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Bacillus thuringiensis Parasporins Functions on Cancer Cells

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ABSTRACT

Bacillus thuringiensis (Bt), a spore-forming gram-positive bacterium that can produce parasporal crystalline inclusions during its sporulation phase, has been widely used for agricultural insect pests control. Cry has unique toxic activities against certain insects, some invertebrates, protozoa, and human cancer cells. Parasporins (PS) have been identified as Cry bacterial proteins and have been divided into six types including PS1, PS2, PS3, PS4, PS5, and PS6. PSs have been found to distinguish and kill certain cancer cells through different mechanism. PS1 was found to induce various cancer cells' death by activating their apoptotic signaling and increasing Ca^{2+}level; PS2 acted on certain cancer cells as a cytolysin by targeting on plasma membrane; PS3 and PS6 acted as a pore-forming toxin and lysis cancer cell plasma membrane; PS4 induced cancer cell death in non-apoptotic way. It has been believed that the variant mechanisms of PSs acting on cancer cells indicated that different PSs may have targeted on different molecules and activated different signal pathway in cancer cells. PSs could be a series of natural bacteria products with potential roles in cancer therapy.

Key words:*Bacillus, Bacillus thuringiensis,Parasporins,Cry protein, Cyt protein, cancer cell.*

INTRODUCTION

The genus Bacillus are rod-shaped, catalase-positive and aerobic or facultative anaerobic[1],which are composed of many saprophytic bacteria and able to produce endospore [2]. Based on spore shape and swelling properties of sporangium, Bacillus are divided into three groups[3]. Group I is characterized by ellipsoidal spores that do not swell the mother cell[4]. This group contains a large number of soil living species such as *B. subtilis, B. sphaericus, B. anthracis, B. cereus, and B. thuringiensis*. *Bacillus thuringiensis(Bt)*, a spore-forming gram-positivebacterium, is first isolated from infected larvae of *Bombyx mori*, the silkworm, which is an entomopathogenic bacterium[5]. It is worthy to note that most of the Gram-positive endospore-forming bacteria play an important ecological role in aerobic decomposition, biodegradation and mineral recycling[2] .

By far, thousands of *Bt* strains are identified to be having a limited host range but a wide range of insecticidal properties including lepidoptera, diptera, coleopteran, and hymenoptera. Moreover, they are cytotoxic to other organisms such as nematodes, mites, and protozoa[6]. Having the advantages of non-polluting residues, high specificity to target insects, safety to non-target organisms such as mammalians, birds, amphibians and reptiles, and relatively low costs of development and application, *Bt* has been employed in modern agriculture commercially to control selected insect pests for approximately 40 years and the microbial insecticides as a sophisticated bio-pesticides have been applied in many agro ecosystems most commonly[7]. Interestingly, the inclusion-body crystals produced by *Bt* are the key components that contribute to its insecticidal action[8,9].These crystals are assembled by one or more insecticidal crystal proteins, delta-endotoxins, produced during sporulation phase in *Bt* growth cycle.

Based on their amino acid sequence homology, delta-endotoxins are classified into Cry and Cyt families[10]. Recent years, a group of non-insecticidal *Bt* strains are isolated from the soil and extensively distributed in nature than insecticidal *Bt* strains. A nematode-killing Cry protein has therapeutic activity against the human and animal hookworm parasite[11]. Furthermore, Parasporin, a group of Cry proteins (http://parasporin.fitc.pref.fukuoka.jp), are identified to present no toxicity against insects , but targeted kill of human cancer cells, a novel biological activity of Cry proteins[12, 13, 14, 15, 16]. These new discoveries of Cry proteins promote further studies on toxin-receptor binding mechanisms. This overview focuses on the structures and the mechanisms of *Bt*parasporins working on various types of cancer cells.

1. The *Bacillus cereus* Group

The *Bacillus cereus*group includes six different species:*B. cereus*, *B. mycoides*, *B. thuringiensis*, *B. anthracis*, *B. pseudomycoides* and *B. weihenstephanensis*[17, 18].The observed difference between *B. thuringiensis* and *B. cereus* is that there are large proteinaceous parasporal inclusions in *B. thuringiensis*. [19]. These inclusion bodies crystals have unique toxic activities against certain insects and some invertebrates[20]as well as unique toxic activities against human cancer cells and pathogenic protozoa[16, 21, 22].

2. *Bacillus thuringiensis*and its crystal proteins

B. thuringiensis was first isolated by Ishiwata as a pathogen from the sotto disease of the silkworm *Bombyx Mori* at the past century[5] and by Ernst Berliner from *Schlaffsucht* disease in flour moth caterpillars.*Bt* is a gram-positive, spore-forming bacerium in the *Bacillus cereus* group.They can grow in a simple culture medium such as nutrient or LB medium. During its sporulation or under aerobic conditions, it can produce a spore along with one or several parasporalcrystals. There are seven stages during sporulation phase. The parasporal protein synthesis starts at stage II or III and the crystal reaches its maximum size (approximately spore size) by the end of stage V[23, 24]. So the crystals were made of proteins varying in size. During the spore maturation, cells will be lysed and release out free spores and crystals into the environment. The crystal inclusions are assembled by one or more crystal proteins known as delta-endotoxins. They are classified into Cry and Cyt families on the basis of their amino acid sequence homology[10]. The Cry is the predominant type and over700 *cry* genes have been identified since the first cry gene was cloned by Schnepf and Whiteley[25](shown at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt /toxins2.html). Depending on their ability to infect an insect, *Bt* strains can be divided into two types: insecticidal and non-insecticidal strains. Insecticidal *Bt* strains are characterized by their ability to produce various types of insecticidal crystal proteins in their life cycle. These proteins can be recognized by several kinds of insects or parasites and kill them. The identified insecticidal crystal proteins include both endotoxins and exotoxins, all of which are produced during sporulation phase of *Bt* life cycle[23, 24]. Endotoxins are δ-endotoxins that are transcript from a single gene located on large transmissible plasmids[26,27,28].The bioactivityis determined by the number and type of δ-endotoxins producedby the*Bt*strain[26, 27]. The most important feature of their proteins is the pathogenicity to insects even though each crystal protein has its distinct host range[26, 27, 28]. Based on their different molecular structureof amino acid homology, the endotoxins are classified into two types: Cry and Cyt proteins. Cry proteins are the predominant type of endotoxins and their toxicity spectrum broadened to a wide range of insects to invertebrates such as nematodes, mites, protozoa, etc[29, 30]. Whereas no pathogenic to mammals found, it makes the extracted Cry toxin commonly used as a reliable biological pesticide to control insect pests for both agricultural and medical importance[31, 32]. The high specificity of Cry proteins to kill insects is supposed to be attributable to specific binding of the proteins to receptors that reside in the mid gut cell membranes of susceptible insects[32] . The second member of δ-endotoxins, Cyt proteins, has been reported with broaden cytolytic activity from Gram-negative bacteria to erythrocytes. Cyt proteins showed high toxicity to mosquito larvae[33] and leaded to lethal to mice after intravenous injection[34]. Except theδ- endotoxins, some *Bt*stains produce insecticidal proteins of α, β and γ-endotoxins[35]. For example, phospholipase C and lecithinase C are α-exotoxins with insecticidal activity and toxin to mice after intravenous injection.

However, β-exotoxins have a broader spectrum of effects against many insects. *Bt* also produces other kinds of toxins such as vegetative insecticidal proteins (VIP) and S-layer protein (SLP). Four classes of VIPs have been identified namedVip1, 2, 3 and 4. Vip1 and 2 are toxic components of a binary toxin that is effective against Coleoptera[36]. SLP is a new group of the parasporal inclusions of *Bt* which is an extracellular protein without exactly crystal structure[36].The protein has been reported to have a remarkably high insecticidal activity versus the coleopteran pest *Epilachna varivestis*[37]. The identified insecticidal and non-insecticidal toxins of *Bt* are summarized in Table1.

3. *Bacillus thuringiensis* andParasporins (PS)

3.1 Identification of Parasporins

As early as 1970s, Prasad *et al*. and Seki *et al*. noted that Lepidoptera-toxic *Bt*crystal proteins had the anti-tumor activities[38]. As early as 1999, Mizuki et al. reported that non-insecticidal *Bt* parasporal inclusions showed an unique activity to in kill the cultured human cancer cells. They found that Crystal parasporal protein exhibited highly cytotoxicity to many types of mammalian cells but not showed the hemolytic activity to rabbit and human erythrocyte. Later Mizuki et al. studied 1744 *Bt*strains from which three parasporal inclusion producing strains (89-T-26-17, 84-HS-1- 11 and 90-F-45-14) were identified. Their parasporal inclusions exhibited no hemolytic activity and insecticidal activity against lepidopteran and dipteran insects as well; however they showed high cytocidal toward leukemia T cells and other kinds of human cancer cells. Especially, the proteins from 89-T-26-17 and 84-HS-1-11 were able to discriminate between normal and leukemia T cells. More interestedly, they can kill the leukemia cells[16]. In 2000, Mizuki *et al.* obtaineda Cry proteinfrom the strain A1190. They found that this Cry protein can be recognized by human leukemic cell and has an anti-cancer cell activity. So they named them parasporin[21]. Then, more and more parasporins are cloned by different groups. Ito et al.found that the parasporal crystal protein from *Bt*strain A1547 had strong cytocidal activity against different human cells and killed the colon and liver cancer cells[39]. Okumura et al.proved that the *Bt* strain 89- T-34-22 can produce at least two novel toxic proteins with cytotoxicity to human cancer cells[40]. Worthy to note that *B. thuringiensis* strain CTC and CTC-like strains were first cloned by Sun et al. 2001[41] and they exhibited low activities against various insect species, which provides a new focus for research of *Bt* non-toxic strains.

The unique characteristic of non-pathogenic *Bt* parasporal proteins that can function on mammalian cancer cells expends the insights of *Bt* and *Bt* crystal protein studies.Up-to-date, 19 parasporins have been identified and divided into six types; PS1, PS2, PS3, PS4, PS5 and PS6,according to their primary structural similarity. Parasporin-1to 4 are designated as Cry31Aa, Cry46Aa, Cry45Aa and Cry41Aa respectively by the *Bt* nomenclature committee,
(http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/index.html).

It's clear that few genealogical relationships existing among parasporins family.
http://parasporin.fitc.pref fukuoka.jp/.The most important quality of these proteinsis heterogeneous in their cytotoxicity and a little is known on how these proteins targeting on the receptor molecules of different types of cancer cells and leading to different anti-cancer activities. More researches are needed to clarify the function of them on human cells.

3.2 Molecular structureand anti-cancer activity properties of each parasporins

After the first report of Yamashita S et.al that the Cry proteins with typical three-domain showed the cytocidal activity preferential for cancer cells[42], more and more studies have uncovered the possible mechanisms of anti-cancer activity of parasporins. Wong RS et al[43] found purified *Bt* 18 parasporal protein could bind to T lymphoblastic leukaemia cell.Krishnam et.al further identified that this protein could bind to the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on human leukaemic T cells (CEM-SS)[44].

Parasporin-1, purified from *Bt* strain A1190, is a 723 amino acid peptide with two trypsin digested sites (Table2), in its N-terminal domainand selected cytotoxicity to human cancer cells such as HeLa, Sawano, HepG2, HL-60or MOLT-4 cells after activated with trypsin treatment[45, 46, 47].It can inhibit protein synthesis and increase Ca^{2+}level , leading to cell death[47].

Parasporin-2 is sequence unique Cry protein with no homology to other existing Cry proteins. Pro-parasporin-2 can be activated by proteinase K digestion at N- and C-terminal regionsand acts as a potent toxin withhighly cytotoxicity to HepG2 and Jurkat human cell lines, but less to the normal hepatocyte (HC) and HeLa cells[39].Kitada S. et.al found that it localizedat the plasma membrane and could bind to lipid raft on the plasma membrane, subsequently inducing cell death[48].

Parasporin-3, purified from *Bt* strain A 1462, is a typical three-domain type toxin with 825 amino acid peptide and can be activated by proteinase K-digestion at N-terminal region as a result of 64-kDa toxic moiety. It acts as a pore-forming toxin on the plasma membrane of cancer cells and increases plasma membrane permeability of target cells[42].

Parasporin-4, purified from stainA1470, comprises of 275 amino acid residues with homologies toboth Cry and aerolysin-type β-pore-forming toxins and has cytotoxicity against CACO-2, Sawano or MOLT-4 human cancer cells[40, 49,50]

Parasporin 4 is composed mainly of β-sheet domains and is a novel cholesterol-independent pore-forming toxin (β-PFT)[51]. Parasporin 4 treatment induced the cell swelling, bleb, nuclear shrinkage, leading the cell plasma membrane burst, efflux of the cytoplasm through the plasma membrane and death in the end. Parasporin 4 could bind nonspecifically to the plasma membrane and form oligomeric complexes in the target cell membranes[50].

Parasporin 5 and 6 are two newly discovered proteins summarized in Table2. Parasporin 6, produced by *Bt* strain M019, is a pore-forming proteinwith anticancer activity against human hepatocyte cancer cells and cervical cancer cells[52]. The cytocidal activities of parasporins to various human cells were summarized in Table3 [54].

Table1. Insecticidal and Non insecticidal toxins of Bacillus thuringiensis

Toxins	Strains types	Effects
Cry protein Delta- endotoxin	Insecticidal *Bt*	Pathogenic to insect pests in cluding Lepidoptera, Dipetra and Coleoptera and even to nematodes, mites, and Protozoa
Cyt protein Delta-endotoxin	Insecticidal *Bt*	Abroad activity against invertebrate and vertebrate cells
VIP	Insecticidal *Bt*	Many ergonomically pests especially to lepidopteravs
Alpha, Beta, Gama endotoxins	Insecticidal *Bt*	Contribute the pathogenicity to insects
Beta exotoxins	Insecticidal *Bt*	a broad-spectrum toxicity in vertebrates and invertebrates
Alpha exotoxins	Insecticidal *Bt*	Insecticidal and have toxic to mice (only when they are injected with the toxin)
Parasporin (Cry protein)	Non-insecticidal *Bt*	Cytotoxic activity toward several human cell lines
S-layer proteins	Insecticidal *Bt*	Some strains have cytotoxic activity toward coleopteran pest; *Bt* CT and CTC-like strains non-toxic to insect

Table2. Digested parasporins and location of cutting

Parasporins	Digested enzyme	Location of cutting	Molecular weight of active protein(KDa)	Reference
Parasporin-1	Trypsin	93,231	56,15	45,47, 53
Parasporin-2	Proteinase k	52	30	39,48
Parasporin-3	Proteinase k	-	64	42
Parasporin-4	pepsin	252	31	50, 51
Parasporin-5	-	-	-	in preparation
Parasporin-6	Trypsin	-	14, 59	52

Table3. Cytocidal activities of parasporins to various human cells [54]

Cell	Characteristics	LD50 (µg/ml) PS1	PS2	PS3	PS4
MOLT-4	Leukemic T cell	2.2	0.022	>10	0.472
JURKAT	Leukemic T cell	>10	0.018	>10	>2
HL-60	Leukemic T cell	0.32	0.019	1.32	0.725
Tcell	Normal T cell	>10	ND	>10	>2
HepG2	Hepatocyte cancer	3.0	0.019	2.8	1.90
HC	Normal hepatocyte	>10	1.1	>10	>2
HeLa	Uterus(cervix) cancer	0.12	2.5	>10	>2
Sawano	Uterus cancer	>10	0.0017	>10	0.245
TCS	Uterus(cervix) cancer	ND	7.8	>10	0.719
UISMC	Normal uterus	>10	2.5	>10	>2
CACO-2	Colon cancer	>10	0.013	>10	0.124

ND: Not done

CONCLUSION

Studies have revealed that insecticidal *Bt*toxins and parasporins are of general properties of stable in alkaline solution and proteolyticdigestion.Of great interest, parasporinsare able to selectively induce human cancer cell death through individual cell-killing mechanisms of binding to the target receptors on the cells. This cytotoxicityis found different depending on the types of protein and kinds of targetcells.It is noteworthy that the cytotoxicity effect of parasporal proteins appears to be highly selective, but the detailed mechanisms by which parasporal proteins targets and kills cancer cells remain unclear. However, tumor progression is a complex, coordinated and environment-dependent event, including cell survival, proliferation, adhesion, invasion and metastasis. Most cancer chemotherapeutic agents target on one or more steps and tumor cells exhibit resistance to the treatment are becoming common.As natural bacteria products, PSs may be novel proteins of potential roles in inhibiting tumor progression during the cancer therapy.

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