A unique morphological feature commonly associated with *Bacillus thuringiensis* serovar *yunnanensis* isolates: An electron microscopic observation

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Abstract

When examined with a phase-contrast microscope, all of the 15 *Bacillus thuringiensis* serovar *yunnanensis* (H20ab) strains, the reference type strain and the 14 Japanese isolates, produced large rhomboidal inclusions in spore-lacking cells but not in spore-forming cells. No envelopes were associated with inclusions. Inclusion matrix consisted of electron-dense homogeneous substances. The frequency of inclusion-forming cells was <1% of the cell population in each strain. Also, phase-dark spherical bodies, much smaller than rhomboidal inclusions, were often associated with cells in sporulating cultures. Electron microscopic studies with the four selected *yunnanensis* strains revealed that: (1) the majority of cells produced spores but not crystalline inclusions, (2) formation of crystalline inclusions was limited to the spore-lacking cells, and (3) electron-dense round bodies, much smaller than rhomboidal crystalline inclusions and putatively assigned to poly- β -hydroxybutyrate inclusions, were often observed in both spore-forming cells and inclusion-forming asporogenous cells. The overall results strongly suggest that the formation of crystalline inclusions in asporogenous cells is an H serotype-specific phenotype characteristic of the *B. thuringiensis* serovar *yunnanensis*.

Key words: *Bacillus thuringiensis* serovar *yunnanensis*; H-serotype 20ab; crystalline inclusion; inclusion-forming sporeless cell

INTRODUCTION

Bacillus thuringiensis is a Gram-positive spore-forming bacterium that is characterized by the formation of proteinaceous parasporal inclusions in sporangia. The parasporal inclusions often exhibit insecticidal and nematocidal activities (Glare and O'Callaghan, 2000). This makes *B. thuringiensis* an ecologically sound biological agent for control of agriculturally and medically important insect pests. Moreover, recent investigators have reported the occurrence of other biological activities in parasporal inclusion proteins of this organism (Akao et al., 1999; Mizuki et al., 1999b, 2000; Kondo et al., 2002).

Currently, 69 flagellar (H) antigenic serotypes are involved in the infraspecific classification scheme of *B. thuringiensis* (Lecadet et al., 1999). *B. thuringiensis* serovar *yunnanensis* (flagellar serotype H20) was established by Yu et al. (1984) on the basis of a Chinese strain recovered from a

larval cadaver of a noctuid lepidopteran, *Spodoptera litura* (Wang et al., 1979). The H-serotype 20 is now divided, by the presence of three H antigenic subfactors (20a, 20b, and 20c), into two subserotypes, H20ab (serovar *yunnanensis*) and H20ac (serovar *pondicheriensis*) (Lecadet et al., 1999).

The occurrence of *B. thuringiensis* belonging to the serovar *yunnanensis* in Japan was first reported by Ohba and Aizawa (1986a) with special reference to an interesting observation that the type strain and a Japanese isolate of this serovar produced huge crystalline inclusions only in asporogenous cells. Subsequent intensive efforts to screen *B. thuringiensis*, effective for microbial control of insect pests, from natural environments of Japan have occasionally provided the organisms belonging to the serovar *yunnanensis* (Ohba, 1996; Mizuki et al., 1999a; Ohba et al., 2000, 2002; M. Ohba, unpublished observations). This paper reports an unusual phenotypic character, the lack of formation of crystalline inclusions in sporogenous cells, com-

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Table 1. *Bacillus thuringiensis* serovar *yunnanensis* isolates used in this study

Isolate ^a	Locality	Source	Reference
Type strain	Yunnan, China	<i>Spodoptera litura</i> (cadaver)	Wang et al., 1979
116-72	Tochigi	Soil	Ohba and Aizawa, 1986a
91-KS-12-5 [†]	Kagoshima (Amami-oshima)	Soil	M. Ohba, unpubl.
91-KS-12-8 [†]	Kagoshima (Amami-oshima)	Soil	M. Ohba, unpubl.
93-Y-28-20	Yamaguchi	Soil	M. Ohba, unpubl.
94-F(M)6-30-5	Fukuoka	Phylloplane	Ohba, 1996
94-F(M)8-20-18	Fukuoka	Phylloplane	Ohba, 1996
97-OK-13-7 [‡]	Okinawa (Iriomote-jima)	Soil	Ohba et al., 2000
97-OK-13-13 [‡]	Okinawa (Iriomote-jima)	Soil	Ohba et al., 2000
97-OK-13-23 [‡]	Okinawa (Iriomote-jima)	Soil	Ohba et al., 2000
97-OK-240-13	Okinawa (Ishigaki-jima)	Soil	Ohba et al., 2000
01-KDT-118-10 [§]	Okinawa (Kitadaito-jima)	Soil	Ohba et al., 2002
01-KDT-118-15 [§]	Okinawa (Kitadaito-jima)	Soil	Ohba et al., 2002
01-KDT-120-1	Okinawa (Kitadaito-jima)	Soil	Ohba et al., 2002
B52	Fukuoka	Phylloplane	Mizuki et al., 1999a

^a Isolates with the same letter were from a single source.

monly retained in these Japanese *yunnanensis* isolates.

MATERIALS AND METHODS

Organisms and culture conditions. Fifteen *B. thuringiensis* strains used in the present study are listed in Table 1. Of these, 14 strains were derived from natural environments of Japan. The type strain of serovar *yunnanensis* was obtained from Dr. W. Wang, Kunming, China. The Japanese strains had been previously identified to the serovar *yunnanensis* (H20ab) by using H antisera specific for 20b and 20c subfactors, respectively.

B. thuringiensis strains were grown at 28°C on nutrient agar, pH 7.8, consisting of meat extract (10 g), polypeptone (10 g), NaCl (2 g), agar (20 g), and distilled water (1,000 ml).

Morphological observations. Three-day-old cultures of the 15 *yunnanensis* strains were examined for morphological features of the sporulating cells under a phase-contrast microscope. Four selected strains were further examined by the transmission electron microscopy (EM). For EM, three-day-old bacterial cultures were prefixed by 1% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.2, at 25°C for 1 h, and postfixed by 1% OsO₄ in PBS at 25°C for 1 h. The fixed samples were dehydrated in an ethanol-propylene oxide series and embedded in Epon 812 resin mixture. Ultrathin sections were made on a Reichert Ultracut micro-

tome, stained with uranyl acetate and lead citrate. Sections were examined with a Hitachi 7200 electron microscope.

RESULTS

When observed with a phase-contrast microscope, there was no significant difference between the type strain of *yunnanensis* and 14 Japanese isolates in morphological features of vegetative cells, sporangia, and spores. All 15 strains formed huge crystalline inclusions in spore-lacking cells but not in spore-producing cells. Inclusion-forming cells accounted for <1% of cell populations in each strain. Figures 1–4 show phase-contrast photographs of the four selected strains: the type strain of *yunnanensis* and the three Japanese strains, B52, 97-OK-13-7, and 01-KDT-118-10. Inclusions were roughly rhomboidal in shape with sizes (3–7 µm long) much greater than the spores that were formed in inclusion-lacking cells. Also, much smaller phase-dark spherical bodies were evident in cells.

Figures 5–12 show electron micrographs of the cells in sporulating cultures of the four selected *yunnanensis* strains. Sporulating cultures of these strains consisted of two cell types in terms of the formation of spores and crystalline inclusions. In each strain, the majority of cells produced spores but not crystalline inclusions. Formation of crystalline inclusions was strictly limited to the spore-

Figs. 1–4. Phase contrast photographs of sporulating cultures of *Bacillus thuringiensis* serovar *yunnanensis* (H20ab) strains. Arrowheads indicate rhomboidal crystalline inclusions formed in spore-lacking cells. Bar=10 μ m. (1) The type strain of serovar *yunnanensis*. (2) The soil isolate 01-KDT-118-10. (3) The soil isolate 97-OK-13-7. (4) The phylloplane isolate B52.

lacking cells, accounting for <1% of the cell population. Usually, multiple inclusions were formed in single asporogenous cells: in most cells, the largest inclusion was roughly rhomboidal in outline, and the smaller crystalline inclusions were varied not only in size but also in shape. Occasionally, narrow and considerably elongated inclusions coexisted with rhomboidal ones (Fig. 12). The inclusions were not enveloped, consisting of an electron-dense homogeneous matrix. As shown in Fig. 6, the smaller inclusions were occasionally embedded into the larger ones.

The four selected *yunnanensis* strains also

produced various-sized round bodies (0.1–0.5 μ m in diameter) different from the above rhomboidal crystalline inclusions (Figs. 5–12). Occasionally, single cells contained multiple round bodies. These bodies were much more smaller than the crystalline inclusions and spores, consisting of highly electron-dense substances. They were observed not only in spore-containing cells but in rhomboidal inclusion-producing asporogenous cells.

DISCUSSION

It has been well established that the ordinary *B.*

Figs. 5–12. Electron micrographs of acrySTALLIFEROUS sporangia and crystalline inclusion-forming asporogenous cells in *Bacillus thuringiensis* serovar *yunnanensis* (H20ab) strains. (C) Crystalline inclusions. Arrowheads indicate putative poly- β -hydroxybutyrate bodies. Bar=1 μ m. (5, 6) The type strain of serovar *yunnanensis*. (7, 8) The soil isolate 97-OK-13-7. (9, 10) The soil isolate 01-KDT-118-10. (11, 12) The phylloplane isolate B52.

thuringiensis strains produce proteinaceous crystalline parasporal inclusions in spore-forming cells. However, this is not the case with the type strain of *B. thuringiensis* serovar *yunnanensis* (H20ab) whose crystalline inclusions are formed in asporogenous cells but not in spore-forming cells (Ohba and Aizawa, 1986a; Srinivas et al., 1997). It appeared from the present results that, like the type strain, all 14 *yunnanensis* strains derived from natural environments in Japan produce large crystalline inclusions in spore-lacking cells but not in sporogenous cells.

Electron microscopic observations revealed that the inclusion matrix of the four selected strains (the type strain and the three Japanese isolates) consists of homogeneous electron-dense substances. Recently, Balasubramanian et al. (2002) reported that the *cry32Aa* gene is responsible for the production of crystal proteins in the type strain of *yunnanensis*. Thus, it is very likely that the electron-dense inclusion matrix observed here in the type strain is composed of the Cry32Aa protein.

Interestingly, Balasubramanian et al. (2002) reported that weak larvicidal activity against the diamondback moth, *Plutella xylostella*, was associated with the Cry32Aa protein expressed in *Escherichia coli* transformants. Our previous studies, however, have demonstrated that the mixtures of spores and inclusions of the *yunnanensis* strains, including the type strain, induced no significant mortality in larvae of the diamondback moth, even at high bacterial concentrations (Mizuki et al., 1999a; Higuchi et al., 2000; Ohba, unpublished observations). It is likely that low-levels of inclusion-forming cells (<1% of the cell populations), coupled with the low toxicity of inclusion proteins, may lead to a failure to detect insecticidal activity of the spore/inclusion mixtures. Previous investigators have also reported that sporulated cultures of the existing *yunnanensis* strains gave no significant mortalities to other lepidopterans and dipterans tested (Wang et al., 1979; Ohba, 1996; Saitoh et al., 1996, 1998; Mizuki et al., 1999a; Higuchi et al., 2000; Ohba et al., 2000, 2002) and a coleopteran (Tsuchiya et al., 2002).

It is noteworthy that the *yunnanensis* strains tested also produced small round bodies in both spore-forming and asporogenous cells. Apparently, these bodies were different in many aspects from crystalline inclusions. Nickerson et al. (1981) made

an interesting observation that the *B. thuringiensis* strain HD-1 (H3abc, serovar *kurstaki*) produced not only proteinaceous crystalline inclusions but also smaller spherical parasporal bodies (microscopically, phase-dark granules), consisting of poly- β -hydroxybutyrate (PHB). This leads to a hypothesis that the small round bodies present in the *yunnanensis* cells are composed of PHB. Chemical composition of these round bodies awaits clarification.

Our findings strongly suggest that the formation of crystalline inclusions in asporogenous cells is a prominent phenotypic character specifically associated with the H-serotype 20ab of *B. thuringiensis*. Our future works will include: (1) identification of *cry* genes in Japanese isolates of *yunnanensis*, (2) determination of whether insect toxicity and/or other biological activities are associated with the *yunnanensis* Cry proteins, and (3) comparative characterization of the crystalliferous *yunnanensis* strains and acrySTALLIFEROUS *Bacillus cereus* isolates possessing the flagellar antigen H20ab (Ohba and Aizawa, 1986b; Shisa et al., 2002).

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