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Review

Parasporin, a New Anticancer Protein Group from *Bacillus thuringiensis*

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Abstract. *Parasporin (PS) is a collection of genealogically heterogeneous Cry proteins synthesized in Bacillus thuringiensis. A prominent feature commonly associated with PS proteins is the strong cytocidal activity preferential for human cancer cells of various origins. The proteins exhibit cytocidal activities only when digested with proteases. Currently, this protein group is classified into four families: PS1, PS2, PS3 and PS4. Marked differences are evident in cytotoxicity spectra and activity levels between the four PS families. Neither hemolytic activity nor insect toxicity is associated with PS proteins. One of the most striking aspects in the events induced by PS1Aa1 is the early and rapid increase of the intracellular Ca^{2+} concentration, with no change in plasma membrane permeability. There is strong evidence that PS1Aa1 kills cancer cells through apoptosis. Unlike PS1Aa1, PS2Aa1 increases plasma membrane permeability of cancer cells. The initial step in cytocidal action of PS2Aa1 is the specific binding of this cytotoxin to a putative receptor located in the lipid rafts, followed by its oligomerization and pore formation in plasma membrane.*

Historical Background

Bacillus thuringiensis is an aerobic Gram-positive spore-former, belonging to the *Bacillus cereus* group. The organism produces unique proteinaceous crystalline parasporal inclusions in sporangia during sporulation. This is the only character that discriminates between the two taxonomically closely related species, *B. thuringiensis* and *B. cereus* (1). *B. thuringiensis* was first isolated in Japan from diseased larvae of the silkworm, *Bombyx mori*, as an entomopathogenic bacterium (2, 3). The extremely high pathogenicity of the organism is solely due to the oral toxicity of crystal (Cry) proteins, contained in crystalline parasporal inclusions, whose activity is highly specific for insects and nematodes. It is now well accepted that the insecticidal activity of Cry protein is induced by its specific binding to the receptor located on the plasma membrane of the mid-gut epithelial cell of susceptible insects (4). This property makes *B. thuringiensis* an environmentally safe and ecologically sound microbial agent in controlling agricultural insect pests (5). The organism has also been used successfully to suppress the population levels of medically important dipteran pests. Examples include the mosquito vectors of malaria, virus diseases (including dengue hemorrhagic fever and West Nile fever) and lymphatic filariasis, and the black-fly that transmits onchocerciasis (6). Of particular interest in recent findings is that a therapeutic activity against the human and animal hookworm parasite is associated with a unique nematode-killing *B. thuringiensis* Cry protein (7).

Historically, it has been believed for many years that *B. thuringiensis* has acquired insecticidal activity in the course of co-evolution with insects through a host-parasite relationship. This hypothesis is attractive for many investigators; however, circumstantial evidence leads to another idea that *B. thuringiensis*, as a species, is merely an environmental saprophyte but not an obligate pathogen of insects. This is supported by the fact that in natural environments *B. thuringiensis* isolates with non-insecticidal Cry proteins outnumber the insecticidal ones (8-11). It is noteworthy that the non-insecticidal isolates often account for >90% of the natural populations from soils (12-15) and phylloplanes (16, 17).

Thus, the important question that naturally arises is whether Cry proteins synthesized in non-insecticidal *B. thuringiensis* have any biological activity which is as yet undiscovered (18). Based on the above historical

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Table I. *Listing of the known parasporins.*

Protein	*Bacillus thuringiensis* source strains	Locality and source of the strain	Reference
Parasporin-1			
PS1Aa1	A1190	Hiroshima, Japan: soil	22
PS1Aa2	M15	Canada: dead mites	27
PS1Aa3	B0195	Fukuoka, Japan: soil	41
PS1Aa4	79-25	Hanoi, Vietnam: soil	42
PS1Aa5	92-10	Hanoi, Vietnam: soil	42
PS1Ab1	B0195	Fukuoka, Japan: soil	41
PS1Ab2	31-5	Hanoi, Vietnam: soil	42
PS1Ac1	87-29	Hanoi, Vietnam: soil	42
Parasporin-2			
PS2Aa1	A1547	Fukuoka, Japan: soil	23
PS2Ab1	TK-E6	Ehime, Japan: soil	43
Parasporin-3			
PS3Aa1	A1462	Tokyo, Japan: soil	24
PS3Ab1	A1462	Tokyo, Japan: soil	24
Parasporin-4			
PS4Aa1	A1470	Tokyo, Japan: soil	25

Parasporin-1, 2, 3, and 4 are assigned to Cry31, Cry46, Cry41, and Cry45, respectively, in the classification scheme of Cry proteins (available at N. Crickmore's website at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/index.html).

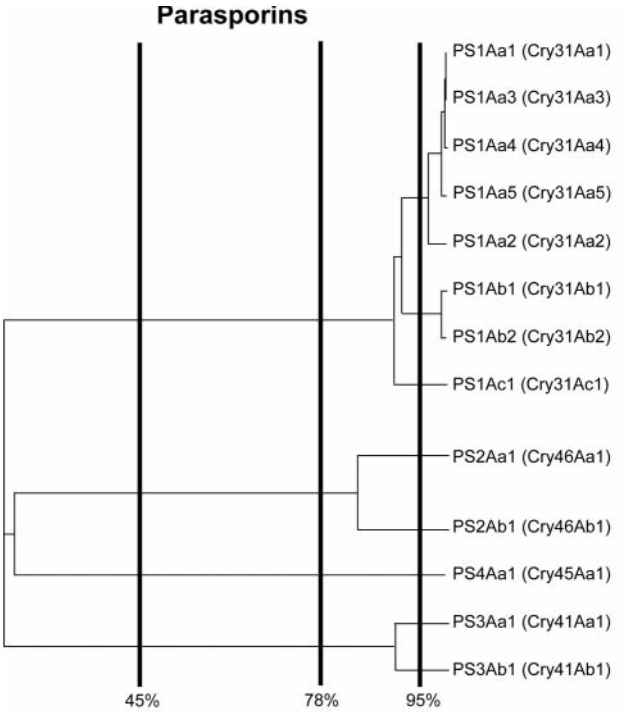

Figure 1. *Dendrogram illustrating the relationships between the existing parasporins belonging to the four families, PS1, PS2, PS3 and PS4.*

background, an extensive screening of *B. thuringiensis* Cry proteins that have novel biological activities other than insect toxicity was commenced in 1996. This has led to the discovery of the unique proteins that target human cancer cells (19) and a human-pathogenic protozoan (20).

Discovery of Parasporin

Mizuki *et al.* (19) were the first to attempt a large-scale screening of *B. thuringiensis* strains whose parasporal inclusion proteins are non-hemolytic but cytocidal to human cancer cells. The screening tests involved protease-digested parasporal proteins of 1,744 *B. thuringiensis* strains, consisting of (i) 1,700 Japanese isolates in the deposits of the *Bacillus thuringiensis* Collection, Kyushu University, and (ii) 44 reference type strains of then-existing *B. thuringiensis* H serovars from the Institut Pasteur, Paris. In a preliminary screening test with sheep erythrocytes, parasporal proteins from 60 strains induced strong hemolysis. It is clear that the hemolysis was caused by broad-spectrum cytolysins (Cyt proteins) active on a wide range of invertebrate and vertebrate cells (21). The other 1,684 strains, lacking hemolytic parasporal proteins, were then examined for *in vitro* cytocidal activity against MOLT-4 cells (human leukemic T-cells). This provided 42 positive strains. It should be noted that the parasporal proteins from these strains retained no insecticidal activities. Finally, three strains were chosen for a further characterization of the anticancer activity associated with parasporal proteins. When tested on MOLT-4, A549 (human lung cancer) and HeLa (human uterus cervix cancer) cells, parasporal proteins of the three strains exhibited strong cytocidal activities with different toxicity spectra and varied activity levels. Of particular interest is that the proteins of the two strains 84-HS-1-11 (later designated A1190) and 89-T-26-17 (A1462) were capable of discriminating between leukemic and normal T-cells, preferentially killing the former.

In 2000, Mizuki *et al.* (22) obtained from the strain A1190 an anticancer Cry protein with a molecular mass of 81 kDa. This led to the creation of a new category of protein, the parasporin, defined as the bacterial parasporal protein which is capable of discriminately killing cancer cells (22). According to the Committee of Parasporin Classification and Nomenclature (website: http://parasporin.fitc.pref.fukuoka.jp/), the 81-kDa protein constitutes the family parasporin-1Aa (PS1Aa) in the current classification scheme (Table I). Subsequent investigations have established three additional families of parasporin: parasporin-2 (PS2) (23), parasporin-3 (PS3) (24), and parasporin-4 (PS4) (25). In addition, recent studies have provided evidence that the organisms with parasporin activities are among the common members in *B. thuringiensis* natural populations occurring in Japan (26), Vietnam (14), Canada (27) and Malaysia (28).

Table II. *Characteristics of parasporins.*

Parasporin[a]	*Bacillus thuringiensis* source strain	Precursor	Active form[b] (kDa)	Remarks (kDa)	Reference
PS1Aa1	A1190	81	15+56[c]	Three-domain Cry protein, activated by *N*-terminal digestion	22, 29
PS2Aa1	A1547	37	30	Non-three-domain type, activated by *N*- and *C*-terminal digestion	23, 44
PS3Aa1	A1462	88	64	Three-domain Cry protein, activated by *N*- and *C*-terminal digestion	24, 45
PS4Aa1	A1470	34	27	Non-three-domain type, activated by *C*-terminal digestion	25, 35

[a]PS1, PS2, PS3, and PS4 correspond to Cry31, Cry46, Cry41, and Cry45, respectively. [b]Generated by protease digestion. [c]Heterodimer.

Structure and Characteristics of Parasporins

As listed in Table I, a total of 13 PS proteins have been isolated from 11 strains of *B. thuringiensis*. Of these, eight proteins are allied to PS1, two to PS2, two to PS3 and one to PS4. Figure 1 shows a dendrogram of the 13 PS proteins. Obviously, few genealogical relationships exist between the four PS families. Table II summarizes the major characteristics of the four reference PS proteins.

The protein PS1Aa1, corresponding to Cry31Aa1, is a polypeptide of 723 amino acid residues with a predicted molecular weight of 81,045. The gene is 2,169 bp long. This protein has a structure of the three-domain type, whose amino acid sequence contains five conserved blocks commonly retained in insecticidal Cry proteins. However, only very low homologies (< 25%) exist between PS1Aa1 and the established classes of Cry and Cyt proteins. As reported by Mizuki *et al*. (22) and Katayama *et al*. (29), PS1Aa1 exhibits cancer cell-killing activity only when digested with proteases. It should be noted that proteolytic processing is also essential for activation of the insecticidal Cry proteins (30). Protease processing of PS1Aa1 (81 kDa) generates a heterodimer protein consisting of 15- and 56-kDa polypeptides, an active form toxic to cancer cells. The digestion occurs on *N*-terminal region of the 81-kDa precursor protein (Table II).

According to Ito *et al*. (23), PS2Aa1 (Cry46Aa1) is a polypeptide of 338 amino acid residues with a predicted molecular weight of 37,446. The gene is 1,014 bp long. Unlike PS1Aa1, PS2Aa1 is not a protein with three domains, lacking the block sequences conserved in insecticidal Cry proteins. This protein shares a low sequence homology only with Cry15Aa (31) among the existing classes of Cry and Cyt proteins of *B. thuringiensis*. It is worth noting that the Cry15Aa protein is highly homologous to the two mosquitocidal toxins (Mtx2 and Mtx3) from *B. sphaericus* (32). Interestingly, these two mosquitocidal toxins show homology to aerolysin of *Aeromonas hydrophila* which produces β-barrel-lined membrane pores (33). Proteolytic processing of the 37-kDa precursor protein yields a 30-kDa cytotoxin active on cancer cells. The protease digestion occurs on both of the *N*- and *C*-terminal regions.

The protein PS3Aa1, corresponding to Cry41Aa1, has a typical three-domain structure with five block sequences commonly conserved in the known insecticidal Cry proteins. It consists of 825 amino acid residues with a deduced molecular weight of 93,689, sharing low homologies with insecticidal Cry proteins. It is of interest to note that the *C*-terminal sequence of PS3Aa1 is similar to that of *Clostridium botulinum* hemagglutinin HA-33 (34). Proteolytic digestion is required for activation of PS3Aa1; the 81-kDa precursor is converted to the 64-kDa toxic moiety by proteolytic processing of both *N*- and *C*-terminal regions.

The fourth parasporin, PS4Aa1 (Cry45Aa1), comprises 275 amino acid residues, with a predicted molecular weight of 30,078. The gene is 828 bp long. None of the five block sequences, commonly conserved in most of the Cry proteins (including PS1Aa1 and PS3Aa1), is retained in PS4Aa1. The three-domain structure is not associated with this protein. Moreover, only low homologies of <30% are evident in amino acid sequences between PS4Aa1 and the existing proteins, including Cry and Cyt proteins.

Anticancer Activity of Parasporins

As mentioned above, none of the PS proteins exhibit hemolytic activity against sheep erythrocytes or *in vivo* insecticidal activity. For *in vitro* cytotoxicity against insect cultured cells, PS1Aa1 was tested on the two cell lines NIAS-AeA1-2, derived from the mosquito (Aedes albopictus), and BM-N from the silkworm, *B. mori* (22). The results showed that these insect cells are not susceptible to PS1Aa1. Another interesting fact is that a goldfish (Carassius auratus) cell line, GF-Scale, is not sensitive to the three crude preparations of parasporal proteins containing PS1Aa1, PS2Aa1 and PS3Aa1, respectively (19).

Table III shows cytotoxicity spectra of the four PS proteins, summarizing the results obtained in recent investigations (23, 24, 29, 35, 37). It involves 13 human cell lines originating from various tissues, nine from tumors and

Table III. *Cytotoxicity spectra of the four parasporins.*

Cell line	Origin	Cytotoxicity[a]			
		PS1Aa1	PS2Aa1	PS3Aa1	PS4Aa1
Human					
HeLa	Uterine cervical cancer	+++	–	–	–
Sawano	Endometrial adenocarcinoma	–	++++	–	+++
TCS	Uterine cervical cancer	–[b]	–	–	+++
UtSMC	Normal uterine smooth muscle	–	++	–	–
MOLT-4	Acute lymphoblastic leukemia	++	++++	–	+++
HL-60	Promyelocytic leukemia	+++	++++	++	+++
Jurkat	T-cell leukemia	–	++++	–	–
T-cell	Normal T lymphocyte	–	+++	–	–
HepG2	Hepatocellular carcinoma	++	++++	++	++
HC	Normal hepatocyte	–	–	–	–
A549	Lung adenocarcinoma	–	+++	–	–
MRC5	Normal fetal lung fibroblast	–	+	–	–
Caco-2	Colorectal carcinoma	–	+	–	+++
Monkey					
Vero	Kidney (African green monkey)	–	NT	–	–
COS-7	Kidney (African green monkey), SV-40 transformed	–	NT	–	–
Rodent					
PC12	Pheochromocytoma (rat)	NT	NT	NT	++
NIH3T3	Embryo (mouse)	–	NT	–	–
CHO	Ovary (Chinese hamster)	NT	NT	NT	–

[a]References: Katayama *et al.* (29), Ito *et al.* (23), Kitada *et al.* (37), Yamashita *et al.* (24), and Okumura *et al.* (35). The levels of cytotoxicity, based on the EC_{50} values in cell proliferation assay (46), were graded as follows: extremely high (++++), high (+++), moderate (++), low (+), and very low / non-toxic (–). NT: Not tested. [b]Mizuki *et al.* (unpublished observation).

four non-cancer normal tissues, and five non-human cells (two monkey and three rodent cell lines). Overall, the four PS proteins have preferential cytocidal activities against human cancer cells. Four non-cancer normal cell lines are resistant to the PS proteins, except that two of these (UtSMC and MRC5) are sensitive to PS2Aa1 at low to moderate levels. Interestingly, PS4Aa1 has a moderate level of cytotoxicity against PC12, a rat cancer cell line.

One of the most striking aspects in the findings is that marked differences are evident in anticancer cytotoxicity spectra and activity levels between the four PS proteins. Two lower molecular weight proteins, PS2Aa1 and PS4Aa1, have relatively broad cytotoxicity spectra, each inducing cell death in six out of nine human cancer cell lines. Interestingly, all of the three leukemic cell lines (MOLT-4, HL60 and Jurkat) are extremely sensitive to PS2Aa1. PS3Aa1, a typical three-domain-type Cry protein, exhibits moderate cytotoxicities against only two cancer cell lines, HL-60 (leukemic) and HepG2 (liver cancer). Thus, PS3Aa1 has the narrowest activity spectrum among the four PS proteins. It is noteworthy that the three cancer cell lines (HeLa, TCS and Jurkat) are mono-sensitive to one of the three proteins, PS1Aa1, PS2Aa1 or PS4Aa1.

Of particular interest is that the PS proteins are also preferentially active on sliced cancer tissues *in vitro*. Ito *et al.* (23) reported that PS2Aa1 selectively destroyed cancer cells but not non-neoplastic cells, chronic inflammatory cells or blood vessels when tested on cultured slices of liver and colon cancer tissues prepared immediately after surgical resection. The results are in good agreement with the observations by the same authors that PS2Aa1 has an extremely high cytotoxicity against HepG2, a liver cancer cell line, but exhibits little activity against HC, a normal hepatocyte cell line (Table III). A similar preferential activity is also associated with PS1Aa1 when tested on tissue slices of colon and liver cancers (Sasaguri *et al.*, unpublished observation).

Mechanism of Cancer Cell-killing Action of Parasporins

Parasporin-1Aa1. Noticeable cytopathy occurs in susceptible cancer cells within 1 h, when tested with PS2Aa1, PS3Aa1 and PS4Aa1 (23,24,35). In contrast, PS1Aa1 induces delayed cytopathy 8-10 h post-administration (22). These findings, together with the facts that the four PS proteins are

genealogically and structurally unrelated to each other, suggest that the cell-killing mechanism of PS1Aa1 is substantially different from those of the three other PS proteins. Recently, Katayama *et al*. (36) have claimed that unlike the insecticidal Cry proteins, PS1Aa1 is not a membrane pore-forming cytotoxin. This is supported by the observations that: (i) neither release of lactate hydrogenase nor penetration of propidium iodide occurs in PS1Aa1-treated HeLa cells, and (ii) there is no alteration of membrane potential in intoxicated cells. A prominent physiological feature in PS1Aa1-treated HeLa cells is the overall decrease in the levels of cellular protein and DNA synthesis. Of great significance is the early and rapid increase of the intracellular Ca^{2+} concentration within 1-3 min after treatment with PS1Aa1. In this regard, it should be noted that suramin, an inhibitor of the trimeric G-protein signaling, suppresses both Ca^{2+} influx and cytocidal activity of PS1Aa1. There is strong evidence that PS1Aa1 induces apoptosis in HeLa cells. Firstly, cytocidal activity of PS1Aa1 is suppressed by synthetic caspase inhibitors. Secondly, PS1Aa1 treatment leads to the degradation of apoptosis-related proteins, pro-caspase-3 and poly (ADP-ribose) polymerase, in HeLa cells.

Parasporin-2Aa1. Recently, the second anticancer Cry protein, PS2Aa1, has been intensively investigated in terms of the mechanism of cell-killing action (37,38). Unlike PS1Aa1, PS2Aa1 increases plasma membrane permeability of the susceptible cells: most of the cytoplasmic lactate hydrogenase leaks from the intoxicated HepG2 cells, while the extracellular propidium iodide enters the cytoplasm. In addition, PS2Aa1 does not form pores in membranes of mitochondria and endoplasmic reticula. The initial step in cytocidal action of PS2Aa1 is the specific binding of the toxin to a putative receptor protein, as yet unidentified, located in the lipid raft of plasma membrane of the susceptible cells. This is followed by the formation of oligomers (>200 kDa) of PS2Aa1 in plasma membranes, leading to pore formation and cell lysis. The oligomerization occurs in the presence of membrane proteins, lipid bilayer and cholesterols. It is noteworthy that a substantial homology exists in amino acid sequences between PS2Aa1 and *Clostridium perfringens* epsilon toxin whose cell-killing mechanism involves the toxin oligomerization in lipid rafts and pore formation in plasma membrane (39).

Other parasporins. Relatively little is known about the cancer cell-killing mechanism of PS3Aa1. PS3Aa1 is a typical three-domain-type Cry protein, conformationally closely related to the insecticidal Cry proteins. This may lead to the hypothesis that, by analogy to insecticidal Cry proteins, PS3Aa1 acts as a pore-forming toxin on the plasma membrane of cancer cells. This is supported, in part, by the fact that PS3Aa1 increases plasma membrane permeability of target cells (24). It is now well accepted that the insecticidal Cry1A proteins induce cell death through pore formation in plasma membrane after initial binding to the GPI-anchored receptors, aminopeptidases and cadherin-like proteins in the mid-gut epithelial cells of the susceptible lepidopteran insects (40).

As mentioned above, PS4Aa1 is different from the three other parasporins in many aspects. Thus, it is conceivable that the mode of action of this protein also differs from those of the others. At present, however, no information is available for understanding the mechanism of preferential activity associated with PS4Aa1. This awaits clarification.

Conclusion

The cytocidal activity preferential for cancer cells makes PS proteins possible candidates for anticancer agents of medical use. It is expected, however, that their direct clinical application will induce undesired immunological responses in patients. The strategy to solve this problem includes identification of PS-specific cell receptors in cancer cells. This may lead to the creation of "magic bullet" drugs targeting the receptors. Work is now progressing towards the final identification of the receptors for PS1Aa1 and PS2Aa1. Parasporal inclusion-forming organisms occur in a variety of aerobic and anaerobic species belonging to several genera, *e.g. Bacillus, Paenibacillus, Brevibacillus* and *Clostridium*. Thus, future work also includes screening tests for novel anticancer parasporal proteins from non-*B. thuringiensis* spore-forming bacteria.

References

1 Logan N: *Bacillus anthracis*, *Bacillus cereus*, and other aerobic endospore-forming bacteria. *In*: Topley & Wilson's Microbiology & Microbial Infections (Tenth Edition). Bacteriology. Borriello SP, Murray PR and Funke G (eds.). London: Hodder Arnold, pp. 922-952, 2005.

2 Ishiwata S: On a kind of severe flacherie (sotto disease). Dainihon Sanshi Kaiho *114*: 1-5, 1901.

3 Aizawa K: Shigetane Ishiwata: His discovery of sotto-kin (*Bacillus thuringiensis*) in 1901 and subsequent investigations in Japan. *In*: Proceedings of a Centennial Symposium Commemorating Ishiwata's Discovery of *Bacillus thuringiensis*. Ohba M, Nakamura O, Mizuki E and Akao T (eds.). pp. 1-14, 2001.

4 Bravo A, Soberón M and Gill SS: *Bacillus thuringiensis* mechanisms and use. *In*: Comprehensive Molecular Insect Science, Volume 6. Gilbert LI, Iatrou K and Gill SS (eds.). New York: Elsevier, pp. 175-206, 2005.

5 Glare TR and O'Callaghan M: *Bacillus thuringiensis*: Biology, Ecology and Safety. Chichester, John Wiley, p. 350, 2000.

6 Becker N: Bacterial control of vector mosquitoes and black flies. *In*: Entomopathogenic Bacteria: From Laboratory to Field Application. Charles JF, Delécluse A and Nielsen-LeRoux (eds.). Dordrecht, Kluwer Academic Publishers, pp. 383-398, 2000.

7 Cappello M, Bungiro RD, Harrison LM, Bischof LT, Griffitts JS, Barrows BD and Aroian RV: A purified *Bacillus thuringiensis* crystal protein with therapeutic activity against the hookworm parasite *Ancylostoma ceylanicus*. Proc Natl Acad Sci USA *103*: 15154-15159, 2006.

8 Ohba M and Aizawa K: Insect toxicity of *Bacillus thuringiensis* isolated from soils of Japan. J Invertebr Pathol *47*: 12-20, 1986.

9 Hastowo S, Lay BW and Ohba M: Naturally occurring *Bacillus thuringiensis* in Indonesia. J Appl Microbiol *73*: 108-113, 1992.

10 Maeda M, Mizuki E, Nakamura Y, Hatano T and Ohba M: Recovery of *Bacillus thuringiensis* from marine sediments of Japan. Curr Microbiol *40*: 418-422, 2000.

11 Lee D-H, Cha IH, Woo DS and Ohba M: Microbial ecology of *Bacillus thuringiensis*: fecal populations recovered from wildlife in Korea. Can J Microbiol *49*: 465-471, 2003.

12 Ohba M, Wasano N and Mizuki E: *Bacillus thuringiensis* soil populations naturally occurring in the Ryukyus, a subtropic region of Japan. Microbiol Res *155*: 17-22, 2000.

13 Ohba M, Tsuchiyama A, Shisa N, Nakashima K, Lee D-H, Ohgushi A and Wasano N: Naturally occurring *Bacillus thuringiensis* in oceanic islands of Japan, Daito-shoto and Ogasawara-shoto. Appl Entomol Zool *37*: 477-480, 2002.

14 Yasutake K, Binh ND, Kagoshima K, Uemori A, Ohgushi A, Maeda M, Mizuki E, Yu YM and Ohba M: Occurrence of parasporin-producing *Bacillus thuringiensis* in Vietnam. Can J Microbiol *52*: 365-372, 2006.

15 Yasutake K, Uemori A, Kagoshima K and Ohba M: Serological identification and insect toxicity of *Bacillus thuringiensis* isolated from the island Okinoerabu-jima, Japan. Appl Entomol Zool *42*: 285-290, 2007.

16 Ohba M: *Bacillus thuringiensis* populations naturally occurring on mulberry leaves: a possible source of the populations associated with silkworm-rearing insectaries. J Appl Bacteriol *80*: 56-64, 1996.

17 Mizuki E, Ichimatsu T, Hwang S-H, Park YS, Saitoh H, Higuchi K and Ohba M: Ubiquity of *Bacillus thuringiensis* on phylloplanes of arboreous and herbaceous plants of Japan. J Appl Microbiol *86*: 979-984, 1999.

18 Ohba M, Yu YM and Aizawa K: Occurrence of non-insecticidal *Bacillus thuringiensis* flagellar serotype 14 in the soil of Japan. System Appl Microbiol *11*: 85-89, 1988.

19 Mizuki E, Ohba M, Akao T, Yamashita S, Saitoh H and Park YS: Unique activity associated with non-insecticidal *Bacillus thuringiensis* parasporal inclusions: *in vitro* cell-killing action on human cancer cells. J Appl Microbiol *86*: 477-486, 1999.

20 Kondo S, Mizuki E, Akao T and Ohba M: Antitrichomonal strains of *Bacillus thuringiensis*. Parasitol Res *88*: 1090-1092, 2002.

21 Knowles BH, White PJ, Nicholls CN and Ellar DJ: A broad-spectrum cytolytic toxin from *Bacillus thuringiensis* var. kyushuensis. Proc Royal Soc London B *248*: 1-7, 1992.

22 Mizuki E, Park YS, Saitoh H, Yamashita S, Akao T, Higuchi K and Ohba M: Parasporin, a human leukemic cell-recognizing parasporal protein of *Bacillus thuringiensis*. Clin Diagn Lab Immunol *7*: 625-634, 2000.

23 Ito A, Sasaguri Y, Kitada S, Kusaka Y, Kuwano K, Masutomi K, Mizuki E, Akao T and Ohba M: A *Bacillus thuringiensis* crystal protein with selective cytocidal action to human cells. J Biol Chem *279*: 21282-21286, 2004.

24 Yamashita S, Katayama H, Saitoh H, Akao T, Park YS, Mizuki E, Ohba M and Ito A: Typical three-domain Cry proteins of *Bacillus thuringiensis* strain A1462 exhibit cytocidal activity on limited human cancer cells. J Biochem *138*: 663-672, 2005.

25 Saitoh H, Okumura S, Ishikawa T, Akao T, Mizuki E and Ohba M: Investigation of a novel *Bacillus thuringiensis* gene encoding a parasporal protein, parasporin-4, that preferentially kills human cancer cells. Biosci Biotechnol Biochem *70*: 2935-2971, 2006.

26 Uemori A, Maeda M, Yasutake K, Ohgushi A, Kagoshima K, Mizuki E and Ohba M: Ubiquity of parasporin-1 producers in *Bacillus thuringiensis* natural populations of Japan. Naturwissenschaften *94*: 34-38, 2007.

27 Jung Y-C, Mizuki E, Akao T and Côté J-C: Isolation and characterization of a novel *Bacillus thuringiensis* strain expressing a novel crystal protein with cytocidal activity against human cancer cells. J Appl Microbiol *103*: 65-79, 2007.

28 Nadarajah VD, Ting D, Chan KK, Mohamed SM, Kanakeswary K and Lee HL: Selective cytotoxic activity against leukemic cell lines from mosquitocidal *Bacillus thuringiensis* parasporal inclusions. Southeast Asian J Trop Med Publ Health *39*: 235-245, 2008.

29 Katayama H, Yokota H, Akao T, Nakamura O, Ohba M, Mekada E and Mizuki E: Parasporin-1, a novel cytotoxic protein to human cells from non-insecticidal parasporal inclusions of *Bacillus thuringiensis*. J Biochem *137*: 17-25, 2005.

30 Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR and Dean DH: *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev *62*: 775-806, 1998.

31 Brown KL and Whiteley HR: Molecular characterization of two novel crystal protein genes from *Bacillus thuringiensis* subsp. thompsoni. J Bacteriol *174*: 549-557, 1992.

32 de Maargd RA, Bravo A, Berry C, Crickmore N and Schnepf HE: Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annu Rev Genet *37*: 409-433, 2003.

33 Parker MW, Buckley JT, Postma JP, Tucker AD, Leonard K, Pattus F and Tsernoglou D: Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. Nature *367*: 292-295, 1994.

34 Tsuzuki K, Kimura K, Fujii N, Yokosawa N, Indoh T, Murakami T and Oguma K: Cloning and complete nucleotide sequence of the gene for the main component of hemagglutinin produced by *Clostridium botulinum* type C. Infect Immun *58*: 3173-3177, 1990.

35 Okumura S, Saitoh H, Ishikawa T, Wasano N, Yamashita S, Kusumoto K, Akao T, Mizuki E, Ohba M and Inouye K: Identification of a novel cytotoxic protein, Cry45Aa, from *Bacillus thuringiensis* A1470 and its selective cytotoxic activity against various mammalian cell lines. J Agric Food Chem *53*: 6313-6318, 2005.

36 Katayama H, Kusaka Y, Yokota H, Akao T, Kojima M, Nakamura O, Mekada E and Mizuki E: Parasporin-1, a novel cytotoxic protein from *Bacillus thuringiensis*, induces Ca^{2+} influx and a sustained elevation of the cytoplasmic Ca^{2+} concentration in toxin-sensitive cells. J Biol Chem *282*: 7742-7752, 2007.

37 Kitada S, Abe Y, Shimada H, Kusaka Y, Matsuo Y, Katayama H, Okumura S, Akao T, Mizuki E, Kuge O, Sasaguri Y, Ohba M and Ito A: Cytocidal actions of parasporin-2, an antitumor crystal toxin from *Bacillus thuringiensis*. J Biol Chem *281*: 26350-26360, 2006.

38 Abe Y, Shimada H and Kitada S: Raft-targeting and oligomerization of parasporin-2, a *Bacillus thuringiensis* crystal protein with antitumour activity. J Biochem *143*: 269-275, 2008.

39 Pepit L, Gibert M, Gillet D, Laurent-Winter C, Boquet P and Popoff MR: *Clostridium perfringens* epsilon-toxin acts on MDCK cells by forming a large membrane complex. J Bacteriol *179*: 6480-6487, 1997.

40 Zhuang M, Oltean DI, Gomez I, Pullikuth AK, Soberon M, Bravo A and Gill SS: Heliothis virescens and Manduca sexta lipid rafts are involved in Cry1A toxin binding to the midgut epitherium and subsequent pore formation. J Biol Chem *277*: 13863-13872, 2002.

41 Uemori A, Ohgushi A, Yasutake K, Maeda M, Mizuki E and Ohba M: Parasporin-1Ab, a novel *Bacillus thuringiensis* cytotoxin preferentially active on human cancer cells *in vitro*. Anticancer Res *28*: 91-96, 2008.

42 Yasutake K, Uemori A, Binh ND, Mizuki E and Ohba M: Identification parasporin genes in Vietnamese isolates of *Bacillus thuringiensis*. Z Naturforsch *63c*: 139-143, 2008.

43 Hayakawa T, Kanagawa R, Kotani Y, Kimura M, Yamagiwa M, Yamane Y, Takebe S and Sasaki H: Parasporin-2Ab, a newly isolated cytotoxic crystal protein from *Bacillus thuringiensis*. Curr Microbiol *55*: 278-283, 2007.

44 Kim H-S, Yamashita S, Akao T, Saitoh H, Higuchi K, Park YS, Mizuki E and Ohba M: *In vitro* cytotoxicity of non-Cyt inclusion proteins of a *Bacillus thuringiensis* isolate against human cells, including cancer cells. J Appl Micribiol *89*: 16-23, 2000.

45 Yamashita S, Akao T, Mizuki E, Saitoh H, Higuchi K, Park YS, Kim H-S and Ohba M: Characterization of the anti-cancer-cell parasporal proteins of a *Bacillus thuringiensis* isolate. Can J Microbiol *46*: 913-919, 2000.

46 Heiss P, Bernatz C, Bruchelt G and Senekowitsch-Schmidtke R: Cytotoxic effect of immunoconjugate composed of glucose-oxidase coupled to an anti-ganglioside (G_{D2}) antibody on spheroids. Anticancer Res *17*: 3177-3178, 1997.

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