Identification of Parasporin Genes in Vietnamese Isolates of *Bacillus thuringiensis*

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Z. Naturforsch. **63c**, 139–143 (2008); received August 7, 2007

Four genes encoding parasporins, cytotoxins preferentially killing human cancer cells *in vitro*, were isolated from four Vietnamese strains of *Bacillus thuringiensis*. Nucleotide sequence analysis revealed that: (1) three genes fall into the two known classes, *ps1Aa* and *ps1Ab*, and (2) another one belongs to *ps1Ac*, a novel gene class established in this study. Upon proteolytic activation, parasporal protein of the organism with *ps1Ac* exhibited strong cytocidal activity against human cancer cells, HeLa and Hep G2, but not to non-cancer normal cells, UtSMC and HC.

Key words: Bacillus thuringiensis, Parasporin, *ps1Ac*

Introduction

Bacillus thuringiensis, a Gram-positive spore-forming organism, was first isolated as a pathogen of the silkworm, *Bombyx mori*, by Ishiwata (1901). The organism is characterized by producing crystalline parasporal inclusions that often contain unique proteins with strong insecticidal activities against several orders of insects of agricultural and medical importance (Glare and O'Callaghan, 2000). At present, these insecticidal proteins fall into two major categories, Cry and Cyt proteins. The former is a collection of 60- to 130-kDa crystal proteins with narrow-spectrum insecticidal activities, and the latter consists of 25- to 30-kDa proteins with broad-spectrum cytolytic activities against invertebrate and vertebrate cells *in vitro*.

Historically, it has long been thought that *B. thuringiensis* has acquired insecticidal activity in the course of co-evolution with insects through a host-parasite relationship. Earlier investigators, however, have claimed that non-insecticidal *B. thuringiensis* isolates outnumbered insecticidal ones in natural environments (Ohba and Aizawa, 1986; Ohba *et al*., 2000; Yasutake *et al*., 2007). Thus, the question that naturally arises is whether parasporal inclusion proteins of non-insecticidal organisms have any biological activity as yet undiscovered or not. Interestingly, Mizuki *et al.* (1999) reported the occurrence of non-insecticidal *B. thuringiensis* strains whose parasporal proteins are preferentially toxic to human cancer cells *in vitro*. This has led to the discovery of parasporin (PS), a functional protein group of Cry proteins with cancer cell-killing activity (Mizuki *et al*., 2000). Currently, PS is divided into four genealogically different groups, parasporin-1 (PS1) to parasporin-4 (PS4) (see the Committee of Parasporin Classification and Nomenclature website at http://parasporin.fitc.pref.fukuoka.jp/index.html).

In a preceding study, we obtained four PS-producing isolates of *B. thuringiensis* from Vietnam (Yasutake *et al*., 2006). Our findings suggested that the four PSs are all allied to the group PS1. The objective of this study was to establish full sequences of the genes for identification.

Materials and Methods

Organisms and plasmids

The four isolates of *B. thuringiensis* used in this study were: 31-5, 79-25, 87-29 and 92-10. The organisms were recovered in our preceding study from urban soils of Hanoi, Vietnam (Yasutake

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et al., 2006). In this study were also used: (1) the type strain of *B. thuringiensis* serovar *israelensis* (de Barjac, 1978) that produces a broad-spectrum cytolysin, Cyt toxin, and (2) *B. thuringiensis* strain A1190, the reference strain producing PS1Aa1 (Mizuki *et al.*, 2000). *Escherichia coli* DH5α, a cloning host, was purchased from Toyobo (Tokyo, Japan) and the plasmid pGEM-T Easy, a cloning vector, from Takara (Kyoto, Japan).

B. thuringiensis and *E. coli* were grown in Luria-Bertani medium at 30 °C and 37 °C, respectively. Ampicilline (50 μg ml^{-1}) was used for selection of *E. coli* transformants.

DNA manipulation and PCR experiments

Plasmid DNAs of *B. thuringiensis* were purified with a QIAprep plasmid midi kit (Qiagen, Hilden, Germany) and those of *E. coli* were isolated with a Labopass mini (Hokkaido System Science, Sapporo, Japan).

Previously, we obtained a half of putative *ps1* gene from each of the four Vietnamese *B. thuringiensis* isolates (Yasutake *et al.*, 2006). In the present study, a primer set was newly designed, on the basis of the specific sequences commonly contained in *ps1Aa* (Mizuki *et al.*, 2000) and *ps1Ab* (Uemori *et al.*, 2007b), to obtain full-length nucleotide sequences of the four genes. The sequence of the forward primer was 5′-ATCTCACCGATTTTGTAG-3′, and of the reverse 5′-TTATGAAACAGGACTAA-3′. The oligonucleotides were purchased from Hokkaido System Science. PCR amplification was done with Takara PCR Thermal Cycler PERSONAL (Takara, Otsu, Japan) as described previously (Wasano and Ohba, 1998). Gene cloning was done with a TA cloning kit (Promega, Madison, WI, USA) as recommended by the manufacturer.

Nucleotide sequences and accession number

Nucleotide sequences were determined directly with the primer walking method as previously described (Wasano *et al.*, 2005). Alignment analysis was done with the DNASIS program (Hitachi Software Engineering, Yokohama, Japan).

The accession numbers at DDBJ (DNA Data Base of Japan) and GenBank for the nucleotide sequences are: AB274825 (the isolate 31-5), AB274826 (the isolate 79-25), AB276125 (the isolate 87-29) and AB274827 (the isolate 92-10).

Parasporal inclusion proteins and protein determination

For cytocidal activity tests, parasporal inclusion proteins were alkali-extracted directly from mixtures of spores and parasporal inclusions. Protein extraction was performed as follows. The mixture was suspended at a concentration of 250 mg (wet weight) ml^{-1} in 50 mmol l^{-1} Na_2CO_3 (pH 10.0) containing 1 mmol l^{-1} EDTA and 10 mmol l^{-1} dithiothreitol at 37 °C for 1 h. Insoluble materials were removed by centrifugation in a 0.45-μm filter tube. The fluid, containing solubilized inclusion proteins, was then treated with proteinase K (60 μg ml^{-1}) for 90 min at 37 °C. Proteolysis was stopped by addition of phenylmethylsulphonyl fluoride.

Protein concentration was determined according to the method of Bradford (1976) with bovine serum albumin as the standard.

Cytopathy

The following human cells used were: HeLa, uterus cervix cancer cells; Hep G2, hepatocyte cancer cells; HC, normal hepatocyte cells; UtSMC, normal uterine smooth muscle cells. Cells were purchased from RIKEN Cell Bank (Tsukuba, Japan) except for UtSMC (Takara, Kyoto, Japan), and cultured under the conditions recommended by the suppliers.

One-dose assay technique (Mizuki *et al.*, 1999) was involved in the qualitative cytocidal activity test. The test was done at a protein concentration of 60 μg ml^{-1}. Cytopathological changes induced by the proteins were monitored using phase-contrast microscopy at appropriate intervals for 24 h.

Results and Discussion

In PCR experiments with *ps1A*-specific primers, DNA fragments of 2.1–2.2 kb were generated from all of the four Vietnamese isolates (data not shown). Table I summarizes the results of identification of the four parasporins. Alignment analysis of the amino acid sequences, deduced from the nucleotide sequences, revealed that the proteins of the two isolates 79-25 and 92-10 were $>$ 99% identical to the reference protein PS1Aa1. Proteins of the two other isolates, 31-5 and 87-29, both shared approximately 87% identity with PS1Aa1. Another recently established protein, PS1Ab1, had 99% and 81% identity to the proteins of 31-5 and 87-29, respectively. Finally, parasporins of the four

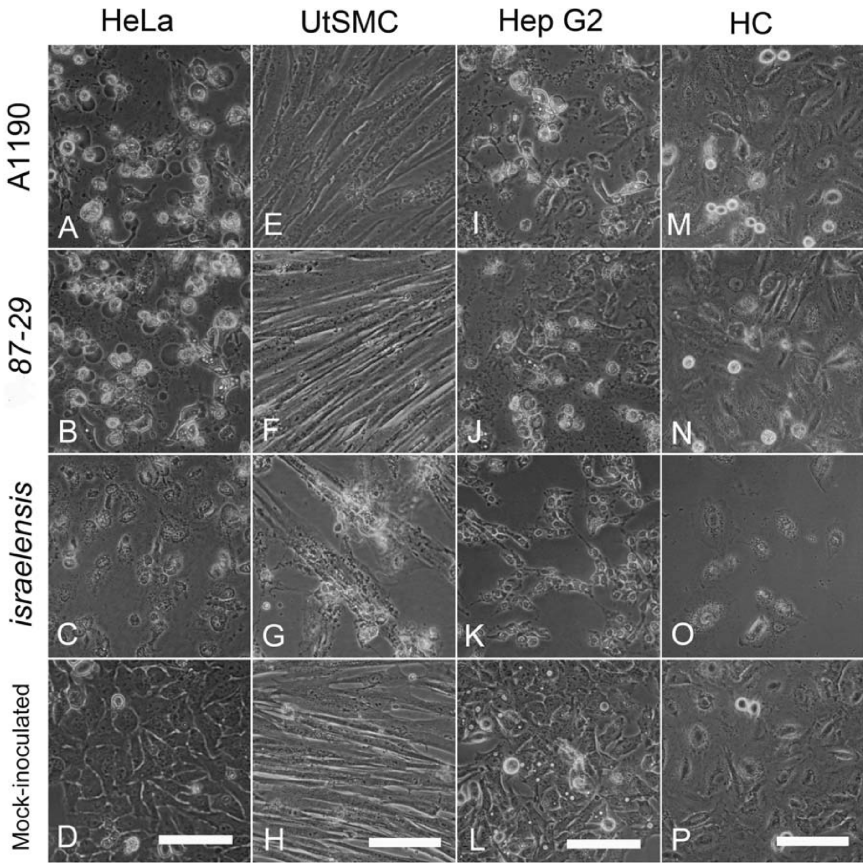

Fig. 1. Cytopathy induced in human cells by proteinase K-activated parasporal inclusion proteins of the three *Bacillus thuringiensis* strains. Parasporal proteins were prepared from: the strain A1190 (PS1Aa1), the Vietnamese isolate 87-29 (PS1Ac1), and the type strain of serovar *israelensis* producing mosquitocidal Cry toxins and broad-spectrum cytolysins (Cyt toxins). Concentration of activated proteins was: 60 μg ml^{-1}. Mock-inoculated control contained proteinase K (30 μg ml^{-1}). Cells: (A–D) HeLa, uterus cervix cancer; (E–H) UtSMC, normal uterine smooth muscle; (I–L) Hep G2, hepatocyte cancer; (M–P) HC, normal hepatocyte. Cytopathy was observed under a phase-contrast microscope 24 h post-inoculation. Bar indicates 50 μm.

Table I. Identification of parasporins from Vietnamese isolates of *Bacillus thuringiensis*.

B. thuringiensis	No. of deduced amino acid residues	Designation of parasporin	% Identity to	
			PS1Aa1	PS1Ab1
A1190 (reference)[a]	723	PS1Aa1	100	86.4
Vietnamese isolates				
79-25	723	PS1Aa4	99.7	86.3
92-10	723	PS1Aa5	99.3	86.8
31-5	726	PS1Ab2	86.9	98.8
87-29	777	PS1Ac1	86.8	81.1

[a] Mizuki *et al.* (2000).
For parasporin names, see the Committee of Parasporin Classification and Nomenclature website

B. thuringiensis isolates fell into the three groups: two existing classes, PS1Aa and PS1Ab, and a novel class, PS1Ac, established in the present study.

When HeLa cells were treated with proteinase K-activated parasporal proteins of the strain A1190 (PS1Aa1) and the isolate 87-29 (PS1Ac1), most cells (> 90%) were damaged in 24 h with

noticeable cytopathy characterized by cell rounding and ballooning (Figs. 1A, B). Unlike HeLa cells, UtSMC cells were not susceptible to the proteins (Figs. 1E, F). When examined on Hep G2 cells, these proteins induced marked detachment of the cells from the surface of the culture plates (Figs. 1I, J), with no sign of cell ballooning. The proteins of the strain A1190 and the isolate 87-29 induced no cytopathological events in HC cells (Figs. 1M, N). The proteins of *B. thuringiensis* serovar *israelensis* exhibited strong cytotoxicity to all of the cells used. The cytopathy induced by the *israelensis* proteins was characterized by early drastic cell lysis without cell ballooning (Figs. 1C, G, K, O).

B. thuringiensis parasporin has been of increasing interest due to its unique ability to kill cancer cells preferentially (Mizuki *et al*., 2000; Ito *et al*., 2004; Yamashita *et al*., 2005; Saitoh *et al*., 2006). Recently, while studying the distribution of *B. thuringiensis* in soils of Vietnam, we obtained four parasporin producers with genes allied to *ps1A* (*cry31A*) (Yasutake *et al*., 2006). In the present study, full nucleotide sequences were given to these genes.

One of the most striking aspects in our results is that a novel class of parasporin, *ps1Ac*, was established on the basis of the gene cloned from the isolate 87-29. Previously, Yasutake *et al*. (2006) reported that the parasporal protein of the isolate 87-29 induced strong cytopathy to HeLa cells. In the present study, this protein killed Hep G2 cells as well as HeLa cells but induced no cytopathy to non-cancer normal cells, UtSMC and HC. It is clear from our observations that PS1Ac is capable of killing cancer cells preferentially, like the other existing parasporins including PS1Aa of the strain A1190. Unlike the proteins of the two strains 87-29 and A1190, the protein of *israelensis* induced marked cytolysis not only in cancer cells but also in non-cancer normal cells. Apparently, this non-specific cytolysis is due to Cyt toxin contained in parasporal inclusions of the *israelensis* strain.

It should be also noted that the present study provided three heterogeneous genes belonging to the class *ps1A*. To date, *ps1A*-related genes have been isolated from *B. thuringiensis* populations inhabiting natural environments in Japan (Mizuki *et al*., 2000; Uemori *et al*., 2007a, b) and Canada (Jung *et al*., 2007). These findings suggest that the *ps1A* genes might be widely distributed in the Pacific Rim regions.

Acknowledgements

The authors thank Dr. A. Ohgushi, Dr. H. Saitoh, Dr. M. Maeda and Ms. K. Kagoshima for invaluable advices and technical assistance.

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