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Formation of Crystals of the Insecticidal Proteins of *Bacillus thuringiensis* subsp. *aizawai* IPL7 in *Escherichia coli*

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***Escherichia coli* JM103 cells harboring expression plasmid pTB1 or pKC6 synthesized the 130- and 135-kilodalton insecticidal proteins, respectively, of *Bacillus thuringiensis* subsp. *aizawai* IPL7, and both products accumulated as cytoplasmic inclusion bodies. Amorphous inclusions which contained contaminating proteins, together with the corresponding insecticidal proteins, were formed in cultures at 37°C, but bipyramidal crystals practically free of contaminants were observed at 30°C. Although 9.8% of the amino acids were substituted between these two proteins, both protein crystals had the same shape as those of the parental *B. thuringiensis* strain, which produced both proteins.**

Bacillus thuringiensis produces proteinaceous crystals toxic to insect larvae (5, 7, 10). Depending on the subspecies, the crystals are specifically toxic to lepidopteran (8, 9), dipteran (12, 16, 17), or coleopterous (2, 4, 6) larvae. *B. thuringiensis* subsp. *aizawai* IPL7 exhibits insecticidal activity toward lepidopteran larvae and synthesizes mainly 130- and 135-kilodalton (kDa) insecticidal proteins (IPs) which form bipyramidal crystals. The two IP-encoding genes were cloned, and the primary structures were deduced from their nucleotide sequences (14, 15). Comparison of the amino acid sequences of the two IPs revealed 90.2% homology and the presence of highly variable regions as well as conserved ones. The 130- and 135-kDa IP genes were also expressed in *Escherichia coli*. *E. coli* JM103 cells harboring expression plasmid pTB1 or pKC6 constantly synthesized the 130- or 135-kDa IP, respectively, under control of the *tac* promoter and the *rrnB* terminator throughout growth to the stationary phase. The products amounted to more than 30% of the total cellular protein (14, 15).

Formation of amorphous and crystallike bodies of 130- and 135-kDa IPs in *E. coli*. *E. coli* JM103(pTB1) cells grown at 37°C in L broth medium (13) were examined for inclusion bodies by phase-contrast microscopy (Fig. 1A). Distinct inclusion bodies were first observed after 8 h of incubation. These continued to grow until the late exponential growth phase and finally reached a length exceeding 0.5 μm (Fig. 1A). At the stationary growth phase, over 95% of the *E. coli* cells contained the inclusion bodies. No phase-refractile inclusions were found in the negative control cells of *E. coli* JM103(pKK223-4) (data not shown). The inclusion bodies were easily recovered from the cells by a combination of freeze-thawing, sonication, and centrifugation (4,500 × *g*, 5 min). Under microscopic observation, the size and shape of the inclusion bodies were not uniform (Fig. 1B). When *E. coli* JM103(pTB1) cells were cultured at 30°C, crystallike inclusion bodies were formed (Fig. 1C) and most were of much the same size (Fig. 1D). *E. coli* JM103(pKC6) cells synthesizing the 135-kDa IP also formed amorphous inclusion bodies at 37°C and crystallike bodies at 30°C. The size of the amorphous bodies was usually larger than that of the crystallike ones with both *E. coli* strains (Fig. 1B and D). The crystals were also formed when IP-specific transcription was accelerated by addition of isopropyl-β-D-thiogalactopyranoside at 30°C but were not formed when IP gene expression was promoted by the weak *lac* promoter. Thus, it is likely that a higher rate of IP biosynthesis is needed for crystal formation in *E. coli*. As previously reported (1, 3). *B. thuringiensis* initiated high-level synthesis of the IPs at the sporulating stage and accomplished crystal formation within 7 to 8 h.

Analysis of amorphous and crystallike bodies formed in *E. coli* by SDS-PAGE. After *E. coli* JM103(pTB1) cells were cultured at 37 and 30°C, both amorphous and crystallike bodies were separately prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 11). The main protein band in the amorphous bodies was the 130-kDa IP, although other protein bands with lower molecular masses were found (Fig. 2A). In Western blot (immunoblot) experiments, degraded IP bands were not observed with the cell extracts of *E. coli* JM103(pTB1) (14). Several washes of the inclusion bodies with 0.5 M NaCl gave similar results, indicating that the lower-molecular-mass proteins were involved in the inclusion bodies. ADC-18 densitometer (Gelman Sciences, Inc.) scanning of the gel indicated that the purity of the 130-kDa IP was about 70%. The crystallike bodies contained lesser amounts of other small proteins, and the purity of the 130-kDa IP was nearly 90%. Similarly, the 135-kDa IP preparation from *E. coli* JM103(pKC6) cells grown at 30°C contained very small amounts of other small proteins (Fig. 2B). Therefore, it is likely that the amorphous bodies incorporated small proteins together with the corresponding IP, whereas the crystallike bodies consisted mostly of IP. Thus, formation of amorphous bodies in *E. coli* cells grown at 37°C appears to be due to incorporation of small proteins. IP newly synthesized at 37°C may not be accurately folded because of altered protein structures. In addition, correct IP-to-IP association may be disturbed by contaminating proteins. Actually, *B. thuringiensis* subsp. *aizawai* IPL7 formed typical bipyramidal crys-

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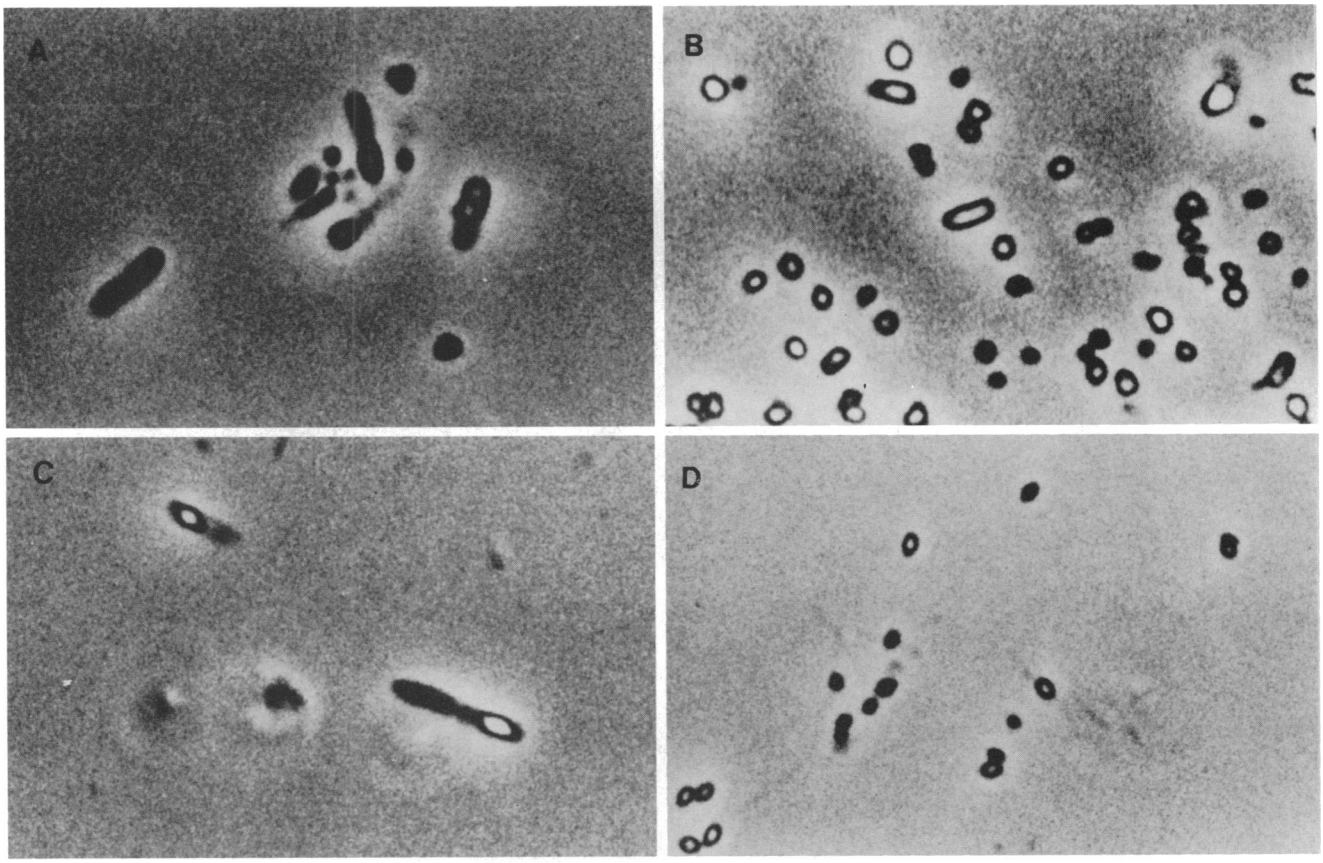

FIG. 1. Microscopic observation of *E. coli* JM103(pTB1) cells synthesizing the 130-kDa IP. *E. coli* JM103(pTB1) cells were cultured at 37 and 30°C to the stationary phase in L broth medium containing 50 μg of ampicillin per ml and examined by phase-contrast microscopy at a magnification of ×1, 350. Inclusion bodies were also examined by phase-contrast microscopy. (A) *E. coli* JM103(pTB1) cells cultured at 37°C. (B) Inclusion bodies from *E. coli* JM103(pTB1) cells grown at 37°C. (C) *E. coli* JM103(pTB1) cells grown at 30°C. (D) Inclusion bodies from *E. coli* JM103(pTB1) cells grown at 30°C.

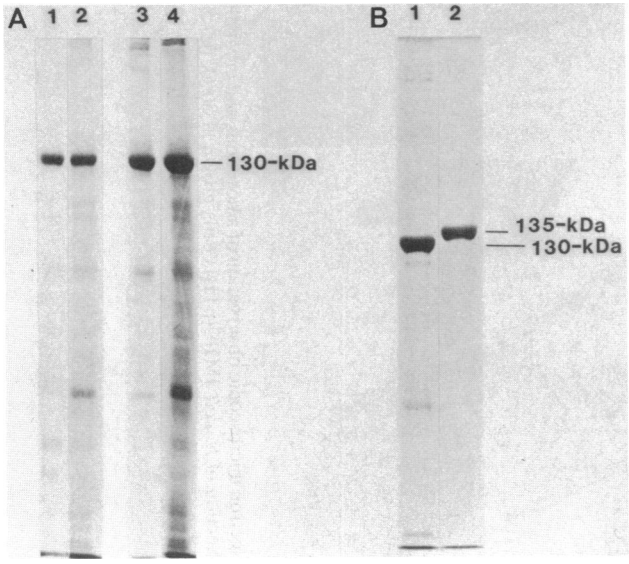

FIG. 2. Analysis of amorphous and crystal inclusion bodies by SDS-PAGE. (A) Inclusion bodies were prepared as described in the legend to Fig. 1 and analyzed by 7.5% SDS-PAGE. Lanes: 1 and 3, about 3 and 10 μg, respectively, of crystallike bodies of the 130-kDa IP from *E. coli* JM103(pTB1) cells grown at 30°C; 2 and 4, about 3 and 10 μg of inclusion bodies of the 130-kDa IP from *E. coli* JM103(pTB1) cells grown at 37°C. (B) The 130- and 135-kDa IPs were similarly purified from *E. coli* JM103(pTB1) and JM103(pKC6) cells, respectively, grown at 30°C as described in the legend to Fig. 1. Lanes: 1, 130-kDa IP; 2, 135-kDa IP. About 5 μg of each IP was analyzed on 7.5% SDS-PAGE.

tals when cultured at 30°C but produced irregular bodies at 37°C (unpublished data).

Electron microscopic observation of amorphous and crystallike bodies formed in *E. coli* cells. The shape and structure of the inclusion bodies formed in *E. coli* JM103(pTB1) and JM103(pKC6) cells were examined by JEOL-1200 electron microscopy (Japan Electron Optics Co., Ltd.). When *E. coli* JM103(pTB1) cells were cultured at 37°C, the inclusion bodies showed dark, amorphous regions (Fig. 3A). Some of the *E. coli* cells were filled with the synthesized protein, as found in the cross-section. When cultured at 30°C, *E. coli* JM103(pTB1) cells formed typical bipyramidal crystals (Fig. 3B). The crystals were approximately 0.83 μm long and 0.27 μm wide. Since the bipyramidal crystals of *B. thuringiensis* subsp. *aizawai* IPL7 were about 1.1 μm long and 0.48 μm wide, they were larger than those formed in *E. coli* JM103(pTB1) cells. The average lengths of the *E. coli* and *B. thuringiensis* cells were about 1.20 and 2.10 μm, respec-

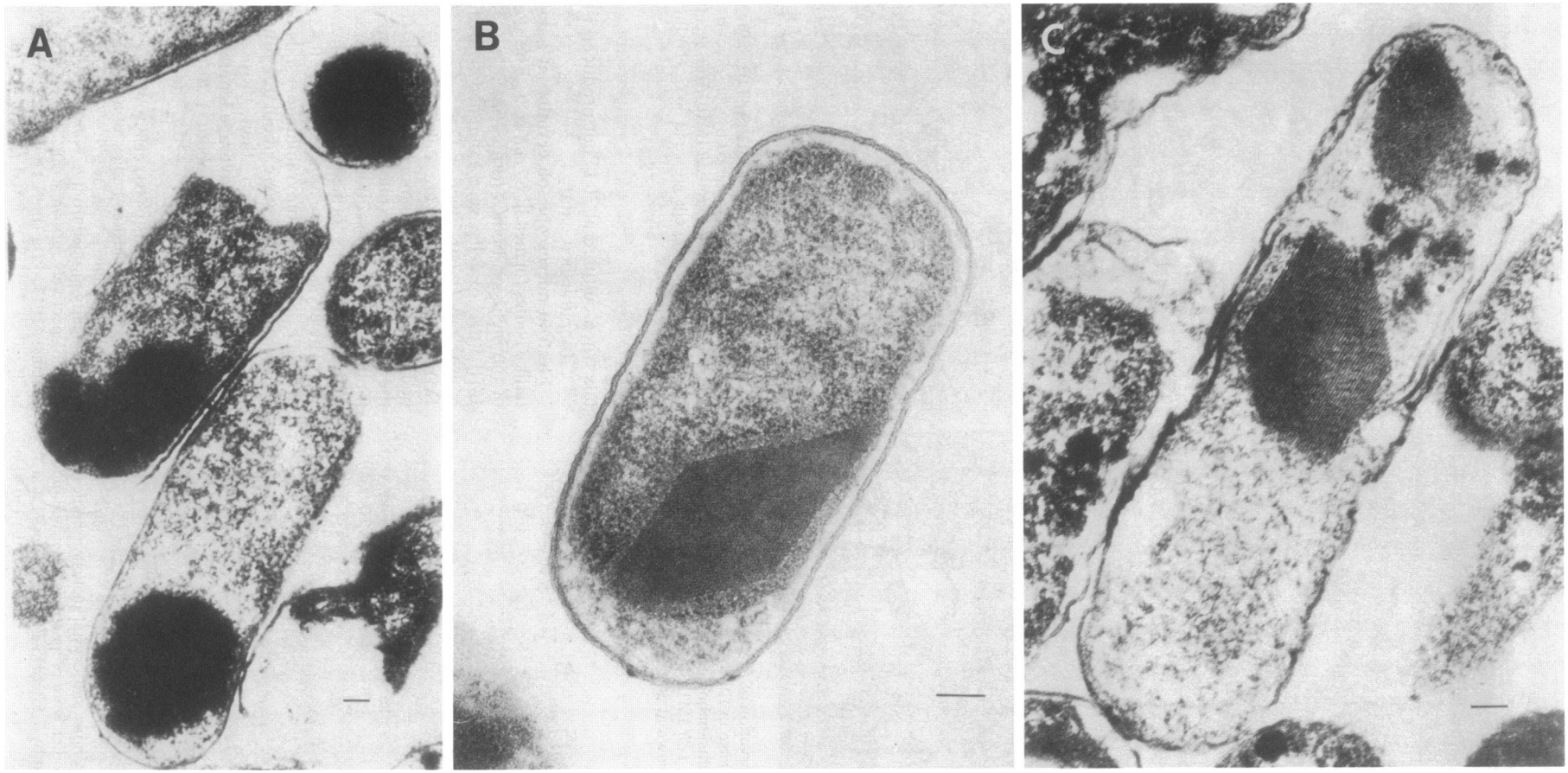

FIG. 3. Electron microscopic observation of amorphous and crystallike inclusion bodies of the 130-kDa IP formed in *E. coli* JM103(pTB1) cells. (A) Section of *E. coli* JM103(pTB1) cells grown at 37°C. (B) Section of *E. coli* JM103(pTB1) cells grown at 30°C. (C) Section of *E. coli* JM103(pKC6) cells grown at 30°C. Bars, 100 nm.

tively. Thus, it is likely that the maximum size of the crystals formed in the cells depends in part on the size of the host cells. When *E. coli* JM103(pKC6) cells were grown at 30°C, bipyramidal crystals were also formed (Fig. 3C). Thus, the 130- and 135-kDa IPs, with 90.2% homology in their amino acid sequences, formed the same bipyramidal crystals in *E. coli* cells as in *B. thuringiensis* subsp. *aizawai* IPL7, which synthesized equal amounts of the 130- and 135-kDa IPs and formed bipyramidal crystals.

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