Biochemical Alterations in *Bacillus megaterium* as Produced by Aflatoxin B_1[1]

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Bacillus megaterium NRRL B-1368 cells and spores were produced on Trypticase Soy Broth (TSB) and Agar (TSA) containing 3.8 µg of aflatoxin B_1 per ml, analyzed for selected chemical constituents, and compared to cells and spores of *B. megaterium* produced on nontoxic Trypticase Soy Media. There was an initial 30% kill of cells after inoculation into toxic TSB and during the first 3.5 hr of incubation followed by a logarithmic growth phase in which the generation time was 75 min as compared to 20 min for the control culture. Chemical analyses revealed an increase in protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) on both a per cell basis and a per cent dry weight basis when *B. megaterium* was grown in toxic TSB. There was a concurrent decrease in the total amounts of cellular protein, DNA, and RNA synthesized in toxic TSB. Amino acid analyses of control and test cell walls showed little, if any, difference in cell wall composition. About 97% sporulation of *B. megaterium* occurred after 3 days on nontoxic TSA although 6 days were required to attain 65% sporulation on toxic TSA. Germination of spores was not inhibited by 4.0 µg of aflatoxin per ml but outgrowth was. No significant differences were observed in the heat resistance, protein, DNA, RNA, or dipicolinic acid content of spores formed on toxic TSA and nontoxic TSA.

Responses of higher animals to the carcinogen, aflatoxin, have been studied to a moderate extent, but less is known concerning the effects of the toxin on bacterial metabolism. Aflatoxin uptake by *flavobacterium aurantiacum* and resulting toxic effects were reported (5, 7). The toxin inhibited deoxyribonucleic acid (DNA) synthesis and, to a lesser extent, ribonucleic acid (RNA) and protein synthesis. Binding capacity studies indicated that aflatoxin has an affinity for bacterial DNA (4, 6) and inhibits DNA polymerase activity (11). There are no reports on the chemical analyses of the aberrant forms of cell walls of *Bacillus megaterium* NRRL B-1368, previously demonstrated to be aflatoxin sensitive (1), produced in the presence of aflatoxin. Analysis of the chemical composition of bacterial spores formed by cells in media containing sublethal aflatoxin concentration has also not been presented. Thus, selected chemical constituents of vegetative cells and spores formed in aflatoxin-containing and nontoxic media were examined. Also presented and discussed are measurements of rates of cell growth and cell division in toxic

and nontoxic media. It was hoped that these data would aid in understanding the toxic effects of aflatoxin upon susceptible cellular species.

MATERIALS AND METHODS

Growth media. *B. megaterium* NRRL B-1368 was cultured on Trypticase Soy Broth (TSB). Five-hundred milliliters of TSB was dispensed into 1,000-ml Erlenmeyer flasks, autoclaved at 121 C for 15 min, and used for the production of control vegetative cells analyzed during the research project. Fifty milliliters of the TSB medium, containing 2% agar (Trypticase Soy Agar TSA), was added to 1,000-ml pharmaceutical bottles, and the agar was allowed to solidify along the flat side surface in order to produce control spore crops by using a surface growth technique.

Besides using the "nontoxic" medium described above to culture *B. megaterium*, the same medium containing 3.8 µg/ml of aflatoxin B_1 (crystalline, dried in situ from chloroform, grade B, lot 940032, Calbiochem, Los Angeles, Calif.), designated as toxic medium, was also used to culture the organism for treated vegetative cell or spore analyses.

Production and harvesting cells and spores. A stock suspension of *B. megaterium* spores was heat shocked for 8 min at 80 C and inoculated into TSB. Transfers were made at 8, 6, and 4 hr into 1,000-ml Erlenmeyer flasks containing 500 ml of toxic or nontoxic media, and incubation was at 32 C in a model G25 incu-

bator gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.). at 150 rev/min. Vegetative cells in the late logarithmic growth phase were harvested by centrifugation at 9,000 $\times$ g. Cells were then washed seven times with 0.1 M phosphate buffer (pH 7.1) and stored at -20 C until they were analyzed for chemical constituents.

Two milliliters of a 2-hr culture of $B.$ $megaterium$ was used to inoculate the surface of toxic or nontoxic TSA and incubated at 30 C for the production of spores. Spores were removed from the nontoxic medium after 3 days and the toxic medium after 6 days with cold 0.1 M phosphate buffer (pH 7.1) and transferred to centrifuge bottles. Spores were washed twice with phosphate buffer, treated 1 hr at 37 C with 0.075% lysozyme, and again washed five times with phosphate buffer. Spore crops were stored at 4 C until chemical, germination, and heat resistance studies were performed.

Direct counts, viable counts, and absorbancy changes. Direct microscopic counts were made on the nontoxic cultures at different times in the growth cycle by using a Petroff-Hausser counting chamber.

Viable counts were made at selected times during the growth cycle by making appropriate dilutions in sterile 0.2% peptone and plating in triplicate on TSA. Incubation was at 30 C, and counts were made 48 to 72 hr after pouring.

Cellular growth as measured turbidimetrically was also correlated with viable counts of $B.$ $megaterium$ cultured on toxic and nontoxic TSA. Absorbancy of the culture at 660 nm was plotted versus time using a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Chemical analyses. Samples of washed vegetative cell suspensions were sonically treated (Heat Systems, Ultrasonics, Inc., Plainview, N.Y.) for 7 min (filamentous cells formed in toxic TSB) or 12 min (control cells formed in nontoxic TSB), and cell walls were isolated and purified by using differential centrifugation procedures followed by repeated washing. Sufficient cell wall material was hydrolyzed and analyzed with a model 120 C (9) Amino Acid Analyzer (Beckman Instruments Inc., Fullerton, Calif.)

Spores were mechanically ruptured by means of a Bronwill Disintegrator (type 2876, Bronwill Scientific Inc., Rochester, N.Y.). The supernate and three washings from glass beads used for disrupting the spores were pooled for chemical analyses. The method of Lowry et al. (8) was used to estimate the protein concentration in the supernatant fluids collected from the cell sonicates and in the spore disintegrates. The RNA procedure described by Clark (2) was modified to determine the RNA content of vegetative cells and spores. Although protein and RNA analyses were performed by using supernatant fluids resulting from sonication of cells or disintegration of spores, calculations were made to express the resultant quantities of each component as a percentage of dry weight of the whole cell or spore. The diphenylamine reaction was used to quantitate DNA in cells and spores. Dipicolinic acid (DPA) analysis was performed by a modified Janssen et al. procedure (3).

Heat resistance, germination, and outgrowth studies.

Decimal reduction times (D values) for spores formed on toxic or nontoxic TSA were calculated by plotting the viable cell count versus time of heating in minutes at 92 C on semilog paper.

Possible effects of aflatoxin on germination and outgrowth were assessed by inoculating heat shocked (60 C for 10 min) spores into toxic and nontoxic TSB and measuring absorbance at 650 nm each 15 min for the first 2 hr and after 3, 14, and 24 hr of incubation at 32 C.

RESULTS AND DISCUSSION

Growth curves. The growth curves of $B.$ $megaterium$ cultured in nontoxic TSB and TSB containing 3.8 μg of aflatoxin B_1 per ml are shown in Fig. 1. There was a decrease in the viable number of cells during the first 3.5 hr of incubation in toxic TSB followed by logarithmic cell division at a rate considerably less than the growth rate of the control culture. Absorbance of treated cells at 660 nm measures the increase in total cellular mass and more closely resembled the control culture although the slope of the control culture was greater. The apparent differences observed upon comparison of the slopes of the rates of growth and rates of cell division of control and treated cells may be partially due to the difference in size of the viable units capable of forming colonies on nontoxic TSA. The aberrant single cell forms have increased length and mass after exposure to aflatoxin B_1 in comparison to cells grown in nontoxic TSB. The initial death of cells during the first 3.5 hr of incubation coupled with an average increased cell size and reduced cell mass result in the observed slope differences. Generation times were calculated to be 75 min for the treated cells and 20 min for the control cells.

Vegetative cell wall composition. It is obvious upon examination of the aberrant cell forms
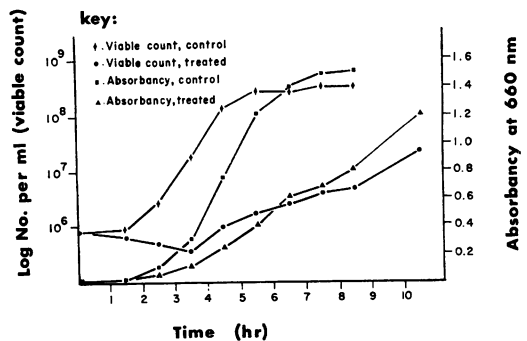
FIG. 1. *Growth curves for Bacillus megaterium cultured in Trypticase Soy Broth (control) and Trypticase Soy Broth containing 3.8 μg of aflatoxin B_1 per ml (treated).*

resulting from growth of *B. megaterium* in toxic TSB that there is more cell wall material per living unit and probably less cell wall material on a dry weight basis than in shorter control cells. This can be concluded by virtue of the shape of the cells assuming there is no difference in cell wall thickness. But there have been no reports on the composition of cell walls of aflatoxin-treated filamentous cell forms. For this reason it was decided that the amino acid composition of cell walls of control and treated cultures should be analyzed. The ratios of alanine, glutamic acid, and lysine, the three amino acids making up the tetrapeptide bridge in most gram-positive bacteria, were not significantly different when the aminograms of control and test cells were compared. Table 1 lists the micromoles of amino acid (lysine, glutamic acid, and alanine) per milligram of protein in the vegetative cell walls of control and test cells. Although amounts of the constituents were greater in cell wall preparations of the control cells, approximate ratios of lysine-glutamic acid-alanine in control (1:2.1:1.9) and test (1:1.9:1.9) cell walls were similar. The apparent higher concentration of the cell wall constituents in control cells was most likely due to improper preparation and cleanup; the presence of intracellular materials would tend to alter the relative concentrations of amino acids.

The constituents *N*-acetylglucosamine, *N*-acetylmuramic acid, and diaminopimelic acid were not run as standards; however, no significantly different peaks were observed. It was concluded that aflatoxin B_1 does not change the chemical composition of cell walls formed in its presence.

Cell and spore composition. Chemical analyses performed on whole cells and spores formed in toxic and nontoxic TSB revealed different results. Table 2 lists the total protein, DNA, and RNA of control and treated cells and spores along with the total DPA content of spores. Analyses of whole cells revealed that the percentages of protein, DNA, and RNA on a dry

TABLE 1. *Concentration in μmoles per mg protein of lysine, glutamic acid, and alanine in cell walls of Bacillus megaterium formed in Trypticase Soy Broth (control) and Trypticase Soy Broth containing 3.8 μg/ml aflatoxin B_1 (treated)*

	Control (μmoles/mg of protein)	Treated (μmoles/mg of protein)
Lysine	3.31	2.73
Glutamic acid	6.97	5.25
Alanine	6.37	5.28

TABLE 2. *Per cent protein, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in vegetative cells and spores and per cent dipicolinic acid (DPA) in spores of Bacillus megaterium formed in Trypticase Soy Medium (control) and Trypticase Soy Medium containing 3.8 μg/ml of aflatoxin B_1 (treated)*

	Vegetative cells		Spores	
	Control	Treated	Control	Treated
Protein	52.0%[a]	73.0%	57.1%	59.4%
DNA	4.1	5.3	2.9	2.7
RNA	11.9	14.6	10.5	10.3
DPA	—	—	6.8	6.3

[a] Per cent of each component is on a dry weight basis.

weight basis were greater when the cells were grown in toxic TSB. At the same time it should be noted that total synthesis of all three components was decreased on a total culture basis. The increase in per cent of protein, DNA, and RNA can be explained in part by considering cell shape. Assuming the concentration of the three components on a cytoplasmic volume basis to be equal in both filamentous and short cells, then the per cent of each component on a dry weight basis would be greater in the filamentous cells due to the decrease in per cent of cell wall material. Cell wall material, of course, would contribute no DNA or RNA and little protein to cell mass.

A similar imbalance in macromolecular biosynthesis was reported in *F. aurantiacum* grown in the presence of 50 μg/ml of aflatoxin B_1 (5). The imbalance, particularly in DNA production, might have been responsible for the decrease in viability of multiplying *B. megaterium* as reported by Lillehoj and Ciegler (6). The decreased total synthesis of DNA, RNA, and protein as reported by Lillehoj and Ciegler is in agreement with the data presented here. No report was made by Lillehoj and Ciegler, however, basing the percentage of these cellular components on a cell dry weight basis. Percentages of DNA, RNA, and protein were found to increase in the test cells in comparison to the controls when the analytical data were expressed in per cent of cell (dry weight)

Age of the cells undoubtedly affects chemical composition. Data presented in Table 2 were obtained from vegetative cells in the late logarithmic growth phase grown in toxic TSB that exhibited an extremely high nucleic acid plus protein content (92.9%) calculated on a dry weight basis. The nucleic acid plus protein

content would have decreased if older cells were analyzed, since cells in the stationary phase of growth contained large and numerous poly-β-hydroxybutyric acid deposits which would result in a decrease of total cellular protein. There have not been reports that expressed the composition of aflatoxin-treated filamentous cells on a dry weight basis; thus, there would appear to be little cell wall material on a dry weight basis in aflatoxin-treated filamentous cells.

Chemical analyses of spores formed in toxic and nontoxic media revealed no significant differences in protein, DNA, or RNA content. Dipicolinic acid, unique to bacterial spores but not found in vegetative cells, was also quantitated in the control and test spores (Table 2). Any difference in composition may be attributed to inefficient cleaning of the test spores as cleaning was particularly difficult due to the abundance of nonsporulating cells. More cell debris in the test spore preparation would tend to decrease the DPA percentage.

Spore studies. Sporogenesis in *B. megaterium* begins when a portion of an axial thread of chromatinic material becomes positioned at one end of the sporangium and a centripetally growing thin septum encloses the chromatin and a small amount of cytoplasm. The sporulation process is believed to be irreversible or committed at this stage. Since one of the morphological alterations in *B. megaterium* grown in toxic TSB was the apparent inhibition of septum formation, a study was made to determine the effect of aflatoxin on the initial septum formation phase of sporulation. Completion of septum formation is compulsory for the development of the mature spore.

Sporulation was followed on toxic and nontoxic TSA. After 3 days, 97% sporulation occurred in the control culture as measured by direct microscopic count. Only 65% sporulation was obtained in the test culture after 6 days in the toxic medium. The 65% figure is only approximate due to the difficulties in counting caused by abnormal length of the filaments and the frequency of spores being formed within these filaments. Old filaments formed septa at close intervals to form a chainlike arrangement of cells the length and width of which were nearly the same. The cells failed to separate perhaps because of incomplete septum formation. Spores were often formed within these irregular partitionings of the filaments. The medium still contained 3.2 μg/ml of aflatoxin after 6 days of incubation which represents only a 16% loss of the 3.8 μg of aflatoxin per ml originally added to the medium. However, it is possible that a localized aflatoxin gradient

may have formed in the immediate area of colony formation due to oxidation and dilution brought about by increased cell mass. The aflatoxin concentration may have been decreased to a level which was not detrimental to either septum formation in dividing cells or septum formation as an initiator of sporogenesis.

An examination of the wet heat resistance of spores formed on control and toxic TSA indicated no significant difference. Survivor curves for spores formed on toxic and nontoxic TSA are shown in Figure 2. The D_{197} [time in min to destroy 90% of the population at 92 C (197 F)] for the control was 17.0 min; the D_{197} for the
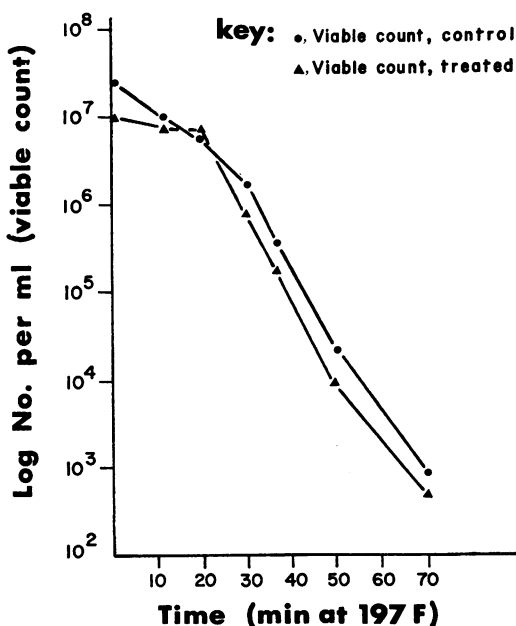

FIG. 2. *Survivor curves at 92 C (197 F) for Bacillus megaterium spores formed in Trypticase Soy Agar (control) and Trypticase Soy Agar containing 3.8 μg of aflatoxin B_1 per ml (treated).*

TABLE 3. *Absorbancy at 650 nm of spore suspensions in Trypticase Soy Broth containing 4.0 μg of aflatoxin per ml B_1 (toxic) and Trypticase Soy Broth (nontoxic)*

Determination	Absorbancy (650 nm) with time							
	0 hr	0.25 hr	0.75 hr	1.25 hr	1.75 hr	3 hr	14 hr	24 hr
Toxic TSB......	.620	.569	.538	.523	.495	.469	.432	.553
Nontoxic TSB......	.638	.569	.538	.523	.495	.469	.854	.957

test culture spores was 16.9 min. The D values are extremely low, indicating little heat resistance.

Germination of spores is characterized by loss in heat resistance, permeability to dilute stains, loss in turbidity or dry matter, and darkening under phase contrast (10). The loss in turbidity and phase darkening were followed to study the effect of aflatoxin on germination of spores which were formed on nontoxic TSA. Initiation of germination is usually brought about by chemical substances and can occur even if environmental conditions are unfavorable to germinal development. Germination initiation involves among other processes the uptake of water with a coincident swelling and decreased refractility of the spore. Germinal development following initiation involves either rupture or adsorption of spore coats followed by the elongation of a germ cell and is generally defined as outgrowth. Table 3 lists absorbancy readings at 650 nm of spore suspensions in toxic TSB and nontoxic TSB at various times during incubation at 30 C. Results of germination studies with normal spores exposed to a suitable germination environment and the same environment plus aflatoxin indicated that the toxin had no effect on germination initiation but suppressed outgrowth for an extended period.

More than 90% of the spores turned phase dark in both control and treated spore suspensions. However, a lag of more than 14 hr was required for outgrowth of treated spores. It would only require a small percentage of spores to progress through the outgrowth stage and begin dividing to increase the turbidity as an indication of growth. Results from the germination studies thus indicate that the catabolic processes occurring during germination initiation are not inhibited by aflatoxin, but the anabolic processes occurring at their maximum rates during the outgrowth phase are inhibited by aflatoxin.

The experiments described are presented as exploratory attempts to define in greater detail the effects of aflatoxin B_1 on the biochemistry of *B. megaterium*. Data derived from the study might be used in conjunction with data collected using higher organisms to more clearly understand the mechanism of action of aflatoxin upon cellular metabolism.

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