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Characterization of Cancer Cell-Killing Activity Associated with Parasporal Proteins of Novel *Bacillus thuringiensis* Isolates

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Parasporal inclusion proteins of three novel *Bacillus thuringiensis* strains isolated in Japan showed strong and rapid cytocidal activities against human uterus cervix cancer cells (HeLa), while showing no cytotoxicity on non-cancer uterine smooth muscle cells (UtSMC). The proteins of the three strains exhibited no insecticidal activities against three lepidopterous and one dipterous insect species. Furthermore, they had no hemolytic activity against sheep erythrocytes. Ouchterlony immunodiffusion tests revealed that the proteins of these strains are immunologically closely related to the group parasporin-1, Cry31Aa. Our results strongly suggest that the parasporin-producing *B. thuringiensis* occurs widely in natural environments in Japan.

Parasporal inclusions of *Bacillus thuringiensis* often contain unique proteins with strong and specific toxicities to insects of several orders (7). Previous studies, however, have provided evidence that non-insecticidal *B. thuringiensis* strains are more widely distributed than insecticidal ones in natural environments (5). Mizuki *et al*. (3) first reported that parasporal proteins of certain non-insecticidal *B. thuringiensis* strains preferentially killed human cancer cells. This finding led to the creation of a new category of parasporal proteins, designated parasporin, defined as the bacterial parasporal protein capable of discriminatively killing cancer cells (4). Recent investigators have demonstrated the existence of at least four phylogenetically different parasporins (2). This study examines the parasporal proteins of three novel *B. thuringiensis* isolates and their cancer cell-killing activities.

The *B. thuringiensis* isolates used in this study were strains A1965, B0186, and B0195. They were recovered from natural environments in three locations in Fukuoka Prefecture, Kyushu, Japan. When observed with a phase-contrast microscope, the three isolates produced large spherical parasporal inclusions that are firmly attached to spores. Electron microscopic observations revealed that the parasporal inclusion occurs within the exosporium membrane. The present isolates were serologically untestable, since vegetative cells were not flagellated, lacking motility.

For *in vitro* cytocidal activity tests, parasporal inclusion proteins were solubilized and degraded by proteinase K according to the method previously described (3). The protease-treated proteins were examined for cytocidal activities against HeLa (human uterus cervix cancer cell) and UtSMC (normal human uterine smooth muscle cell) by the methods of Mizuki *et al*. (3, 4). Cytopathic effects of the proteins on HeLa and UtSMC cells were observed under phase-contrast microscopy for 24 h. The proteins from all three strains induce strong cytopathy against HeLa cells, but not against UtSMC. The cytopathy in HeLa cells was characterized by cell-ballooning, followed by cell-shrinking. Cell proliferation tests with a MTT assay also showed that the proteins are highly toxic to HeLa cells, but not active on UtSMC cells. When tested on sheep erythrocytes according to the method of Ohgushi et al. (6), no hemolytic activities were associated to the proteins of all three isolates.

We then examined the three isolates for larvicidal activities of the mixes of spores and parasporal inclusions against four insect species: *Bombyx mori, Spodoptera litua, Plutella xylostella,* and *Aedes aegypti.* The tests were done with one-dose assay technique as described previously (1). The results revealed that the parasporal inclusions of all three isolates were non-insecticidal.

Ouchterlony double immunodiffusion tests were done to

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examine the relationships between these three proteins and the four known parasporins (2). The proteins of the three isolates formed precipitin lines against parasporin-1 antibodies, but not against antisera to the three other parasporins.

Our findings provide ample evidence that the parasporal proteins from the three novel isolates of *B. thuringiensis* are related to parasporin. First, they have cytocidal activities preferential for cancer cells. This fits the definition of "parasporin" proposed by Mizuki et al. (4). Second, they are immunologically closely related to the parasporin-1. The present results also suggest that parasporin-producing organisms are widely distributed in natural environments of Japan. It is of interest to note that the parasporin-producing *B. thuringiensis* have recently been isolated from soils of Vietnam (8).

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