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Typical Three-Domain Cry Proteins of *Bacillus thuringiensis* Strain A1462 Exhibit Cytocidal Activity on Limited Human Cancer Cells

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***Bacillus thuringiensis* strain A1462 produced two parasporal inclusion proteins with a molecular mass of 88 kDa that were converted to 64-kDa toxins when activated by proteinase K digestion. Both toxins exhibited strong cytocidal activity against two human cancer cell lines, HL60 (myeloid leukemia cells) and HepG2 (liver cancer cells), while low or no toxicities were observed against 11 human and three mammalian cell lines, including four non-cancer cell lines. The cytotoxicity of both toxins on susceptible cells was characterized by rapid cell swelling. Gene cloning experiments provided two novel genes encoding 88-kDa Cry proteins, Cry41Aa and Cry41Ab. The amino acid sequences of the two proteins contain five block regions commonly conserved in *B. thuringiensis* insecticidal Cry proteins. This is the first report of the occurrence of typical three-domain Cry proteins with cytocidal activity preferential for cancer cells.**

Key words: *Bacillus thuringiensis*, cell swelling, Cry protein, cytocidal activity, parasporin-3.

Abbreviations: BIS, *N,N′*-methylene-bis-acrylamide; CAPS, *N*-cyclohexyl-3-aminopropanesulfonic acid; DIG, digoxigenin; EC_{50}, 50% effective concentration; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; ORF or orf, open reading frame.

Bacillus thuringiensis, a Gram-positive bacterium, produces parasporal inclusions during sporulation. The parasporal inclusion contains δ-endotoxins that often exhibit insecticidal activity against several orders of insect. The δ-endotoxins are currently classified into two families, Cry and Cyt proteins. The Cry proteins are solubilized and proteolytically activated by midgut proteases of the susceptible insects under alkaline conditions. It has been well established that this proteolytic processing is essential for the activation of Cry toxins (*1*). Strong and specific insecticidal activity of the Cry proteins makes this organism an environmentally sound microbial agent for controlling insect pests of agricultural and medical importance (*2*).

The insecticidal properties of the Cry proteins have also suggested that *B. thuringiensis* is an obligate entomopathogen that has co-evolved with insects through host-parasite relationships. It should be noted, however, that non-insecticidal *B. thuringiensis* is far more widely distributed in the natural environment than insecticidal ones (*3, 4*). Moreover, recent studies have provided evidence that parasporal inclusion proteins that are preferentially active on human cancer cells occur in non-insecticidal *B. thuringiensis* (*5–13*). We proposed a new family of proteins, parasporin, defined as *Bacillus thuringiensis* and related bacterial parasporal proteins that are non-hemolytic but capable of preferentially killing cancer cells (*6*, *7*).

In a previous study (*10*), we reported that the parasporal inclusion proteins of non-insecticidal *B. thuringiensis* strain A1462 (formerly 89-T-26-17) exhibit selective cytocidal activity against certain human cancer cells when activated by proteolytic degradation. The objectives of this study were: (i) to identify the inclusion proteins responsible for the cytocidal activity of strain A1462, (ii) to examine various cancer and normal cells for susceptibility to the toxins, (iii) to clarify the cytocidal action of the toxins on sensitive cells, and (iv) to characterize the genes encoding the toxin proteins.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—B. thuringiensis isolate A1462 (formerly 89-T-26-17) was derived from a soil sample collected in Tokyo, Japan (*10*). For cloning of the toxin genes, *Escherichia coli* XL-1 Blue MRF', SOLR and the vector Lambda Zap II were obtained from Stratagene (La Jolla, CA, USA). The pBluescript SK(–) was excised from Lambda Zap II. The cloned genes were expressed in an acrystalliferous *B. thuringiensis* mutant strain BFR1 (*6*) using the *E. coli–B. thuringiensis* shuttle vector pHT3101 (*14*).

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Table 1. **Cytocidal activity of the two 64-kDa proteins, P2 and P3, against various cultured cells.**

Cell	Origin	EC_{50} (μg/ml)	
		P2	P3
Human			
MOLT-4	Leukemic T cell	>10	>10
Jurkat	Leukemic T cell	>10	>10
HL60	Myeloid leukemia	1.32	1.25
HeLa	Uterus cervix cancer	>10	>10
TCS	Uterus cervix cancer	>10	>10
Sawano	Uterus cancer	>10	>10
HepG2	Hepatocyte cancer	2.80	1.86
A549	Lung cancer	>10	>10
CACO-2	Colon cancer	>10	>10
T cell	Normal T cell	>10	>10
UtSMC	Normal uterus	>10	>10
HC	Normal hepatocyte	>10	>10
MRC-5	Normal lung	>10	>10
Simian			
Vero	African green monkey kidney	>10	>10
COS-7*	African green monkey kidney	>10	>10
Murine			
NIH3T3-3	Mouse embryo	>10	>10

*SV40 transformed.

Cells and Culture Conditions—Cells used in this study are listed in Table 1. Most cells were purchased from RIKEN Cell Bank (Tsukuba, Japan). UtSMC and HC were obtained from Cambrex Bio Science (Walkersville, MD, USA) and Applied Cell Biology Research Institute (Kirkland, WA, USA), respectively. They were maintained under the conditions recommended by the suppliers. Media were supplemented with 10% fetal bovine serum and kanamycin (30 μg/ml).

Normal human T cells were freshly prepared by the method previously described (*5*) and were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and kanamycin (30 μg/ml) at 37°C.

SDS-PAGE and Protein Determination—SDS-PAGE was performed in 10% separating gels as described by Laemmli (*15*) or by Kagawa *et al*. (*16*) with an acrylamide/*N*,*N*′-methylene-bis-acrylamide (BIS) concentration of 30:0.8 or 30:0.135, respectively. After electrophoresis, the gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO, USA). The molecular masses of proteins were estimated by comparison with molecular standards (Bio-Rad Laboratories, Hercules, CA, USA).

Protein concentration was determined by the method of Bradford (*17*) using a Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories).

N-Terminal Amino Acid Sequencing—The 88-kDa protoxins and the 64-kDa activated proteins were separated on SDS-PAGE. The proteins were then transferred to a polyvinylidene difluoride membrane, and the membrane was stained with 0.2% Coomassie Brilliant Blue R-250. N-terminal amino acid sequences of the proteins were determined using an automatic sequencer Model 473A (Applied Biosystems, Foster, CA, USA).

Peptide Mapping—V8 protease digestion of proteins was done by the method of Cleveland *et al*. (*18*), and the protein digests were subjected to SDS-PAGE using 15% separating gels.

Purification of Toxins—Parasporal inclusions of A1462 were purified by the biphasic separation technique of Goodman *et al*. (*19*). The purified inclusions of A1462 were solubilized in 50 mM Na_2CO_3/1 mM EDTA for 60 min at 37°C. Proteins were also alkali-extracted directly from sporulated cultures of the transformants. The solubilized proteins were treated with proteinase K (10 μg/ml) in the presence of 10 mM DTT for 90 min at 37°C. The proteinase K–digested proteins were then treated with 1 mM phenylmethylsulfonyl fluoride to inhibit the protease activity. Toxins were separated from proteolysis products by anion-exchange column chromatography on RESOURCE Q (Amersham Biosciences, Freiburg, Germany) at a flow rate of 2 ml/min with increasing stepwise gradient of NaCl (0 to 1 M) in 10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS)-NaOH (pH 10.4). Purified toxins were concentrated using a Vivaspin (Vivascience AG, Hannover, Germany), and subjected to gel filtration on Superdex 75 HR (Amersham Biosciences) using 25 mM Tris-HCl (pH 8.8)/150 mM NaCl at a flow rate of 0.5 ml/min.

Cytotoxicity Assay—Cytotoxicity assays were performed in 96-well microtest plates. Each well received 90 μl of cell suspension containing 2×10^4 cells (2×10^5 cells in the case of normal T cells), and the plate was incubated at 37°C for 16 h. This was followed by the addition of 10 μl of activated protein solution to each well. The cytopathic effect was monitored under a phase-contrast microscope 1 h and 24 h post-administration. HepG2 cells were used for the trypan blue exclusion test to detect damaged cells. One hundred microliters of 0.4% trypan blue (Life Technologies, Rockville, MD, USA) was added to each well. After incubation for 10 min at room temperature, the supernatant was removed from the wells and the stained cells were monitored under a phase-contrast microscope.

The levels of cytotoxicity of the activated proteins were also assessed by the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay, a cell proliferation assay (*20*, *21*) using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) or a Premix WST-1 Cell Proliferation Assay System (Takara Shuzo, Kyoto, Japan). The cell survival rate was determined by comparing the absorbance value with that of the control without activated proteins. Experiments were done in duplicate. The 50% effective concentration (EC_{50}) values were determined by probit analysis.

For the assay of cell lysis, the release of lactate dehydrogenase (LDH) into the culture medium (*22*) was measured with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Each well of a 96-well microtest plate received 90 μl of cell suspension containing 2×10^4 HepG2 cells. After incubation at 37°C for 16 h, A1462 proteins (10 μg/ml each) were added to each well. The amount of LDH release was determined by comparing the absorbance with that of the control without activated proteins (0% release) or 0.1% Triton X-100 treatment (100% release). Experiments were done in duplicate.

Gene Cloning—A genomic library of the strain A1462 was constructed from a total DNA preparation as described previously (*6*). Immunopositive plaques were selected with polyclonal antibodies against whole inclusion proteins of

strain A1462, and DNA clones were cut out as pBluescript SK(–) plasmids by *in vivo* excision.

Of the DNA clones obtained, four (designated C15, C17, C18 and C25) contained similar *cry*-like sequences. For the construction of a PCR-digoxigenin (DIG) probe, PCR was done with oligonucleotides p20-F (5′-TCCGGTAGATAT-TACGCTGAATC-3′) and p20-R (5′-TCACAACATTTTCTT-CCCCTATT-3′) using a PCR DIG Probe Synthesis kit (Roche Diagnostics, Mannheim, Germany). The primer set was designed on the basis of the sequence in the upstream region of C25. The PCR-DIG probe (199 bp) was used for plaque hybridization against a genomic A1462 library. Positive plaques were selected using DIG Nucleic Acid Detection kits (Roche Diagnostics) according to the manufacturer's instructions, and three additional DNA clones, C103, C128 and C129, were obtained.

To determine the arrangement of clones, PCRs were done against total DNA of A1462 with forward primers C103-F (derived from C103: 5′-TGAGGATCCAGCGCAAG-TCCA-3′) or C129-F (from C129: 5′-AAAACTCGAAAAG-GGGGAAAT-3′), and reverse primers C15-R (from C15: 5′-CCGTTGGCTATTACTGTCGTTTT-3′) or C15/C18-R (a common sequence between C15 and C18: 5′-GCTATAA-TGGCATCCACAACC-3′).

DNA Sequencing—DNA sequences were determined with an automatic sequencer Model 373S using Dye Terminator Cycle Sequencing FS Ready Reaction Kits (Applied Biosystems), and were analyzed by the DNASIS program (Hitachi Software Engineering, Kanagawa, Japan). The deduced amino acid sequences were compared with the sequences of existing proteins using FASTA.

Expression of the cry Genes—The plasmid pC25-15 was generated by inserting the 3.9-kb *Sma*I–*Sph*I fragment from pC25, containing the *cry41Aa1* gene, into the *Sma*I–*Sph*I site of pC15. Then, the plasmid pC128 was digested with *Eco*RV and *Nco*I, and the 1.8-kb *Eco*RV–*Nco*I fragment was ligated into the *Sma*I–*Nco*I site of pC25-15. This plasmid, designated pC128-25-15, was digested from the *Eco*RI/*Not*I adapter site with *Not*I, and the 6.6-kb fragment was re-inserted into the *Not*I site of pBluescript SK(–). The plasmid was named pC128-25-15*. For gene expression, pA1462-*cry41Aa1* was finally constructed by inserting the 6.6-kb *Sac*I–*Xba*I fragment of pC128-25-15* into the *Sac*I–*Xba*I site of pHT3101.

The plasmid pC17-18 was obtained by inserting the 2.3-kb *Sal*I–*Sph*I fragment from pC17 into the *Sal*I–*Sph*I site of pC18. The 3.6-kb *Aat*II–*Sac*I fragment from the plasmid pC17-18 was ligated into the *Aat*II–*Sac*I site of pC103. This plasmid containing the *cry41Ab1* gene was named pC103-17-18. pC103-17-18 was digested from the *Eco*RI/*Not*I adapter site with *Not*I, and the 6.7-kb fragment was re-inserted into the *Not*I site of pBluescript SK(–). This was designated pC103-17-18*. For gene expression, pA1462-*cry41Ab1* was constructed by inserting the 6.7-kb *Sma*I-*Sac*I fragment of pC103-17-18* into the *Sma*I–*Sac*I site of pHT3101.

The plasmids were introduced into the BFR1 by electroporation, followed by selection of transformants according to the method of Mizuki *et al*. (*6*) or Lereclus *et al*. (*14*). The transformants were grown on Brain Heart Infusion (Difco, Becton Dickinson Microbiology Systems, Sparks, MD, USA)-agar plates containing erythromycin (50 μg/ml) at 28°C for 4 days.

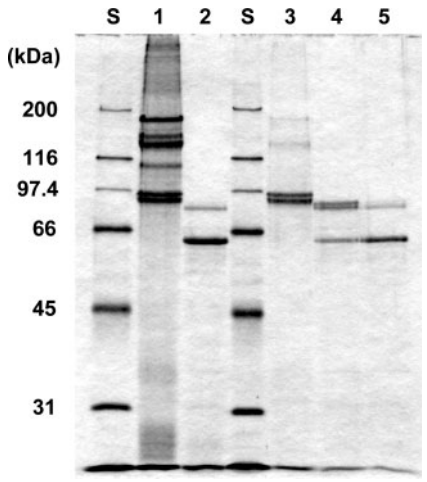

Fig. 1. **Protein analysis of *B. thuringiensis* strain A1462.** SDS-PAGE was done using a low concentration of BIS. Each lane contained 5 μg of the parasporal inclusion proteins. Lane S, molecular standards. Lane 1, alkali-solubilized proteins in the presence of DTT. Lane 2, alkali-solubilized and proteinase K–treated proteins with DTT. Lane 3, alkali-solubilized proteins in the absence of DTT. Lane 4, proteinase K–treated 88-kDa proteins without DTT. Lane 5, proteinase K–treated 88-kDa proteins with DTT.

RESULTS

Solubilization and Proteolytic Processing of the 88-kDa Proteins—As shown in Fig. 1, parasporal inclusions of the strain A1462 contained major proteins of 180, 150, 120, 100 kDa and a doublet of approximately 88 kDa. Proteinase K digestion of the solubilized inclusion proteins generated two proteins: a major protein of 64 kDa and an 80-kDa minor protein (Fig. 1, lane 2).

When the inclusions were alkali-solubilized in the absence of DTT, only the doublet proteins of ca. 88 kDa were detected in the supernatant (Fig. 1, lane 3), suggesting that the other proteins remained insoluble. These two proteins were very similar in their N-terminal amino acid sequences: the upper one with the sequence of MNQN (Y/C)NNNGY and the lower one with MNQ(N/S)(Y/C) NNNGY. The 88-kDa proteins were degraded to a single band of 64 kDa by processing with proteinase K, and the presence of DTT significantly enhanced the processing (Fig. 1, lane 5). The 64-kDa proteins obtained here exhibited cytocidal activity against MOLT-4 cells (data not shown).

Purification of the 64-kDa Proteins—To purify the cytocidal 64-kDa toxins, the 88-kDa proteins were treated with proteinase K in the presence of DTT and subjected to anion-exchange column chromatography. As shown in Fig. 2A, the elution profile consisted of a flow-through fraction and three protein peaks. The 64-kDa proteins were found in the second and the third peaks (Fig. 2B). Hereafter, the 64-kDa proteins of the second and the third peaks are referred to as P2 and P3, respectively (Fig. 2). When examined by V8 protease digestion, the proteolytic profile for P2 was similar to that for the lower protein of the 88-kDa doublet (Fig. 1, lane 3), while P3 was similar to the upper protein of the doublet (data not shown). There was high similarity in the N-terminal amino acid sequences between P2 and P3; the sequence of the former was

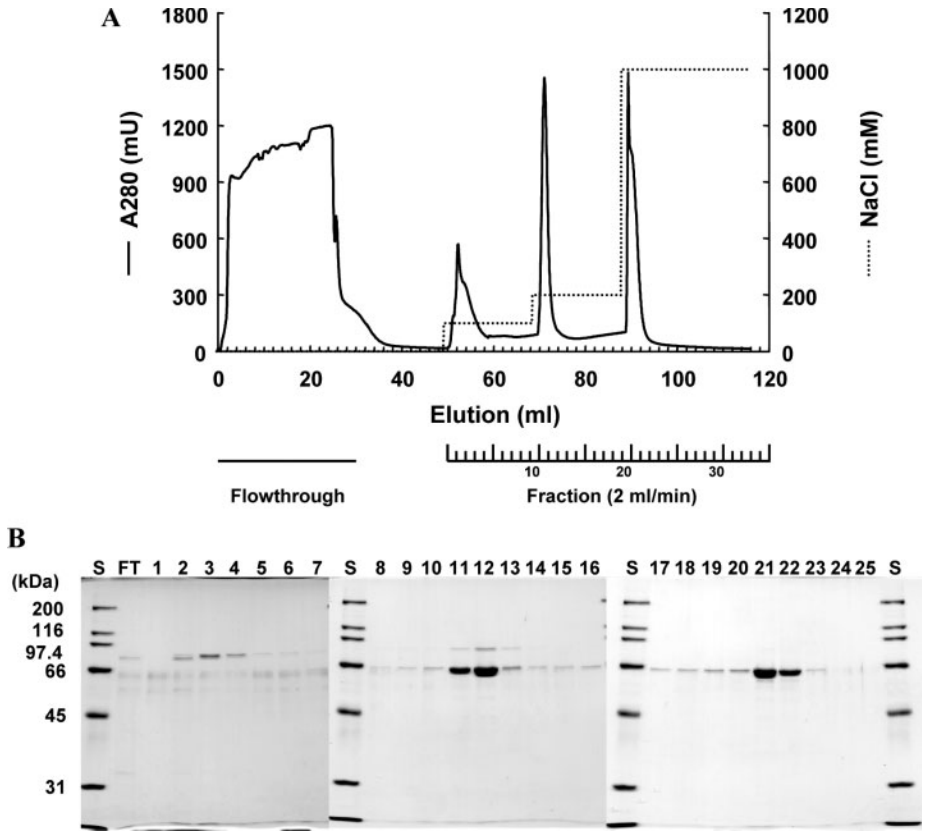

Fig. 2. **Purification of two cytocidal 64-kDa proteins of *B. thuringiensis* strain A1462.** (A) Elution pattern of the A1462 toxins on anion-exchange chromatography. (B) Analysis of each fraction (5 μl) by SDS-PAGE. Lane S, molecular standards. Lane FT, flow-through fraction from the column. Lane numbers correspond to fraction numbers.

DVKDAVITSXNIASY (X: an uncertain amino acid) and the latter DVRDAVITSINIASY.

Cytotoxicity of the Purified 64-kDa Proteins—The cytocidal activities of the 64-kDa proteins, P2 and P3, were examined against various cultured cells by the MTT assay (Fig. 3, Table 1). Two 64-kDa protein preparations from different peaks showed similar dose-dependent activities with narrow cytotoxicity spectra. HL60 and HepG2 cells were highly susceptible to both proteins. The proteins also exhibited cytotoxicity against several other cell lines; however, the activity levels were substantially low even at high protein concentrations (EC_{50} > 10 μg/ml). Slight differences were evident between P2 and P3 in the degree of cytotoxicity against Jurkat, TCS, and HepG2; in particular P2 had no cytocidal activity against Jurkat cells. Four human normal cells were not susceptible to the proteins.

Figure 4 shows the cytopathological changes induced in HepG2 cells by P2 and P3. Early cytopathy, induced in 1 h, was characterized by cell rounding and cell swelling. However, most of the cells excluded trypan blue. At 24 h post-administration, gross cell damages were observed; cell membranes of the swollen HepG2 cells seemed to have become fragile, and cells were stained with trypan blue.

Figure 5 shows the results of the time course study of the cytotoxicity on HepG2 cells. The survival rate was measured by the MTT assay, while the degree of membrane damage was assayed by monitoring LDH release from the cells. In both assays, the activity of P3 was significantly higher than that of P2. Cell death and membrane damage induced by the A1462 toxins were time-dependent.

Gene Cloning—As shown in Fig. 6, this study provided two gene complexes. Complex A, which was derived from the four DNA clones (C129, C128, C25 and C15), contained three open reading frames (orfs): *orf1a*, *orf2a* and *orf3a*. These orfs were closely located and oriented in the same direction. Complex A also contained various insertion sequence (IS)–related domains (Fig. 6A), although these domains were incomplete (data not shown). Complex B was derived from the three DNA clones, C103, C17 and C18, and consisted of three orfs, *orf1b*, *orf2b* and *orf3b*. These genes were also closely located and oriented in the same direction. All *orf*s had putative ribosome binding sites (5′-GGAGG-3′ or 5′-GAAGA-3′) in their upstream regions and no transcriptional terminator sequence was evident between the *orf1*s and *orf2*s, or between the *orf2*s and *orf3*s (data not shown). In the case of gene complex A, a terminator sequence was identified downstream of *orf3a* (data not shown).

ORF1a and ORF1b consisted of 180 amino acid residues with predicted molecular weights of 19,587 and 19,610, respectively. As shown in Fig. 7A, there was a high degree of homology (87.2%) between the two proteins. The proteins ORF2a and ORF2b consisted of 825 and 829 residues, with predicted molecular weights of 93,689 and 93,837, respectively. A high homology of 88.1% was observed in the sequences of these two ORF2 proteins. The N-terminal amino acid sequences of the 88-kDa doublet proteins (upper, MNQNCNNNGY and lower, MNQSCNNNGY) were contained in the deduced sequences of ORF2a and ORF2b, respectively (Fig. 7B). Furthermore, N-terminal amino acid sequences of the

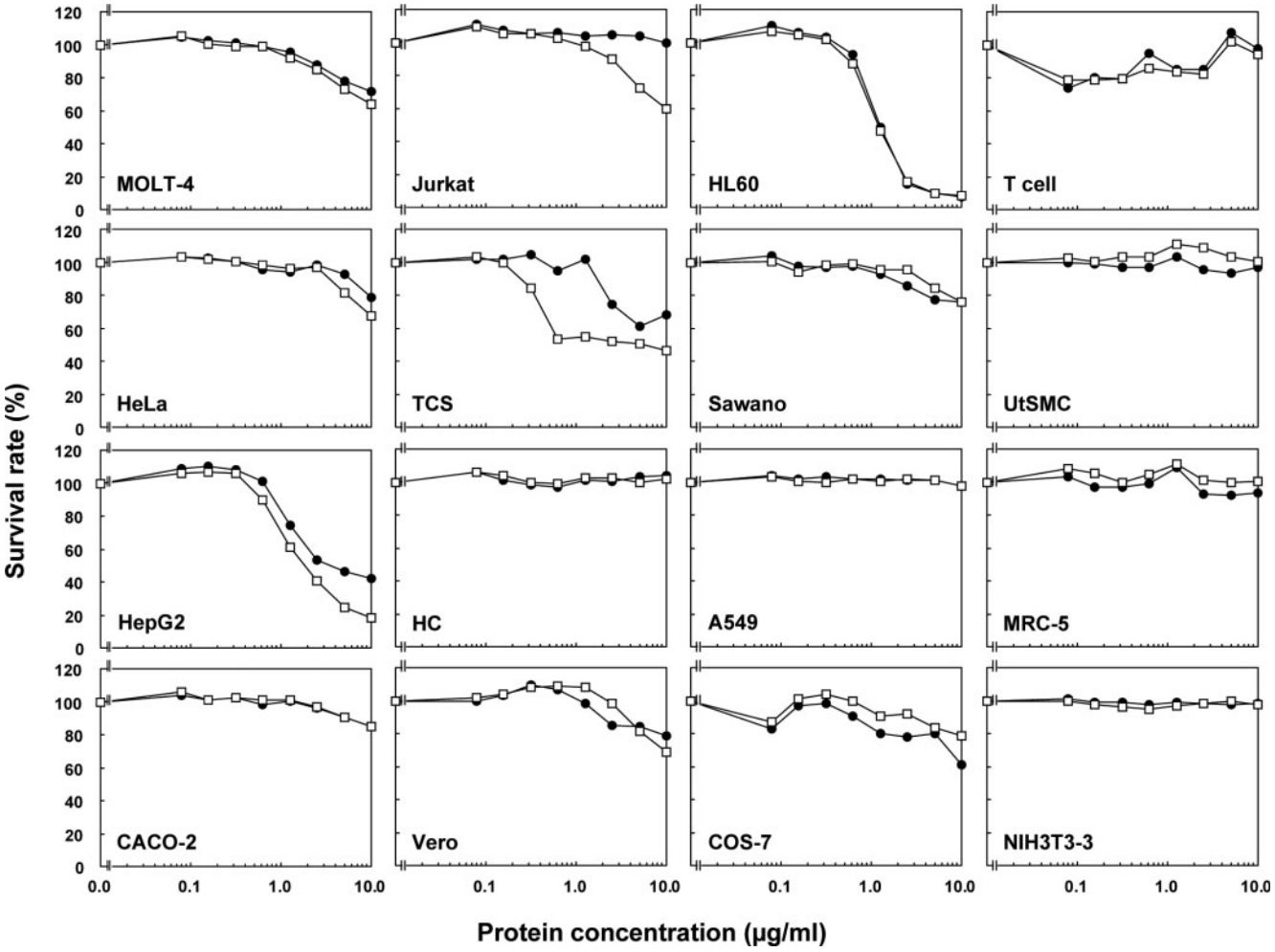

Fig. 3. **Dose-response curves for the two purified 64-kDa proteins, P2 and P3, of *B. thuringiensis* strain A1462 against various cultured cells.** The levels of cytotoxicity of P2 (closed circles) and P3 (open squares) were assessed using the MTT assay 24 h post-administration.

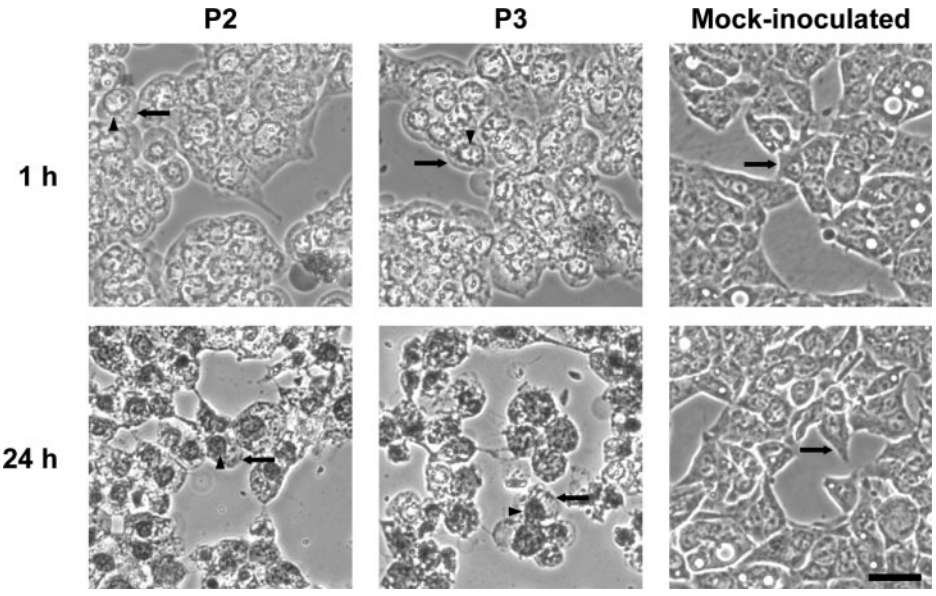

Fig. 4. **Cytocidal activity of purified toxins of *B. thuringiensis* strain A1462.** Cytopathic effects of the A1462 toxins (final concentration, 10 μg/ml each) on HepG2 cells were observed by phase-contrast microscopy 1 h and 24 h post-administration. Trypan blue staining was used to detect membrane damaged cells. Arrows and arrowheads show cell and nuclear membranes, respectively. Bar = 50 μm.

purified 64-kDa proteins (P2, DVKDAVITSINIASY and P3, DVRDAVITSINIASY) were detected in ORF2b and ORF2a, respectively. These ORF2 proteins retained five conserved block regions (blocks 1 to 5) commonly contained in known Cry proteins (*1*). However, these showed only low homologies between ORF2 proteins and the known Cry proteins; Cry3Ba (*23*) showed 27.0% homology with ORF2a and 29.1% with ORF2b. A comparative study of the C-terminal sequences showed that the present ORF2s have sequences similar to that of *Clostridium botulinum* hemagglutinin HA-33 (*24*).

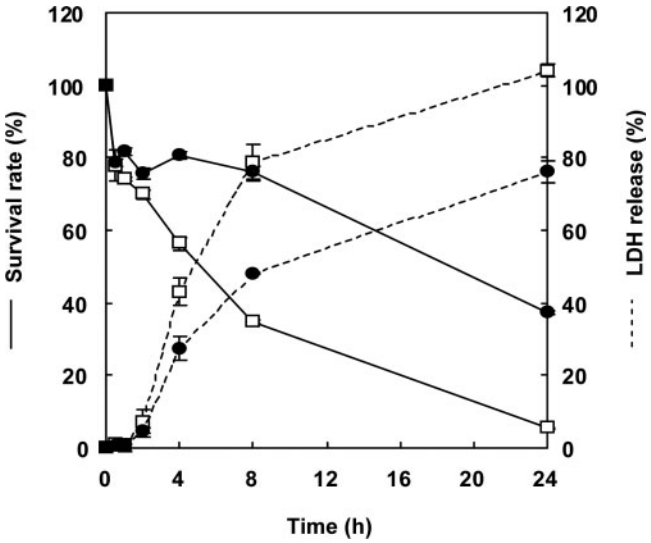

Fig. 5. **Time dependent cell death induced by the A1462 toxins of *B. thuringiensis* strain A1462 on HepG2 cells.** HepG2 cells were treated with P2 (closed circles; final concentration, 10 μg/ml) or P3 (open squares; 10 μg/ml). The levels of cytotoxicity of activated proteins (straight lines) were assessed using the MTT assay. LDH release (dotted lines) was measured by comparison with the amount of release induced by Triton X-100 (0.1%). Error bars represent standard deviation.

Figure 7C shows the deduced amino acid sequences of ORF3a and ORF3b; the former protein consisted of 737 residues with a molecular weight of 82,225 and the latter of 735 residues with a molecular weight of 82,141. The ORF3s contained the same N-terminal sequences (MNYNVTKAREAVQAL) as the 120-kDa protein, which was comprised in parasporal inclusions of the strain A1462 (*10*). The two proteins shared a high homology of 99.3%, with three conserved regions (blocks 6 to 8) of Cry proteins (*1*).

Expression of the orf2a and orf2b Genes—When the *orf2a* and *orf2b* genes were introduced through plasmid vectors into the acrystalliferous *B. thuringiensis* strain BFR1, parasporal inclusions were not formed. As shown in Fig. 8A, however, the production of 88-kDa proteins was evident in both transformants. These proteins were not synthesized in strain BFR1 containing the shuttle vector pHT3101.

The alkali-treated proteins showed no cytotoxicity against HepG2 cells (Fig. 8B). Proteinase K treatment of the recombinant 88-kDa proteins generated 64-kDa proteins (Fig. 8A, lanes 6 and 7) with cytocidal activity against HepG2 cells (Fig. 8B). When these 64-kDa proteins were subjected to anion-exchange column chromatography, the proteins from *orf2a* and *orf2b* were eluted with 1 M and 200 mM NaCl, respectively (data not shown).

Figure 9 shows cytopathological changes and dose-dependent cytotoxicity induced in Jurkat, TCS and HepG2 cells by the recombinant 64-kDa proteins.

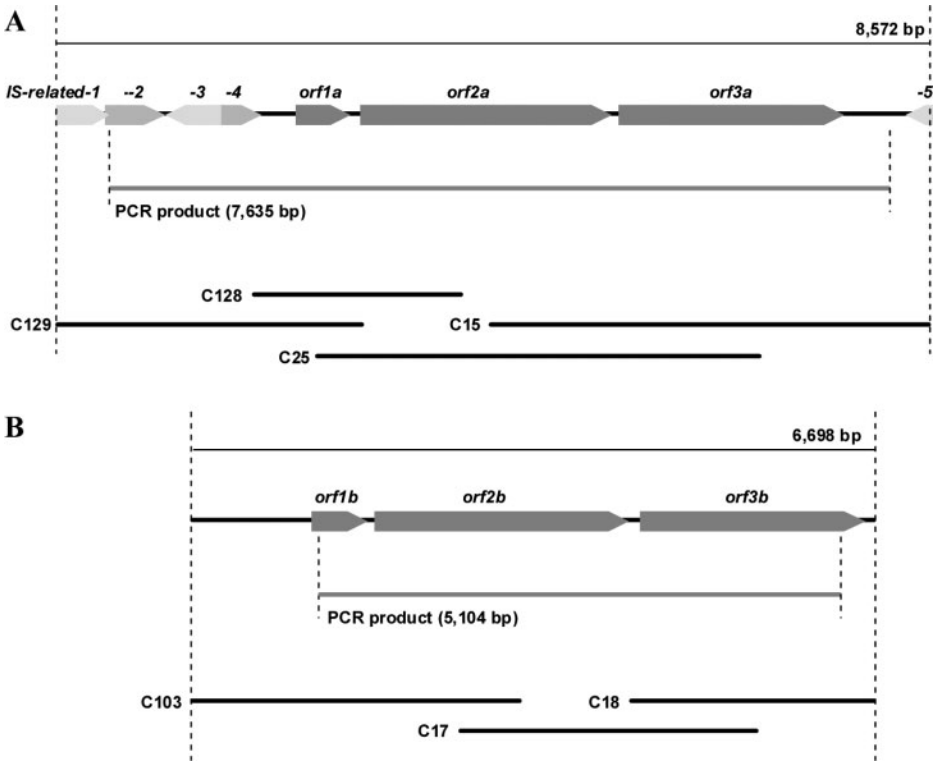

Fig. 6. **Structural organization of gene complexes in *B. thuringiensis* strain A1462.** DNA clones were obtained from a genomic library of the strain A1462. Arrangement of clones was determined using PCR against the total DNA of A1462 (see "MATERIALS AND METHODS"). Arrows show the position and direction of putative open reading frames.

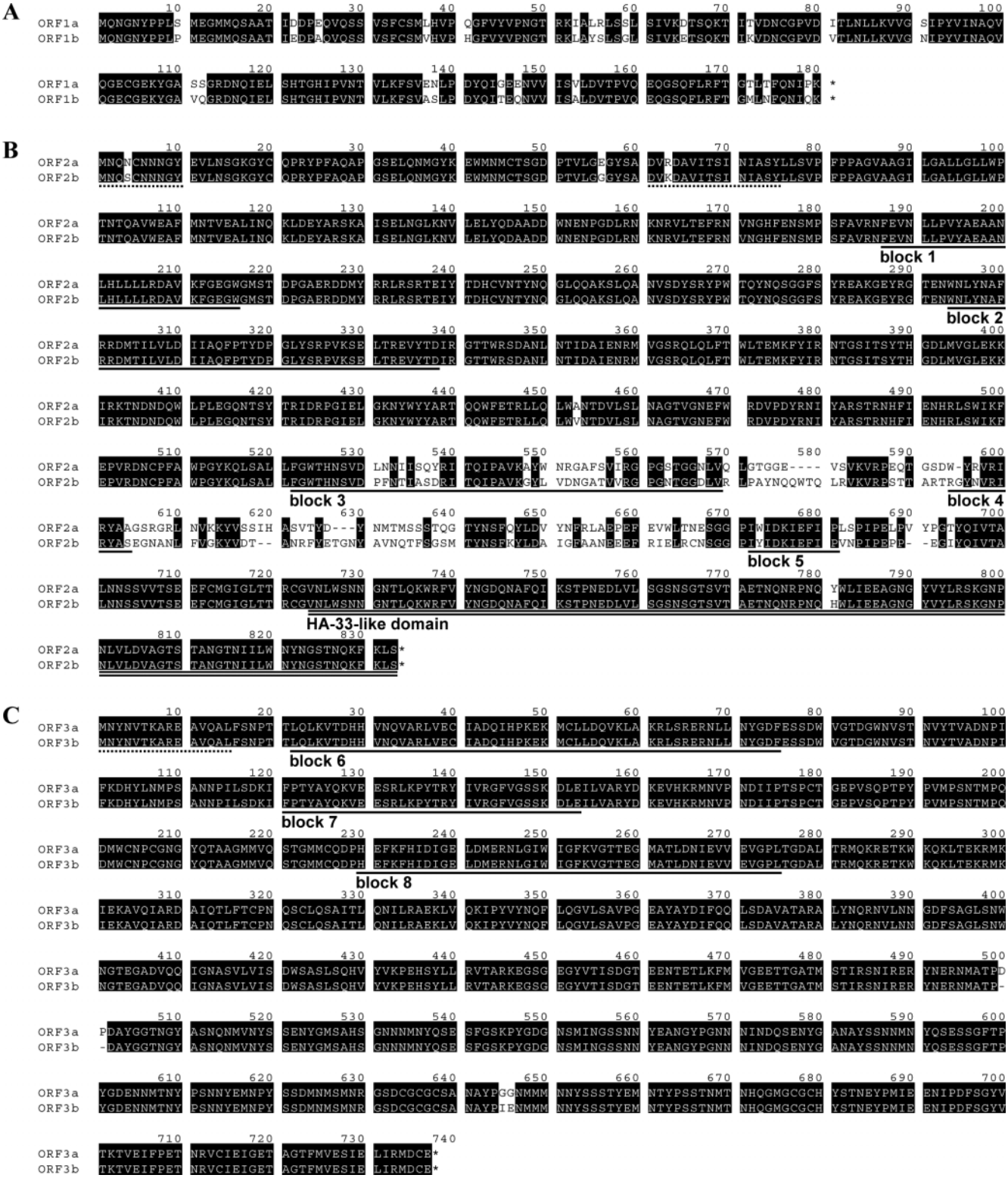

Fig. 7. **Comparison of deduced amino acid sequences.** (A) ORF1s are the hypothetical 20-kDa proteins. (B) ORF2a and ORF2b, named Cry41Aa1 and Cry41Ab1, respectively, are new short-type Cry proteins. (C) ORF3s are similar to the C-terminal half of 130-kDa Cry proteins. Conserved residues are highlighted. Eight conserved block regions (blocks 1 to 8) are underlined. The N-terminal amino acid sequences of the A1462 inclusion proteins and the activated 64-kDa proteins are underlined by dotted lines. A HA-33–like sequence of ORF2s is double underlined.

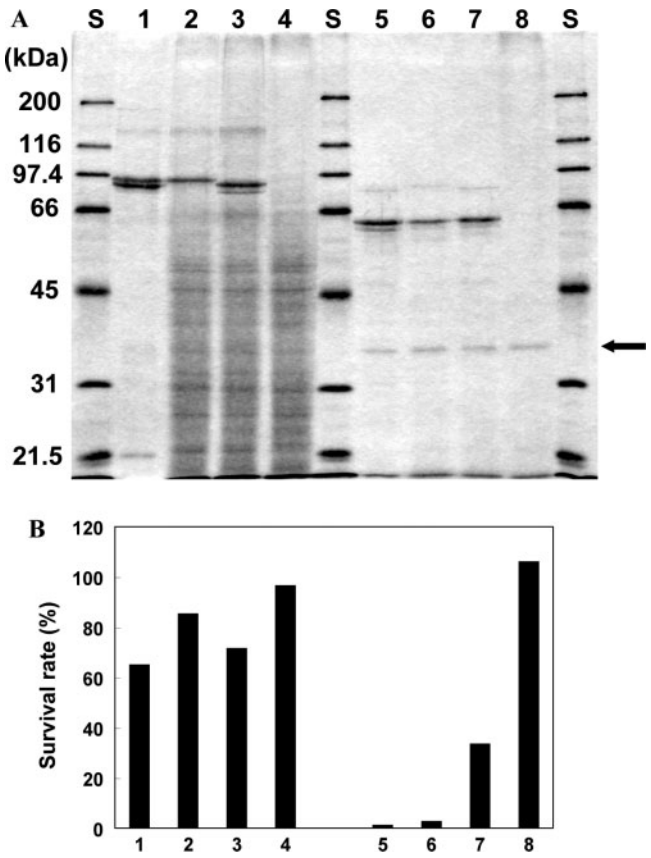

Fig. 8. **SDS-PAGE profiles of the cloned proteins.** (A) SDS-PAGE was done using a low concentration of BIS. Lane 1, alkali-solubilized inclusion proteins (1 μg) of the wild-type A1462 in the absence of DTT. Lanes 2–4, 5 μg each of alkali-solubilized transformant BFR1(pA1462-*cry41Aa1*), BFR1(pA1462-*cry41Ab1*) and BFR1(pHT3101) without DTT. Lanes 5–8, proteinase K–treated 88-kDa proteins of lanes 1–4, respectively, with DTT. Lane S, molecular standards. Arrow shows the position of proteinase K. (B) Cytocidal activities of the proteins on HepG2 cells. The levels of cytotoxicity were assessed using the MTT assay 24 h post-administration.

Damaged cells became swollen 1 h after administration of the 64-kDa proteins. Both proteins were highly toxic to HepG2 cells, but showed moderate or low toxicity to Jurkat and TCS cells. In particular, the ORF2b protein exhibited no cytocidal activity against Jurkat cells. In general, the cytocidal activity of the ORF2a protein was greater than that of the ORF2b protein.

DISCUSSION

This is the first report of the occurrence of a typical three-domain Cry protein that exhibits cytotoxicity against mammalian cells. The results strongly suggest that the toxins of *B. thuringiensis* strain A1462 with strong cytocidal activities against limited human cancer cells, HL60 and HepG2, have structural and functional similarities to insecticidal Cry proteins.

Previously, Mizuki *et al*. (*5*) reported that the parasporal proteins of the *B. thuringiensis* isolate 89-T-26-17 (A1462 in this study) exhibited neither insecticidal nor hemolytic activities. Subsequently, Yamashita *et al.* (*10*) showed that the proteins of the isolate have cytocidal activities against MOLT-4 and HeLa, but not against normal T cells, and that the 64-kDa protein, a proteolysis product of inclusion proteins, is the toxin preferentially killing cancer cells. In this study, protein purification and gene cloning experiments provided evidence that the 64-kDa protein is a mixture of two distinct populations with the same molecular size of 64 kDa. It is clear that the two 64-kDa proteins are derived from the 88-kDa doublet proteins present in native parasporal inclusions. This is supported by the fact that there exist identical homologies in internal amino acid sequences between the 64- and 88-kDa proteins.

The two 64-kDa proteins, encoded by the *orf2a* and *orf2b* genes, share a high sequence homology of 88%. It is noteworthy that both proteins contain five conserved block regions common in known Cry protein families. Nevertheless, there are low sequence homologies between the present proteins and existing Cry proteins. Thus, the ORF2a and ORF2b proteins have been designated Cry41Aa1 and Cry41Ab1, respectively, by the *Bacillus thuringiensis* Toxin Nomenclature Committee (see N. Crickmore's nomenclature website at http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/index.html).

It has been well accepted that the insecticidal Cry proteins initially bind to specific receptors on the cell membrane of susceptible insect cells, leading to early cell death with marked cytopathological damage, including cell swelling (*1*, *25*, *26*). In the present study, the cancer cell–killing action of the two Cry41A proteins was relatively slow when monitored by the trypan blue exclusion test, MTT assay, and LDH assay. It should be noted, however, that these two proteins also induced marked cytopathy, characterized by cell swelling, in susceptible cancer cells. This observation, coupled with the fact that the Cry41A protein has a three-domain structure, leads to the hypothesis that the Cry41A protein kills cancer cells by a receptor-mediated mechanism similar to that of insecticidal Cry proteins.

The two Cry41A proteins exhibited similar cytocidal activity on susceptible cells, but the activity level of Cry41Ab1 was lower than that of Cry41Aa1. Interestingly, our data also show a marked difference between the two proteins in amino acid sequence of domain III (block 3 to block 5). Thus, it is likely that the difference in the domain III structure may lead to the difference in toxicity levels. It is unclear whether and how domain III of the Cry41A protein plays a role in cancer cell–killing activity. This awaits clarification.

The Cry41A proteins are eligible to be members of the "parasporin" family (*5*) due to their unique biological activity. Katayama *et al*. (*7*) proposed the name of "parasporin-3" for the A1462 protein. Our present findings show the occurrence of subgroups in parasporin-3, tentatively named parasporin-3Aa and -3Ab for Cry41Aa and Cry41Ab, respectively. Interestingly, previous studies have revealed that the three other parasporins are genealogically unrelated to one another (*6–9*, *11*, *12*). These findings, coupled with the present results, suggest that the four parasporins have different cytocidal mechanisms due to differences in protein structure. In fact, the four parasporins induce different cytopathological events and have different cytotoxicity spectra (*6–12*). It is very likely that each parasporin has a specific receptor molecule on

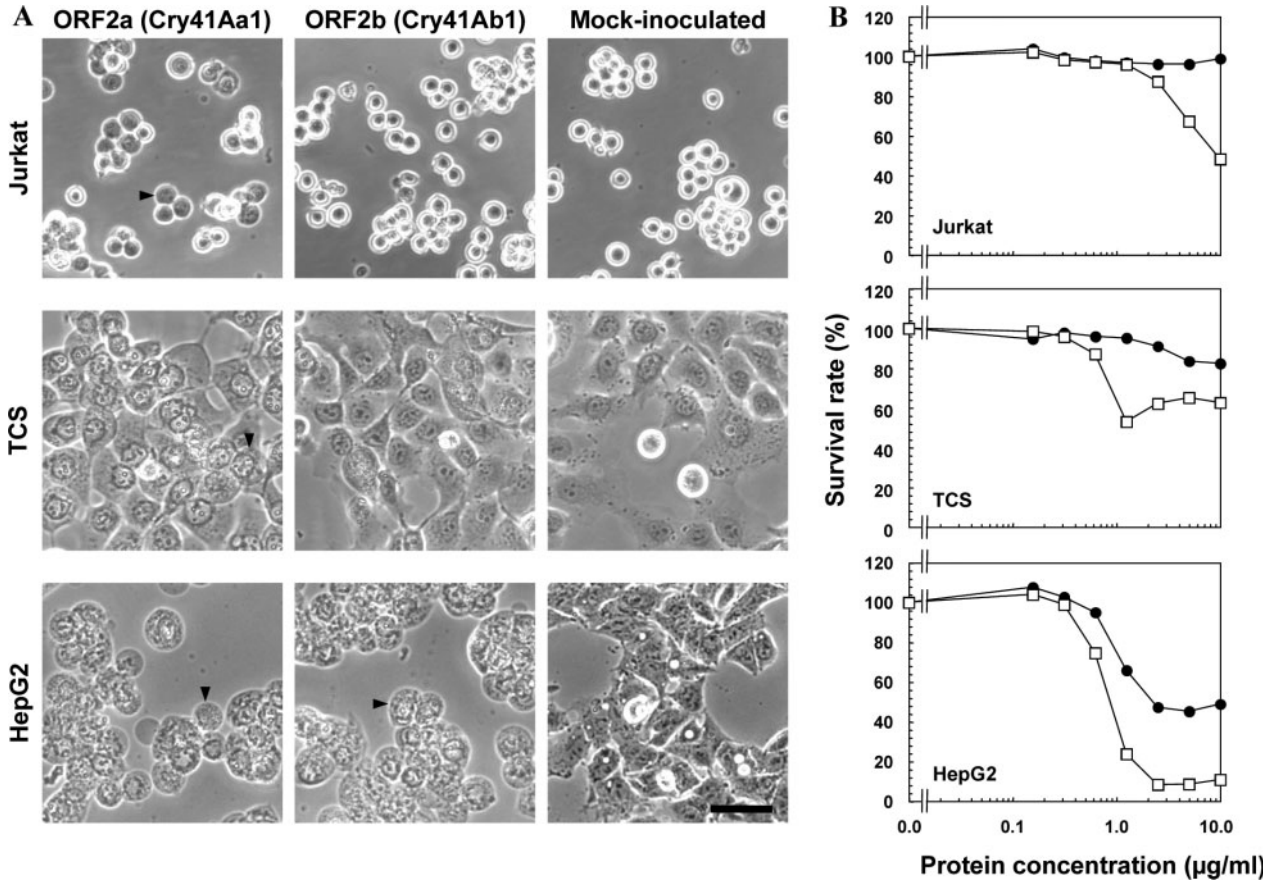

Fig. 9. **Cytotoxicity of the proteinase K–activated 64-kDa proteins of *B. thuringiensis* transformant BFR1(pA1462-cry41Aa1) and BFR1(pA1462-cry41Ab1) on Jurkat, TCS and HepG2 cells.** (A) Cytopathic effects of ORF2a (Cry41Aa1) and ORF2b (Cry41Ab1). Cells were treated with activated proteins (final concentration, 10 μg/ml). Phase-contrast microscopic observation was done 1 h post-administration. Arrowheads indicate swollen cells. Bar = 50 μm. (B) Dose-response analysis of ORF2a (Cry41Aa1, open squares) and ORF2b (Cry41Ab1, closed circles). The levels of cytotoxicity were assessed using the MTT assay 24 h post-administration.

susceptible cancer cells. Future work will include the isolation and identification of the receptor for parasporin-3, which is one candidate for the medical use of Cry proteins.

Nucleotide sequences obtained here have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB116649 (gene complex A) and AB116651 (gene complex B).
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