Morphological Alterations in *Bacillus megaterium* as Produced by Aflatoxin B₁

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Morphologically abnormal cells were produced by *Bacillus megaterium* NRRL B-1368 in response to aflatoxin B₁. Filamentous forms were characterized by early granulation and unusually large and numerous deposits of poly-β-hydroxybutyric acid within the cells. Pantoyl lactone was without effect as a reversing agent for the observed inhibition of cell septum formation. *B. megaterium* cells and spores produced on toxic (3.8 µg of aflatoxin B₁ per ml) and nontoxic Trypticase Soy Broth and Trypticase Soy Agar (TSA) were observed by using phase contrast and electron microscopy. Transfer of aberrant forms to nontoxic TSA yielded macrocolonies with daughter cells morphologically indistinguishable from untreated cells. Agar slide cultures of filamentous cells transferred to nontoxic TSA indicated that normal cells were formed. Electron photomicrographs showed a decreased number of mesosomes in filamentous cells as compared to control cells. There were no observable morphological differences in spores formed on toxic or nontoxic TSA.

The inhibition of cell division in bacteria by aflatoxin B₁ with the resultant development of aberrant forms has been reported (7, 8). The possibility of Chang liver cell enlargement has been inferred from studies in which a decrease in cell division was found to occur with a concomitant increase in the protein, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) contents per cell (4). Since inhibition of septum formation in bacteria by aflatoxin B₁ is obvious upon examination by phase contrast microscopy, electron microscopy was chosen to determine whether unusual organelles were formed in aflatoxin-treated cells and spores or, conversely, to determine if the numbers and kinds of cellular components were similar to those in control cells and spores. The ability of aberrant forms to revert to normal growth and division when placed on a nontoxic medium was also examined as well as the effect of pantoyl lactone on cell division. Pantoyl lactone has been used as a reversing agent in septum formation inhibition studies (5).

The results of these studies on the toxic effects of aflatoxin upon cellular structure coupled with studies on its toxic effects on cellular chemical composition could provide information as to the site of action of aflatoxin upon susceptible cells.

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MATERIALS AND METHODS

Cells and spores of *Bacillus megaterium* were produced and harvested as previously described (1). Pantoyl lactone, a septum-inducing agent, was added to *B. megaterium* cultures growing in Trypticase Soy Broth (TSB) containing 3.8 µg of aflatoxin B₁ per ml (toxic) to determine its effects on cell septum formation. The chemical was added to the medium at a concentration of 0.075 M initially or after 1.5 hr of growth. Fifty milliliters of growth medium was added to 250-ml Erlenmeyer flasks and incubated at 30°C in a model G25 shaker-incubator (New Brunswick Scientific Co., New Brunswick, N.J.) at 150 rev/min. Absorbance at 660 nm and viable counts using Trypticase Soy Agar (TSA) were made throughout the growth cycles. Results of these growth studies indicated that pantoyl lactone had neither an inhibitory nor stimulatory effect on the rates of growth or division in control and treated cultures. The apparent lack of effect may be due to the inability to distinguish formation of septa by turbidity and viable counts. Observations by phase contrast microscopy, however, did not reveal usual septum formation.

Division and morphological development of *B. megaterium* cells grown in either toxic or nontoxic TSB were observed with either a microscope (American Optical Co., Buffalo, N.Y.) or a Leitz Ortholux microscope, both of which were fitted with photomicrographic equipment. Photographs were taken at several magnifications and times during the growth cycle.

The ability of elongated vegetative cells formed in toxic TSB to revert to normal cellular division and morphology when placed on nontoxic TSA was
studied with phase contrast microscopy. Observations of cell division in one or more stationary *B. megaterium* cells were made on agar slides. A layer of liquified TSA (4% agar) was placed near the center of a standard 3 by 1 inch glass slide and allowed to harden and dry for about 2 hr. The edges of the agar layer were trimmed to about 22-mm square and a loop of the vegetative cells was placed on the medium; a cover slip was placed on top of this and sealed with warm paraffin to prevent further drying. Agar temperature was maintained at 30 ± 1 °C with a 250-watt infrared brooder lamp and was measured potentiometrically. Photographs were made throughout cell division.

Aberrant filamentous cells produced by *B. megaterium* in toxic TSB were also surface plated on nontoxic TSA and incubated at 32 °C. Colony formation was visually examined and morphology of daughter cells from different areas in the colony were examined by phase contrast microscopy to determine the permanence of aflatoxin-induced phenotypic changes.

The results of phase contrast examination of filamentous cells formed in the presence of aflatoxin B₁ and normal *B. megaterium* cells are shown in Fig. 1. Filaments often reached 50 μm and intertwined to such an extent that they made direct microscopic counts extremely difficult.

![Fig. 1. Normal and aberrant forms of Bacillus megaterium produced during growth in Trypticase Soy Broth and Trypticase Soy Broth containing 3.8 μg of aflatoxin B₁ per ml, respectively. (A) Control at 3.5 hr of incubation, (B) 3.8 μg of B₁ per ml at 3.5 hr of incubation, (C) control at 5.5 hr of incubation, (D) 3.8 μg of B₁ per ml at 5.5 hr. Micrographs taken through a phase microscope. × 500.](image-url)
induced mutation was unlikely due to the reversion of filamentous cells formed in toxic TSB to normal morphology when transferred to nontoxic medium (Fig. 2). Visual examination of colonies of daughter cells formed from filamentous cells streaked on TSA indicated no morphological differences from cells never exposed to aflatoxin. Transfer of filamentous cells to nontoxic TSB resulted in growth patterns identical to the control cells. Thus, there also did not seem to be permanent damage in the filamentous cells formed in toxic TSB.

Reports of the use of electron microscopy to detect possible changes in the morphology of bacterial cells and spores formed in aflatoxin-containing media have not appeared. Gross examination of cells immediately reveals the inhibitory nature of aflatoxin on vegetative cell division. However, with phase contrast microscopy no detectable differences were found in spores formed in the presence or absence of aflatoxin. Cells and spores were next examined by using electron microscopy. Organelles of specific interest were transverse septa and mesosomes in vegetative cells and mesosomes in sporangia.

Cell types were fixed for electron microscopy by adding sufficient cell or spore suspension to screw-cap test tubes (13 by 100 mm) to yield pellets about 0.5 mm thick upon centrifugation. Both cell and spore pellets were treated identically. The pellets

Fig. 2. Time-lapse phase contrast micrographs of dividing filamentous Bacillus megaterium cells which were formed in Trypticase Soy Broth containing 3.8 μg of aflatoxin B<sub>1</sub> per ml and then transferred to nontoxic Trypticase Soy Agar. Note the formation of normal-sized daughter cells. (A) Zero hr of incubation at 30 C, (B) 1 hr, (C) 2 hr, and (D) 8 hr. Micrographs taken through a phase microscope. ×1,950.
FIG. 3. Ultrathin sections of Bacillus megaterium cells formed in Trypticase Soy Broth. Cells are in different stages of cell division. Abbreviations: CW, cell wall; CM, cell membrane; PHB, poly-β-hydroxybutyric acid; S, septum; M, mesosome; and NM, nuclear material. The marker represents 1.0 μm.
Fig. 4. Ultrathin sections of Bacillus megaterium cells formed in Trypticase Soy Broth containing 3.8 μg of aflatoxin B₁ per ml. Abbreviations: S, septum; M, mesosome; PHB, poly-β-hydroxybutyric acid; CW, cell wall; CM, cell membrane. Markers represent 1.0 μm.
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**Fig. 5.** Ultrathin sections of *Bacillus megaterium* spores formed in Trypticase Soy Agar. Abbreviations: *P*, protoplast; *PCW*, primordial cell wall; *CX*, cortex; *ISC*, inner spore coat; *OSC*, outer spore coat. Markers represent 1.0 μm.
were suspended in a 50% aqueous solution of glutaraldehyde diluted to 6.25% with Sorensen's phosphate buffer, pH 7.2, and kept at room temperature for 2 hr. The samples were then washed three times in 1 hr with Sorensen's buffer and suspended in a 2% aqueous solution of osmium tetroxide diluted to 1% with Sorensen's buffer. The samples were dehydrated after 1.5 hr at room temperature for 10 min each in 25, 50, 75, and 95% ethanol and twice for 15 min each in 100% ethanol. The samples were then embedded by using the method of Luft (9) by suspending them in the complete epoxy-resin mixture (Epon 812, D. LECHOWICH APPL. MICROBIOL.

Fig. 6. Ultrathin sections of Bacillus megaterium spores formed in Trypticase Soy Agar containing 3.8 μg of aflatoxin B₁ per ml. Abbreviations: P, protoplast; PCW, primordial cell wall; CX, cortex; ISC, inner spore coat; and OSC, outer spore coat. Markers represent 1.0 μm.
Ladd Research Industries, Inc., Burlington, Vt.) and kept overnight in a desiccator. Approximately 2 drops of the sample were placed on top of fresh Epon complete mixture in gelatin capsules, the desiccator was evacuated, and the samples were permitted to settle to the bottom of the capsule overnight. Hardening was accomplished by heating the samples for 12 hr at 35°C, 12 hr at 45°C, and 12 hr at 65°C. The gelatin was removed from the hardened Epon upon cooling, and ultrathin sections were made on an LKB Ultratom microtome and examined with an EM-100 electron microscope (Philips Electronics & Pharmaceutical Industries Corp., St. Joseph, Mo.)

Mesosomes are intracytoplasmic membranes contiguous to the cytoplasmic membrane and contain enzymes involved in terminal electron transport and may function as control points in DNA replication. Other functions attributed to mesosomes include involvement in the formation of spore septa and transverse septum formation during cell division (6).

Figures 3 and 4 show electron micrographs of vegetative cells grown in nontoxic and toxic TSB, respectively. Structural components are pointed out. Two normally dividing cells are shown in Fig. 3, one in the early stages of centripetal deposition of a new septum and the second in a later stage. Filamentous cells are shown in Fig. 4. Poly-β-hydroxybutyric acid deposits are more abundant, but cell septa are apparently lacking in the longer cell (Fig. 4). Mesosomes are located near the areas of septum formation in control cells but were not seen as distinct or abundantly in the filamentous cells.

No significant differences were observed in the structures of control and test spores nor was the spore structure different than that reported by Freer and Levinson (3) for B. megaterium. Photographs showing the structural components of control and test spores are shown in Fig. 5 and 6, respectively. Both control and test spores exhibited light and dark protoplasts as shown in the bottom portions of the figures.

RESULTS AND DISCUSSION

The binding of aflatoxin to DNA has been documented (2, 10). It is possible that aflatoxin B₁ is binding to the particular locus on the DNA molecule responsible, in part, for the initiation of mesosomal development, or mesosomal control of cross plate or septum formation, or both. Inhibition of mesosomal function in this manner might still leave the cell capable of synthesizing other cellular components and thus to form filaments.

As for the apparent lack of effect of aflatoxin on spore structure, it is theorized that the toxin concentration decreased in the medium in the immediate area in which growth occurred to a level which eventually permitted delayed and reduced spore formation (1). Aflatoxin analysis of the total sporulation medium after sporulation had occurred indicated that 3.2 μg/g of aflatoxin B₁ were present (only a 16% loss).

Thus, chemical and heat resistance (1) as well as electron microscopy studies reveal no differences between control and test spores, but a definite inhibition of sporulation of B. megaterium was observed in the toxin-containing medium.

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