Biochemical and Biophysical Research Communications **272,** 218–223 (2000)
doi:10.1006/bbrc.2000.2765, available online at http://www.idealibrary.com on IDEAL®

Noninsecticidal Parasporal Proteins of a *Bacillus thuringiensis* Serovar *shandongiensis* Isolate Exhibit a Preferential Cytotoxicity against Human Leukemic T Cells

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Received April 20, 2000

A *Bacillus thuringiensis* isolate, 89-T-34-22, belonging to the serovar *shandongiensis* (H22) produced noninsecticidal and nonhemolytic proteins crystallizing into irregular-shaped parasporal inclusions. The proteins showed *in vitro* cytotoxicity to human cells, including cancer cells, only when activated by protease treatment. The human leukemic T (MOLT-4) cells were >100 times more susceptible than HeLa and normal T cells to the proteins of 89-T-34-22. The cytotoxicity was dose dependent and the median effective concentration for the MOLT-4 was 3.5 μg/ml. The cytopathy induced by the 89-T-34-22 proteins was characterized by remarkable condensation of the nucleus and cell-ballooning. Five major parasporal proteins of 89-T-34-22, with molecular masses in the range of 16–160 kDa, shared no similarity with the previously reported proteins in terms of the N-terminal sequence. © 2000 Academic Press

***Key Words: Bacillus thuringiensis;* cytotoxicity; human T cell; leukemic T cell; HeLa cell.**

Bacillus thuringiensis is a gram-positive bacterium producing insecticidal proteins against agricultural and medical pests during sporulation. Recent isolation of many insecticidal *B. thuringiensis* strains from various environments (1–3) has broadened the toxicity spectrum of this organism to a wide range of insects and other invertebrates including nematodes, mites, and protozoa (4). It is noteworthy, however, that the observations have also provided the evidence for a wide distribution of non-insecticidal *B. thuringiensis* in natural environments (2, 3, 5, 6). These non-toxic isolates have attracted less attention than insecticidal ones and their biological activities, if any, have not been clarified for long (7). Recently Mizuki *et al.* (8) obtained, in a mass-screening test, an interesting result that human cancer cell-killing activities are associated with noninsecticidal parasporal inclusions of certain *B. thuringiensis* strains.

Here we report the results of characterization of a novel isolate, designated 89-T-34-22, whose inclusions are not insecticidal but highly cytocidal to human leukemic T cells.

MATERIALS AND METHODS

Bacterial strains and culture media. The bacterial strains used in this study were the type strain of *B. thuringiensis* serovar *israelensis* H14 (9) and the soil isolate 89-T-34-22 belonging to the serovar *shandongiensis* (H22). The latter strain was derived from a soil sample collected in the city of Hino, Tokyo, Japan (8). The organisms were grown on nutrient agar (pH 7.6) consisting of meat extract (10 g), polypeptone (10 g), NaCl (2 g), agar (2 g) and distilled water (1000 ml) at 28°C.

Human cells and culture condition. Human cells used were: MOLT-4, leukemic T cell; HeLa, uterus cervix cancer cell; and normal T cell. MOLT-4 and HeLa cells were provided by RIKEN Cell Bank (Tsukuba, Japan) and cultured under the condition recommended by the supplier. Normal T cells were prepared from buffy coats obtained from Fukuoka Red Cross Blood Center (Fukuoka, Japan). They were separated from lymphocytes by the method previously described (8) and cultured at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum and 30 μg/ml of kanamycin.

Electron microscopy (EM). The strain 89-T-34-22 was grown on nutrient agar for 3 days. For transmission EM, ultrathin sections were prepared by the method of Higuchi *et al.* (10).

Proteolytic processing and cytotoxicity of parasporal inclusion proteins. Parasporal inclusions were separated from spores by the method of Goodman *et al.* (11) and further purified by discontinuous sucrose density gradient centrifugation as described previously (12). Purified parasporal inclusions were stored at −20°C prior to use. The inclusions were solubilized in 50 mM Na_2CO_3 (pH 10.0) at 37°C for 1 h in the presence of 10 mM DTT and 1 mM EDTA. Solubilized proteins (7 mg/ml) were treated at pH 8.0 with either of proteinase K (final conc.: 0.1, 1, 10, and 100 μg/ml), trypsin (final conc.: 0.01, 0.1,

0006-291X/00 $35.00

1, and 10 mg/ml), and chymotrypsin (final conc.: 0.01, 0.1, 1, and 10 mg/ml) at 37°C. After incubation for 90 min, proteolytic reaction was stopped with 1 mM phenylmethylsulfonyl fluoride and the proteolysis products were examined for both SDS–PAGE profiles and cytopathic effect (CPE) on MOLT-4, HeLa and normal T cells. The CPE was monitored under a phase contrast microscope for 20 h after administration of the proteins and the degree of CPE was graded on the basis of the proportion of damaged cells as described previously (8).

Dose–response study. Aliquots (90 μl) of cell suspension containing 2×10^4 cells were added to each well of a microtest plate and preincubated at 37°C for 16 h. The well then received 10 μl of proteinase K (50 μg/ml)-treated proteins prepared in 10-fold serial dilutions in 50 mM Na_2CO_3 (pH 10.0) + 10 mM DTT + 1 mM EDTA. The test was repeated at least three times. To assess the levels of cytotoxicity, a cell proliferation test with a MTT assay (13, 14) was done 24 h post-inoculation using a Premix WST-1 kit (Takara Co., Kyoto, Japan) and the absorbance was measured at 450 nm. The average of absorbance values in mock-inoculated negative control was used as a blank value. The arbitrary survival rate was determined on the basis of the relative value of absorbance to the blank. The median effective concentration (EC_{50}) values were calculated by using a log-probit program.

Protein determination and SDS–PAGE. Protein concentration was determined by the method of Lowry *et al.* (15) with bovine serum albumin as the standard. SDS–PAGE was performed as described by Laemmli (16), using 4% stacking and 12% resolving gels. After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie blue R250 (Sigma Co.). The molecular masses of parasporal inclusion proteins were determined by using molecular standards purchased from Bio-Rad Co., CA.

N-terminal sequencing of proteins. The proteins of the isolate 89-T-34-22, resolved on SDS–PAGE, were transferred to a PVDF membrane (Bio-Rad Co.) for determination of their N-terminal sequences in an automatic sequencer Model 473A (Applied Biosystem, Foster, CA). The amino acid sequences obtained were compared with those of the existing proteins, deposited in SWISS-PROT and PIR, by using FASTA (17).

RESULTS AND DISCUSSION

Parasporal inclusions. The *B. thuringiensis* isolate 89-T-34-22 produced irregular-shaped, roughly round parasporal inclusions during sporulation when observed under a phase-contrast microscope (Fig. 1A). As observed by transmission EM, the size of parasporal inclusions varied from 0.9 to 1.2 μm in diameter and the inclusion matrix seemed homogeneous (Fig. 1B).

The results showed the overall similarity in inclusion morphologies between the isolate 89-T-34-22 and the type strain of serovar *shandongiensis* (H22) (18). A similarity has also been found between these two *shandongiensis* strains in having no insecticidal activities (8, 18). Interestingly, however, Mizuki *et al.* (8) observed that the human cell-killing activity is associated with the 89-T-34-22 inclusions, but not with the inclusions of the type strain of *shandongiensis.*

Protein profiles of parasporal inclusions and N-terminal sequencing. Figure 2 shows the SDS–PAGE profile of parasporal inclusion proteins of 89-T-34-22 and their N-terminal amino acid sequences. Inclusions of the isolate 89-T-34-22 consisted of five major proteins with molecular masses of 160, 60, 34, 32, and 16 kDa. This is in marked contrast to the profile, observed in the type strain of serovar *shandongiensis,* consisting of the two proteins, 144 and 60 kDa (18).

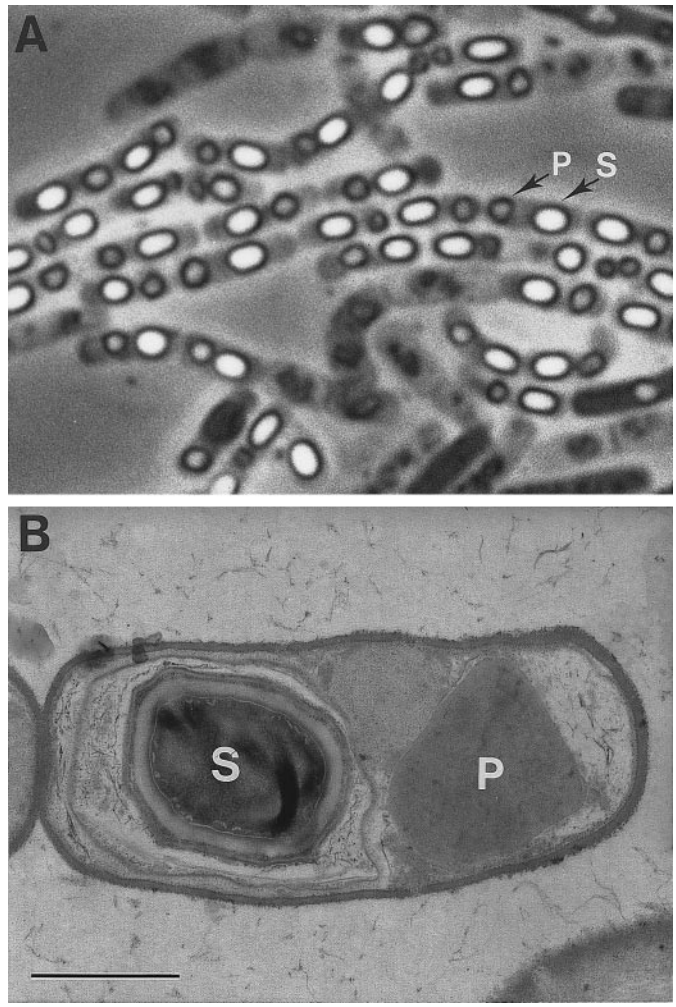

FIG. 1. Parasporal inclusions of the isolate 89-T-34-22 belonging to *Bacillus thuringiensis* serovar *shandongiensis* (H22). (A) Phase-contrast micrograph of a sporulating culture. (B) Transmission electron micrograph of a matured sporangium containing a spore and a parasporal inclusion. S, spore. P, parasporal inclusion. Bar, 1 μm.

N-terminal sequences of the five proteins were dissimilar to those of the inclusion proteins of the type strain (18). Also, they showed no significant similarity or homology with those of the existing Cry/Cyt proteins of *B. thuringiensis.* Among the sequences of 89-T-34-22 proteins, the sequences of 160- and 60-kDa proteins started with methionine; however, the first residue of the others was alanine or aspartic acid, suggesting that they are the degradation products from higher-molecular-mass proteins. It is of interest to note that the two proteins, 34 and 32 kDa, had the identical sequence in the first five amino acids. It is likely that, if any, these two proteins may have similar biological activities.

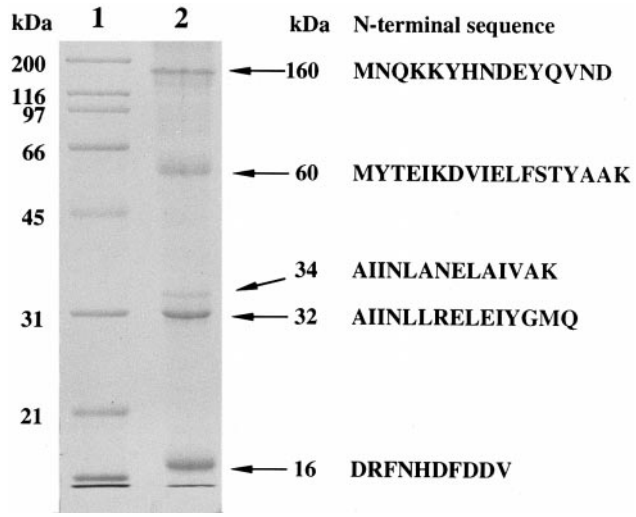

FIG. 2. SDS–PAGE profile and N-terminal sequences of parasporal inclusion proteins of the *Bacillus thuringiensis* isolate 89-T-34-22. Lane 1, molecular size marker. Lane 2, solubilized inclusion proteins (6 μg) of the isolate 89-T-34-22.

Proteolytic processing and cytotoxicity. To examine the proteolytic processing of inclusion proteins of the isolate 89-T-34-22, alkali-solubilized proteins were treated with proteinase K, trypsin, and chymotrypsin, respectively. As shown in Fig. 3, there was a similarity in proteolysis profiles between proteinase K-treated and chymotrypsin-treated inclusion proteins. These profiles, however, considerably differed from that obtained in trypsin-treated proteins. Also, proteinase K and chymotrypsin degraded the 160-kDa protein more efficiently than trypsin.

A marked cytotoxicity was induced when solubilized inclusion proteins were treated with proteases (Fig. 3). The non-treated proteins exhibited a low-level toxicity against MOLT-4 cells but no activity on HeLa and normal T cells. The three proteases were all effective for toxin activation and the three cell species used were all susceptible to the activated toxin. The concentration of trypsin and chymotrypsin, required for toxin activation, was approximately 100-fold greater than that of proteinase K (Fig. 3). The greatest cytotoxicity against MOLT-4 cells was obtained when processed by proteinase K at a concentration of 100 μg/ml and the protein profile was composed of two major proteins with molecular masses of 45 and 28 kDa (Fig. 3A). The data also showed that the 28-kDa protein is substantially resistant to the three proteases. At present, however, no information is available for determination of the ultimate toxic molecule.

It is clear from the results that the proteolytic processing is essential for activation of the 89-T-34-22 proteins as in the cases with insecticidal Cry/Cyt proteins of *B. thuringiensis* (19). The low-level cytotoxicity against MOLT-4 cells, retained in alkali-solubilized proteins, is likely due to the proteolysis products generated by the action of endogenous protease. It is of interest to note that the major protein of 28 kDa is similar to that of the Cyt protein, a hemolytic inclusion protein, of serovar *israelensis* in molecular size (20). In our previous study (8), however, protease-digested inclusion proteins of the strain 89-T-34-22 exhibited no hemolytic activity against vertebrate erythrocytes.

Cytopathic effect. As shown in Fig. 4, the proteinase K-digested proteins of serovar *israelensis* and 89-T-

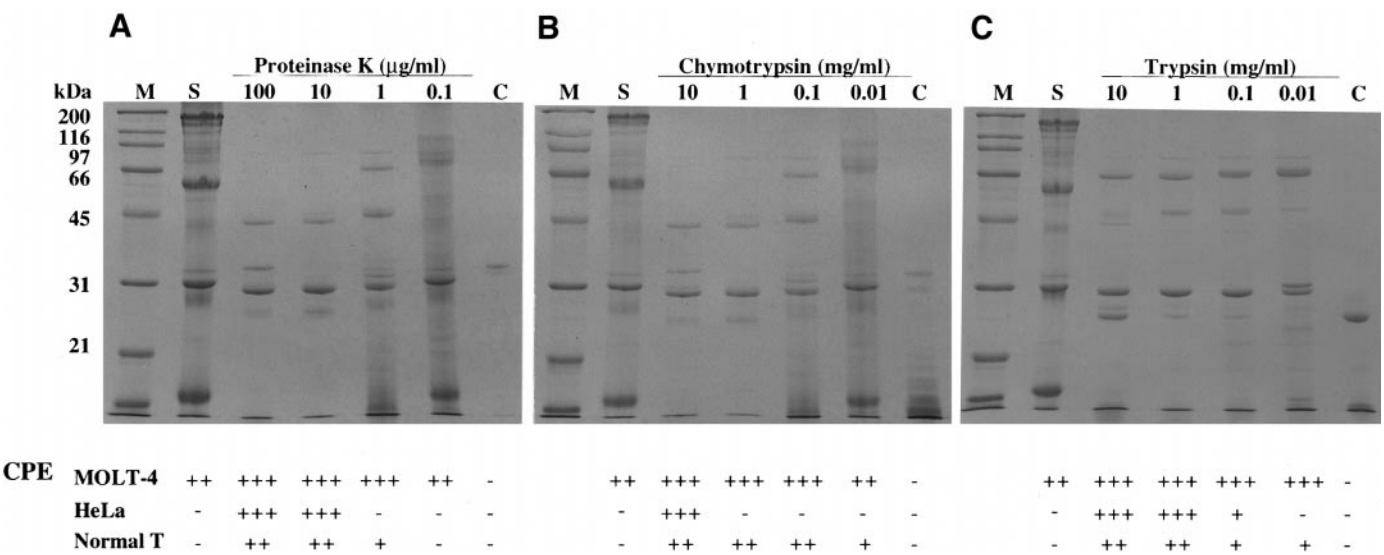

FIG. 3. Proteolytic profiles of parasporal inclusion proteins of the *Bacillus thuringiensis* isolate 89-T-34-22. (A) Proteinase K digestion. (B) Chymotrypsin digestion. (C) Trypsin digestion. Each lane contained 10 μg of inclusion proteins. M, molecular size marker; S, solubilized proteins; C, protease (proteinase K, 100 μg/ml; chymotrypsin, 10 mg/ml; trypsin, 10 mg/ml) in the absence of inclusion proteins. The degree of CPE, graded as described previously (8), is shown under each lane: +++, high; ++, moderate; +, low; −, no cell damages.

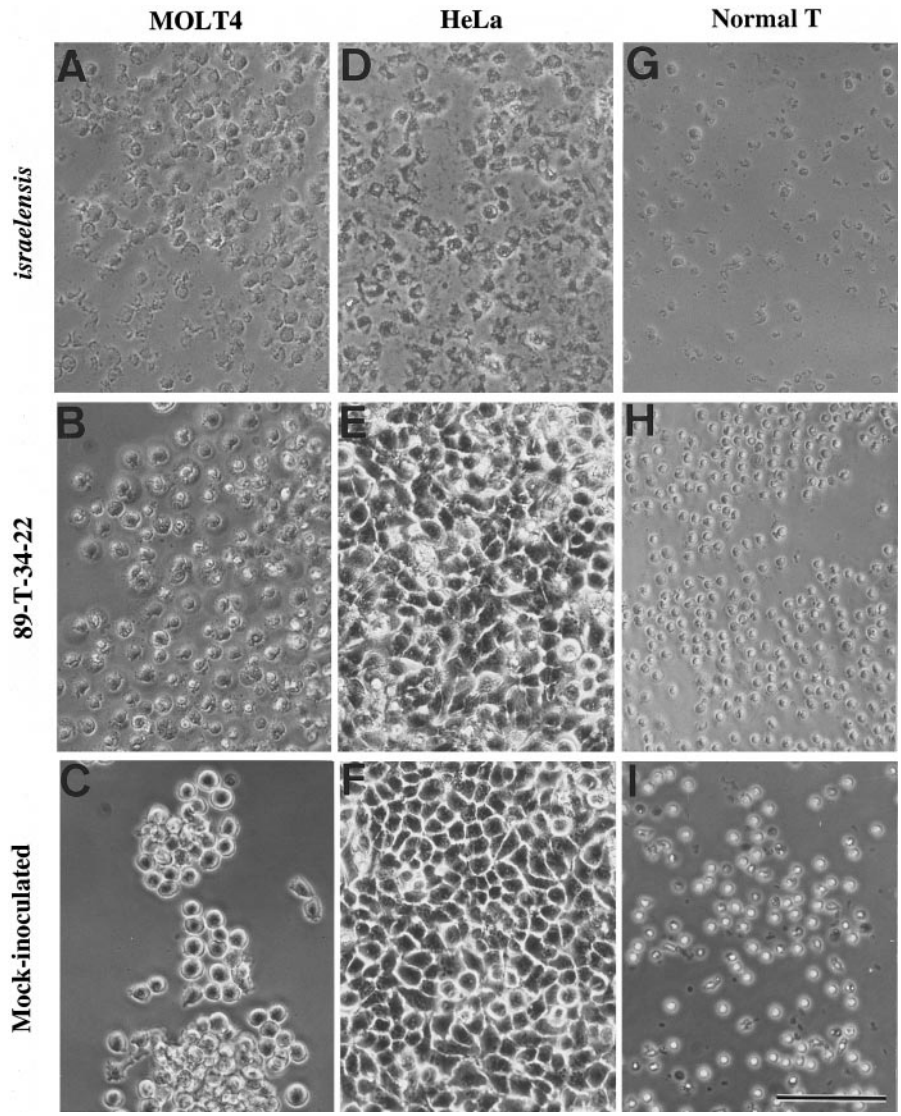

FIG. 4. Cytopathic effect of proteinase K-treated inclusion proteins on MOLT-4, HeLa, and normal T cells. Inclusion proteins: A, D, and G, *Bacillus thuringiensis* serovar *israelensis* (H14); B, E, and H, *B. thuringiensis* isolate 89-T-34-22; C, F, and I, mock-inoculated control. Phase-contrast microscopy at 24 h postinoculation. Bar, 80 μm.

34-22 both showed CPE on all of human cells tested. The proteins of 89-T-34-22 produced early and strong cytopathy in MOLT-4 within 1 h after administration, while slow CPE occurred in HeLa and normal T cells 16 h post-treatment. The cytopathological change, induced by the 89-T-34-22 protein, was characterized by condensation of the nucleus and cell-ballooning (Figs. 4B, 4E, and 4H). In contrast, the *israelensis* proteins induced in 1 h drastic cell-disruption without cell-ballooning (Figs. 4A, 4D, and 4G). No cytopathological change was detected in mock-inoculated cells (Figs. 4C, 4F, and 4I).

It has been well established that the Cyt1 protein of *israelensis* has a broad-spectrum cytolytic activity against invertebrate and vertebrate cells (20). Thus, *israelensis*-induced drastic cytopathy, observed in the present study, is attributable to the action of Cyt1 protein. It should be noted that the feature of cell-ballooning, caused by the proteins of 89-T-34-22, is very similar to that of the cytopathy induced by insecticidal Cry toxins in lepidopteran insect cells *in vivo* (21) and *in vitro* (22). Interestingly, our recent studies have also demonstrated that a marked cell-ballooning occurs commonly in human cancer cells, when treated with inclusion proteins from the three other non-insecticidal strains (8, 23, 24).

Dose-response study. Dose-responses of proteinase K-activated proteins of 89-T-34-22 and *israelensis* against human cells were monitored with MTT assay. As shown in Fig. 5, the cytotoxicities of proteins were dose dependent. The EC_{50} value of the 89-T-34-22 pro-

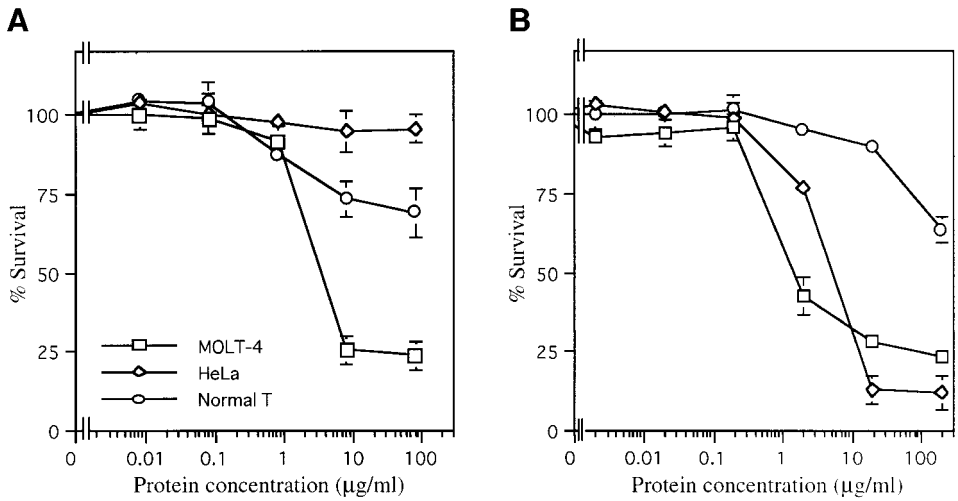

FIG. 5. Dose–response curves of cytocidal activity associated with proteinase K-treated inclusion proteins of (A) the *Bacillus thuringiensis* isolate 89-T-34-22 and (B) *B. thuringiensis* serovar *israelensis* on MOLT-4, HeLa and normal T cells.

teins was 3.5 μg/ml for MOLT-4, while >300 μg/ml for HeLa and normal T cells. The results clearly showed that MOLT-4 cells are >100 times more susceptible than HeLa cells and normal T cells. The EC_{50} values of the *israelensis* proteins were 3.1, 4.7, and >450 μg/ml for MOLT-4, HeLa and normal T cells, respectively.

One of the most striking aspects in our study is that the proteins from two strains, 89-T-34-22 and *israelensis*, had each own preference in killing human cells; in particular, the former exhibited a preferential cytotoxicity for leukemic T cells. Recently, we have reported the occurrence of parasporal proteins with cytocidal activity against human cancer cells in three non-insecticidal *B. thuringiensis* isolates of Japan (8, 24). Of particular interest is that an 81-kDa protein, designated the *parasporin*, from a *B. thuringiensis* soil isolate kills leukemic T cells and HeLa cells but not normal T cells (23). Our data, however, clearly show that the proteins of 89-T-34-22 are different in many aspects from those, including the *parasporin*, of the three other strains. Experiments are currently underway to identify the leukemic cell-killing protein(s) in the isolate 89-T-34-22 and the cell receptor, if any, for the toxin.

ACKNOWLEDGMENT

The authors thank Dr. T. Kawarabata, Kyushu University, for invaluable advice for the research.

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