic effects to the insects, such as Lepidoptera, Coleoptera, Hymenoptera and Diptera, and nematodes also (Bravo et al., 2007).

The discovery of insecticidal activity of cry protein was found to be the isolation of spores and crystals from dead flour moth caterpillars, and there is no effect when direct contact with spores and crystals, but it died when feed spores and crystals coated leaves (Sanahuja et al., 2011). When the insect ingests the crystals, the cry protein is solubilized in the alkaline environment of its midgut and it is cleaved by proteases in the gut. Then it binds to cell membrane receptor of the gut epithelium resulting in the cell disruption which leads to the death of the insect (Bravo et al., 2007). Recently, a novel protein having cytotoxicity against human cancer cells has been reported from the parasporal inclusions produced by *B. thuringiensis* strains. These cytotoxic proteins are neither insecticidal nor hemolytic. They are structurally different from Cry and Cyt proteins. Hence these proteins become a new family; the members of the family express preferential cytotoxicity to human cancer cells and name as parasporin (Hayakawa et al., 2007).

A novel medicine goes through various stages of development, including drug discovery, pre-clinical and clinical development, and regulatory approval. In vitro and in vivo experiments on cell lines and animals are the first steps in the identification of a new medicine. It then moves on to the clinical research phase, where the major goal is to test the efficacy and safety in people. A new drug must undergo about 15 years of development before it can be sold to consumers (Santos et al., 2022).

2.2 General characteristics of Bacillus thuringiensis

Bacillus thuringiensis is a member of *the Bacillus cereus* group which includes five other species: *B. cereus*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis. Bacillus thuringiensis* is genetically and phenotypically closely related to the *Bacillus cereus* group. The difference between these two species is the release of crystal parasporal inclusions during sporulation period of *Bacillus thuringiensis* (Charles et al., 2000).

Asano *et al*. (2003) on his work with *Bacillus thuringiensis* he reported that it could culture on a simple culture medium, produce spores after depletion of nutrients in the medium. Seven stages were observed in the sporulation period. Crystals started to synthesize the second or third stage and reach its maximum size by the fifth stage. These crystals with varying size are called δ endotoxin. There are two types of δ-endotoxins named Cry and Cyt proteins, and both are located on large transmissible plasmids. They are different structurally and functionally (Asano et al., 2003)

2.3 Bacillus thuringiensis Cry protein.

Ancient time poisonous chemical pesticides were used to protect agricultural and industrial products from insects. This is adversely impacting and harmful to farmworkers. More cancer cases are reported from field workers when compared to other fields. Usage of pesticide causing killing of worms and microorganisms in the soil, which results in destroying fertility characteristics of soil and also the content of pesticide remains in fruit and vegetables even after washing and peeling. It also contaminates drinking water. Hence, it's urgent to replace synthetic pesticide for the survival of the ecosystem. The most relevant alternatives were found from crystal protein released during the sporulation period of entomopathogenic microorganism named *Bacillus thuringiensis* as an environmentally friendly pesticide. *Bacillus thuringiensis* seems to be indigenous to many habitats, including soil, insects, stored products, deciduous and coniferous leaves, etc. were reported by Shishir *et al*. (Shishir et al., 2014). Another study was concluded by Lone *et al*. in 2016, using *Bacillus thuringiensis* to improve the environmental conditions, and to build up chemical-free earth. Environment-friendly pest control needs to be developed instead of humanmade pesticides because the pesticide also affects nontarget insects such as predators, parasites, etc. The problem was solved by a good alternative from *Bacillus thuringiensis* (Rampersad and Ammons, 2005). Cinar *et al*. in 2008 isolated *Bacillus thuringiensis* strain from the olive environment, a study in which he observed that the isolate contains different crystal shapes like spherical, cuboidal, bi-pyramidal and irregular shapes. Among these irregular shaped crystals producing *Bacillus thuringiensis* were found more frequent (24%) in the olive environment (Cinar et al., 2008).

Cry proteins are insecticidal protein, classified based on their host specificity and amino acid homology. The crystal proteins are seen in different structures such as bipyramidal (Cry1), cuboidal (Cry2), flat rectangular (Cry3A), irregular (Cry3B), spherical (Cry4A and Cry4B), and rhomboidal (Cry11A) (Schnepf *et al*. 1998) (Schnepf et al., 1998). Bravo *et al*. 2011 explained in his work about the first practical application of *Bacillus thuringiensis* done by Huisz in 1928 who isolated *Bacillus thuringiensis* strain from Ephestia and tested it on European corn bores. The work eventually led to the first commercial product sporeine, which was produced in France in 1938. There are many subspecies and serotypes of *Bacillus thuringiensis* with a range of well-characterized insecticidal proteins or *Bacillus thuringiensis* toxins that kill insects among the Lepidoptera, coleopteran, and nematodes (Bravo et al., 2011). In 2008 Pigott *et al*. investigated cry protein with thousand *Bacillus thuringiensis* strains, in their investigation, they found that most of the cry proteins are toxic to lepidopterans, dipterans and coleopterans whereas some are also toxic to hymenopterans and nematodes. Pigotts also report the effect of cry toxin in the insect's gut. The cry toxin needs to treat with enzymes to be functional when the insect ingested the toxin it solubilizes in the midgut pH and produce protoxins. It exposes the cleavage site, which cut by host proteases to release

activated core. Then the toxin bind to midgut epithelial surface and disrupt the membrane, allow ion and water to enter the cell, the cell swell and lysis leads to the death of the insect (Pigott et al., 2008).

3d cry toxins are very useful to target insect and kill few numbers of species. 3d Cry toxins are globular molecules having three domains joined in single linkers. In Cry toxin family 3d Cry toxin is the largest family having 53 subgroups. An essential character of this protein is the presence of protoxins having two different lengths, such as 65 and 130 kD. The mechanisms of action proposed was that the 3d Cry toxin promote the formation of nonselective channels allowed cations, anions and neutral solutes, leads to colloidal osmotic lysis of the cells in the midgut, result cell swelling and lysis (Pardo-López et al., 2013).

2.4 Bacillus thuringiensis Cyt protein.

In 2001, Guerchicoff *et al*. studied on hemolytic Cyt genes family produced by *Bacillus thuringiensis*. Cyt toxins also show similar activity like pore formation, disruption of insect midgut. There was no specific receptor identified for Cyt toxin. In their experiment, they found a positive correlation between Cyt1 & Cyt2 proteins with mosquitocidal activity. In their work, they also concluded that Cyt genes are widely distributed among a range of *Bacillus thuringiensis* (Guerchicoff et al., 2001).

Cyt or cytolytic protein that exhibits hemolytic activity, this class of δ-endotoxins differs in amino acid composition and action mechanism from Cry toxins. These toxins act synergistically with mosquitocidal Cry toxins. The Cyt proteins present only in Dipteran strains, but cry protein present various *Bacillus thuringiensis* strain with different host range. Cyt toxins some time used to overcome insecticide resistance and to increase the activity of microbial insecticides (Juárez-Pérez et al., 2002). The structure of three Cyt protein Cyt1Aa, Cyt2Aa and Cyt2Ba, all are showing the same topology having an α-β domain with two outer layers of α helix hairpin covered by the β sheet (Soberón et al., 2013). Nair *et al.* 201, isolated *Bacillus thuringiensis* from Qatar and named QBT229. They reported a new Cyt1A protein with higher cytotoxicity towards lung cancer cells. It was because of the replacement of consecutive amino acid in the β-8 sheet of the Cyt1A protein (Nair et al., 2018).

2.5 Bacillus thuringiensis Vip protein

Palma *et al*. 2014. in his study with *Bacillus thuringiensis,* he introduces another insecticidal protein released by *Bacillus thuringiensis* during the vegetative growth phase, named vegetative insecticidal protein (Vip) and secreted an insecticidal protein (Sip). Vip protein shows insecticidal activity against lepidopterans and divided into four families Vip1-4, respectively. Sip protein toxic to coleopterans (Palma et al., 2014). Vegetative insecticidal proteins (Vip), which are released into the growth media during vegetative growth, are one of the crucial *Bt* protein families. Vip proteins fall into three subfamilies. Many Coleopteran and Hemipteran pests are insecticidal to Vip1 and Vip2 heterodimer poisons. There have been reported and named 15 Vip1 proteins, 20 Vip2 proteins, 111 Vip3 proteins, and 5 Vip4 proteins to date. (Syed et al., 2020).

2.5.1 Vip 1/Vip 2 protein

Yu *et al*. 2011 characterized the Vip protein and reported that Vip1&2 are binary toxins and act against coleopterans, whereas Vip3 toxins show activity against lepidopterans. Vip protein classified based on amino acid sequence similarity into three groups, eight subgroups, twenty five classes and 82 subclasses. According to studies based on their genetic homology, Vip1 and Vip2 behave similarly to many mammalian toxins as binary toxins of the A + B type. Vip1 shares relatively little structural similarities with the CdtB toxin of Clostridium dificile, the protective antigen of Bacillus anthracis, or Clostridium spiroforme toxin. Vip2 and the active domain of C. difficile's CdtA toxin are structurally related. The C2 toxin produced by Clostridium botulinum and the domain Ia of the iota toxin produced by Clostridium perfringens are both comparable to the VIP1 and VIP2 toxins. Overall, data suggests that Vip2 functions as an ADP-ribosyltransferase toxin that prevents the polymerization of actin filaments, leading to anomalies in the cytoskeleton and the death of insect cells. It is hypothesized that Vip1 functions as a B toxin (Binding domain) that transports Vip2 inside the midgut cells of insects. Vip2 is a cytotoxic A toxin with a binary toxin response, and when used alone, it has no deleterious effects on insects (Syed et al., 2020).

2.5.2 Vip 3 protein

They also found novel Vip genes (Vip3Aa28, Vip3Aa29, Vip3Aa30, Vip3Aa31, VipAa32 and Vip3Aa33) (Yu et al., 2011). Chakroun *et al*. 2016 published an article about Vip proteins, and he said that Vip 1&2 proteins are also found in *Bacillus cereus, Bacillus sphaericus* and *Brevibacillus laterosporus.* Both of these are located on the same operon with different open reading frames divided by an intergenic spacer of 4 to 16 bp within a 4 to 5kb genomic sequence. The insecticidal activity starts with the uptake of toxin by the larva and activated by the midgut enzyme (Chakroun et al., 2016). A study about Vip1Ac shown that the activated monomer form oligomers containing seven Vip1 molecules and identify the receptors present in the midgut brush border membrane, and toxin was inserted into the membrane.

Vip 3 protein only found in *Bacillus thuringiensis*. The transcription start point was located 101 bp upstream of the start codon, promoter region -35 and -10 were similar to the promoter of *Bacillus subtilis* which are controlled by σ^E holoenzyme. This concluded that the Vip 3Aa16 gene transcribed by σ^E holoenzyme. When an insect takes the protein leads to loss of gut peristalsis and overall paralysis. The midgut was damaged, disrupted, lysed epithelial cells and leakage of cellular material

into the lumen. There was no damage occur in hindgut and foregut(Leuber et al., 2006).

2.5.3 Vip 4 protein

Vip4 is the least characterized toxin of the Vpb class. Only five Vip4 proteins have been identified to date. The first reported Vip4 toxin was Vip4Aa1 (now named Vpb4Aa1), isolated from Bt strain Sbt009, with no insecticidal activity against any pests. Its molecular mass is ~108 kDa and it possesses 965 amino acids. The main region spanning from 47 to 77 amino acids is a PA14 domain, and the region from 218–631 residues is named the bacterial Binary_ToxB domain. This novel protein shares 34% identity with the Vip1Aa1 protein and 65% with the Ia domain of the Iota toxin of B. cereus, specifically to the B component of the binary toxin (Ruiz de Escudero et al., 2014). The length of the vip4Aa1 gene is 2895 bp, and its deduced amino acid sequence contains 965 residues. According to in silico analysis, the molecular mass of the compound is 108 kDa (Palma et al., 2014; Chakroun et al., 2016). According to Palma et al. (2014), it shares 34% of the amino acids in its predicted protein sequence with the Vip1Aa1 protein. An analysis of the protein sequence identified a signal peptide sequence (1-28) as well as two conserved domains, the PA14 domain (a protective antigen against anthrax) at 45–179 amino acids and the Binary ToxB exotoxin bacterial domain (218–631 amino acids), which is often seen in binary Vip1. Vip4Aa1 is shown to be phylogenetically much more closely connected with Vip1 proteins than Vip2 and Vip3 proteins, albeit its insecticidal action and host range are still unknown (Leuber et al., 2006).

2.6 Bacillus thuringiensis Sip protein

The secreted insecticidal protein (Sip) is the first and only member of the secreted protein family that belongs to the *Bacillus thuringiensis* insecticidal genus and has been shown to be poisonous to coleopteran larvae. The Sip protein, also known as Sip1Aa1, was first isolated from culture supernatants of the *Bacillus thuringiensis* strain EG2158 (Palma et al., 2014).

Bacillus thuringiensis strain EG2158 was shown to have larvicidal efficacy against Colorado potato beetle (*Leptinotarsa decemlineata*) larvae by Donovan's bioassay screening of *Bacillus thuringiensis* culture supernatants. Using ion-exchange fractionation of the EG2158 culture supernatant, a protein with the name Sip1A (secreted insecticidal protein) of about 38 kDa with activity against the Colorado potato beetle (CPB) was discovered. The sip1A gene was isolated using an oligonucleotide probe based on the N-terminal sequence of the purified Sip1A protein. The cloned sip1A gene's sequence was used to infer the 367 residues (41,492 Da) of the Sip1A protein The sip1Aa1 gene has a length of 1104 bp and produces a protein with 367 amino acids and a mass of 41 kDa. Sip1Aa1 has a 30 amino acid long, typical anticipated Gram-positive consensus secretion signal. The protein was discovered to have undergone N-terminal processing, with the first 43 amino acids having been removed by active proteases identified in the culture media. It exhibits a weak but considerable similarity to the 36-kDa Mtx3 mosquitocidal toxin from Ls, which is a toxin belonging to the ETX_MTX2 family. This homology strongly implies that Sip1Aa1 toxicity may be brought on by the development of pores, but the exact mechanism of action is still unknown (Donovan et al., 2006).

2.7 Bacillus thuringiensis S layer protein

The S-layer, which makes up to 15% of the total protein in a cell, is an organized structure of proteinaceous paracrystalline arrays that completely covers the surfaces of many bacteria and archaea. S-layer proteins' (SLP) purpose has not yet been fully established. These proteins might serve a role in maintaining the integrity and form of cells, according to a theory. Additionally, they are the outermost component of the cell membrane, it has been theorized that they may be involved in macromolecule exchange with the environment (Beveridge et al., 1997). According to Pena et al.'s 2006 study report, an S-layer protein was discovered by testing *B. thuringiensis* strains for efficacy against the coleopteran pest *Epilachna varivestis* (Mexican bean beetle; Coleoptera: Coccinellidae). They analysed two collections of *B. thuringiensis* strains for unidentified Cry proteins as well as strains recovered from dead insects. Some of the B. thuringiensis strains tested for toxicity against *E. varivestis* displayed a moderate level of toxicity. However, a strain of B. thuringiensis (GP1) that was isolated from a dead insect shown an incredibly strong insecticidal activity. When the parasporal crystal made by the GP1 strain was purified, it was discovered to have insecticidal efficacy only against *E. varivestis*, not against the lepidopterans *Manduca sexta* or *Spodoptera frugiperda*, nor against the dipteran Aedes aegypti. Cloning and sequencing of the gene encoding this protein. It was an S-layer protein that resembled the SLPs from *Bacillus licheniformis* (OlpA) and Bacillus anthracis (EA1) that had been previously discovered (Peña et al., 2006).

2.8 Bacillus thuringiensis Parasporin protein

Parasporin is a non-insecticidal protein produced by the bacterium *Bacillus thuringiensis*. This protein is showing high cytotoxic activity against human cancer cells when treated with the enzyme. There are currently six different classes of parasporin (PS1, PS2, PS3, PS4, PS5, and PS6), based on amino acid homology and level of activity. Out of the 6 PSs group, the First 4 PSs were studied in detail. Various studies reported that the effect of these proteins towards transformed cells led to the overexpression of targeted receptor molecules than in healthy cells. Then the protein identifies the transformed cells and kills them correctly, and this made them potential candidates of cancer treatment (Chubicka et al., 2018).

Mizuki *et al*. 1999 introduced a new variety of cry toxins during the sporulation time of *Bacillus thuringiensis* that exhibit cytocidal activity against human cancer cells instead of insecticidal activity. He focused on this and later in 2000, designate this protein as parasporin (Mizuki et al., 2000). Several studies occur based on this protein and identified different types of

parasporin. Based on amino acid homology and activity spectrum, it divided into six main classes named parasporin1-6, respectively. Currently, 19 parasporins are available parasporin nomenclature site. The site was organized in 2006 to construct a taxonomical system based on the amino acid identity. (http//parasporin.fitc.pref.fukuoka.jp/ index.html).

Cry, Cyt and parasporin toxins are categories based on available structural similarities of *Bacillus thuringiensis* toxins and pore-forming toxin (PFT) are, three-domain type α-PFTs, Cyt toxin type β-PFTs and aerolysin type β-PFTs respectively. Here α-PFTs are produced pores as a bundle of α-helices, on colicins, exotoxin A, diphtheria toxins, etc. The β-PFTs produce β-barrel transmembrane pores on aerolysin, epsilon, cholesterol-dependent cytolysin, α-hemolysin, etc (Xu et al., 2014).

2.8.1 Parasporin *1 protein*

In 1999, Mizuki *et al*. isolated 81 kDa protein having cytolytic activity, from *Bacillus thuringiensis* (84-HS-1-11) isolates of Hiroshima Prefecture, Japan. Later in 2000, they characterized the protein and also cloned the novel cytotoxic gene. It shows activity only after treatment with enzymes like protease, trypsin, etc. There was no activity in the alkalized environment. They show similarity with the Cry31 gene and classified under Cry31. This protein designated as Parasporin 1 (Mizuki et al., 2000). Katayama *et al*. 2005 also isolated the 81 kDa protein from A1190 formerly 84-HS-1-11 and named pro parasporin 1 then activated with trypsin and named parasporin 1. In their work, they activate pro parasporin to parasporin with trypsin, purified the activated parasporin with column chromatographic technic and also defined the molecular structure and cytotoxic activity of parasporin 1(Katayama et al., 2005). Jung *et al*. 2007 reported novel Cry protein Cry31Aa2 (PS1Aa2) with cytotoxic activity when study with *Bacillus thuringiensis* (M15) isolated from two-spotted dead spider (Jung et al., 2007). Later Uemori *et al*. 2008 isolated *Bacillus thuringiensis* (B015) strain which carrying two parasporin gene PS1Aa3 and PS1Ab1 (Cry31Aa3 and Cry31Ab1 respectively) from Japan soil. He reported that he isolated PS1Aa3 having 81 kDa with 2,169 base pair long, 100% percentage similarity with PS1Aa1. PS1Ab1 having 82 kDa with 2178 base pair long, it shows 86.4% similarity with PS1Aa1 reference protein (Uemori et al., 2008). PS1 related four new protein genes named PS1Aa4, PS1Aa5, PS1Ab2 and PS1Ac1 were isolated by Yasutake *et al.* in 2008 from *Bacillus thuringiensis,* 79-25, 92-10, 31-5, and 87-29 respectively urban soil of Hanoi, Vietnam. In their work, they establish a new class PS1Ac having the cytotoxic activity to HepG2 by specific gene cloned from *Bacillus thuringiensis* 87-29 isolates (Yasutake et al., 2008). Nagamatsu *et al*. 2010 identified three cry toxin from two strains of *Bacillus thuringiensis* named M019 and CP78A, CP78B and CP84. PS1Aa6 (CP78A), PS1Ad1 (CP78B) and PS6Aa1 (CP84) (Nagamatsu et al., 2010).

1n 2010 Poornima *et al*. isolated Parasporin 1 protein carrying *Bacillus thuringiensis* (LDC-391) from the soil of urban and semi-urban area of Madurai district, Tamilnadu. They isolated small spherical parasporal inclusion different from large parasporal non-insecticidal inclusions that are commonly reported. The anticancer activity they were observed that strong activity towards HCT-250 and moderate activity towards U-391 cells (Poornima et al., 2010).

2.8.2 Parasporin 2 protein

Itoh *et al*. 2004 isolated *Bacillus thuringiensis* strain 94-F-45-14 (later A1547) from the soil. They identified non-insecticidal nonhemolytic protein showing good anticancer activity towards human cancer cells. In their study, they observed that the protein shows high cytotoxicity to HepG2 and Jurkat cells. They also observed cytotoxic action of the protein with cells swelling, slowly the cell damage when apoptotic process occur (Ito et al., 2004). Brasseur *et al*. 2015 studied on *B*. *thuringiensis* serovar *dakota* strain 4R2, which was purchased from Ohio State University, Columbus, OH, USA. In their research they observed PS2Aa1 was very cytotoxic to many cancer cells, they explore the mechanism using selected cancer cells from different tissue (HepG2-hepatocyte cancer, PC-3-prostate cancer and MCF-7-breast cancer) they identified that apoptosis cell death was occurring via caspases and poly (ADP-ribose) polymerase (PARP) cleavage. The anticancer activity was assessed by the MTT assay method (Brasseur et al., 2015).

2.8.3 Parasporin 3 protein

Parasporin 3 was first isolated from a soil sample of Tokyo, Japan *Bacillus thuringiensis* A1462 formerly 89-T-26-17 Yamashita *et al*. 2005, they reported that the typical three-domain Cry protein show cytotoxic activity towards HL60 and HepG2. During their work, they purified protein with two 64 kDa showing similar homologies in internal amino acid sequences. Both proteins have common five conserved block regions same as Cry protein family and the orf2a and orf2b genes encode them, later designated as Cry41Aa1 and Cry41Ab1 respectively. CryAb1 is less toxic to the cancer cell (Yamashita et al., 2005).

2.8.4 Parasporin 4 protein

Lee *et al*. 2000 were observed cytotoxic parasporal inclusion from *Bacillus thuringiensis* strain 89-T-34-22 (A1470) soil isolate. Thus they focused on the parasporal inclusion and reported that it contains five different proteins with molecular masses of 160, 60, 34, 32, and 16 kDa. After solubilized with alkali and treat with proteinase k, trypsin and chymotrypsin. 160 kDa protein show more activity after proteinase K and chymotrypsin treatment. The study also showed that the protein profile contains two major protein have 45 and 28 kDa molecular mass. 28 kDa show similarity with cyt protein, of *server israelensis* protein of 28 kDa is similar to that of the Cyt protein, a hemolytic inclusion protein, of *serovar israelensis* in molecular size (Lee et al., 2000). Later in 2008, Okumara *et al*. analyze the two Cytotoxic proteins of 28 kDa produced by A1470 strain of *Bacillus thuringiensis* and they reported that one of the 28 kDa protein is new and designated as Cry45Aa

(parasporin 4). During their study cloned the protein gene into *E.coli* and observed the cytotoxicity by MTT assay and reported that it shows high cytotoxicity towards Sawano, CACO2, MOLT-4, HL 60 and TCS cells (Okumura et al., 2008). After two years (2010) they purified the parasporin 4 protein and noticed that the cancer cells swelling, nuclear shrinkage, and burst the cells within 24 hours when treated with the protein. They were also done PSI-BLAST with protein gene, which shows similarity with *Bacillus thuringiensis* Cry protein and aerolysin-type β-pore-forming toxin. From this, they concluded that parasporin 4 was rich in β-sheet (Okumura et al., 2011).

2.8.5 Parasporin 5 protein

Parasporin 5 was first isolated by Ekino *et al*. 2014, in their study, they isolated a 30-kDa protein having cytocidal activity to leukemic cells from parasporal inclusions of *Bacillus thuringiensis serovar tohokuensis* strain A1100, and they reported that the protein shares little amino acid sequence homology with any other proteins, including Cry and Cyt proteins from *Bacillus thuringiensis*. Hence, it constitutes a new class of Cry protein family, represented as Cry64Aa1 (parasporin-5Aa1). The mode of action of this protein was unknown, but it was predicted to act as a β-PFT similar to parasporin 4 (Ekino et al., 2014). After one year (2015) Ammons *et al*. reported two novel parasporin 5 like genes (PS5-1 and PS5-2) from the *Bacillus thuringiensis* isolated from manure, which directly collected rectum of cattle. The PS5-1 show 51% similarity with Cry64Aa1 and PS5-2 show 41 or 45% similarity with Cry64Aa. In their study, they also isolated *Bacillus thuringiensis* from the Caribbean island of Trinidad and reported the presence of parasporin 1 and 6 (Ammons et al., 2016). Okassov *et al*.2015 analyze the parasporin protein and in his review, he said that sequence analysis of parasporin 5 reveals it is an Epsilon/Mtx like toxin. There was no more report on this mammalian Cry toxin (Okassov et al., 2015).

2.8.6 Parasporin 6 protein

Nagamatsu *et al*. 2010, reported the parasporin 1 like gene from M019 strain of *Bacillus thuringiensis* later he also purified protein with 84 kDa which is also showing cytotoxic activity against human cancer cell, and similarity with Cry63Aa1 and named as parasporin 6. The swelling of the cells and the formation of vacuoles in the cytoplasm occurred when treatment with the protein (Nagamatsu et al., 2010). There was no more report on this mammalian Cry toxin (Okassov et al., 2015)

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