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Some Effects of Diflubenzuron on Growth and Sporogenesis in *Streptomyces* spp.†

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Diflubenzuron, an insect growth regulator that blocks chitin deposition in insect cuticles, was tested for its effects on morphogenesis of *Streptomyces* spp. Use of diflubenzuron resulted in reduced dominance of spore hairs, reduced the width of the outer wall, and prevented formation of the inner spore wall in *S. bambergiensis*. In *S. coelicolor*, diflubenzuron altered the structure of the fibrillar pattern of spore envelopes. Exposure to diflubenzuron resulted in small increases in exported protein and in a ca. 20% increase in chitinase in both *Streptomyces* spp.

Streptomyces spp. are ubiquitous, spore-forming, filamentous bacteria that are major constituents of soil and sediment microbial communities (4, 9, 13, 19–21). It is known that pesticides such as Mansate D, Benlate T, Penogen PX, Vitaflo, and Arasan 75 inhibit gowth of nontarget organisms such as *Streptomyces* spp. (10). A new class of pesticides, chitin synthetase inhibitors, are being considered for use as insecticides, with a potentially large environmental distribution, in an attempt to control agricultural insect pests (6, 9, 14). Since chitin is produced by *S. coelicolor* A3(2) during sporogenesis (17) and is a component of the spore hairs of *S. bambergiensis* (16), a study was initiated to determine the effect(s) of diflubenzuron (DFB), one of the most potent of these pesticides, on growth and sporulation of these two species of *Streptomyces*.

MATERIALS AND METHODS

Organisms. *S. bambergiensis* (ISP no. 5590) and *S. coelicolor* A3(2) were obtained from E. B. Shirling. *S. bambergiensis* and *S. coelicolor* were maintained on chitin medium (19) and glycerol-asparagine (15), respectively.

Media. Crab (*Callinectes sapidus*) chitin was purified by sequential treatments with 2 N HCl, 2 N NaOH, acetone, and distilled water and then was lyophilized (3). DFB [1-(4-chlorophenyl)-3-(2,6-di-fluorobenzoyl)urea, Th60-40; Dimilin, trademark of Phillips-Duphar B.V., Amsterdam, The Netherlands] (5) was dissolved in acetone. Serial dilutions were prepared from a stock solution of 0.8 g/ml. Chitin-agar was prepared from precipitated chitin in trace salts and phosphate buffer (pH 7.4) (19). DFB was diluted in acetone (0.5 ml), mixed with 20 ml of the molten chitin agar at 55°C, and poured into sterile disposable petri dishes. Control plates were prepared by adding only acetone to the agar. Plates were stored at room temperature for 36 h to permit evaporation of the acetone. The plates were inoculated with spores and incubated for 10 days at 27°C.

Submerged cultures for determining the effects of DFB on protein export and antibiotic pigment production were made with 2% (wt/vol) flake chitin (Calbiochem-Behring, La Jolla, Calif.) in 0.05 M Tris-buffered medium (pH 7.6) containing 134 mg of K_2HPO_4, trace salts per liter (15), and 0.01% yeast extract (Difco Laboratories, Detroit, Mich.). In this case 25 ml of medium was placed into 100-ml Erlenmeyer flasks. Dimethyl sulfoxide (DMSO) was used as a carrier for DFB in these studies. DMSO only controls (0.5 ml of DMSO per 25 ml culture) and DFB-DMSO media were run in duplicate flasks with preincubation for 24 h at 30°C. Flasks were inoculated with spores washed from appropriate slants and incubated at 30°C for 85 h with 150 reciprocal aeration (1-in. [2.54-cm] oscillations). Media were clarified by centrifugation (5,000 × g) prior to determination of cell-free chitinase production and antibiotic-pigment production. *S. coelicolor* pigment production was quantified at 350 nm with 1-cm cuvettes.

Chitinase assay. Cell-free chitinase was determined by monitoring the enzymatic release of ^{3}H-soluble products from ^{3}H-labeled chitin (11). Each assay contained 0.5 ml of enzyme and 122 μg of [^{3}H]chitin (specific activity, 3,808 dpm/nmol of *N*-acetylgalactosamine). Hydrolysis was permitted to continue at 30°C for 60 min. Enzymatic activity was terminated with 200 μl of 10% trichloroacetic acid. Protein was determined by the dye-binding assay described by Bradford (1).

Electron microscopy. Intact colonies were excised from the agar plates and fixed by a modified procedure described by Luft (8) with ruthenium red to enhance visualization of acidic polysaccharides. Triton X-100 (final concentration, 0.1% [wt/vol]) was added to the glutaraldehyde-ruthenium red fixative to promote fixative penetration into spores. The osmium-ruthenium red fixation was maintained at 25°C for 1 h and then at 5°C for 14 h. Specimens were dehydrated by a series of treatments with ethanol and embedded in Epon 812 (7). Thin sections were cut with a diamond knife, collected on Formvar-coated copper grids, and poststained with 2% (wt/vol) aqueous uranyl acetate and 2% lead citrate (13). Portions of the same 10-day-old growth of *S. bambergiensis* on chitin agar that was used for thin sectioning were also used to make spore impressions. Spore impressions were taken from agar lawns at multiple sites by making surface impressions with Formvar-carbon-coated copper grids. The samples were shadowed with platinum-carbon and examined by transmission electron microscopy. In many cases the classic shadowed image was not observed because the spore hairs elevated spores from the grid surface.

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FIG. 1. Electron micrographs comparing whole mounts of aerial spores of *S. bambergiensis* grown on chitin agar in the presence of DFB. (A) Control; (B) 10-ppm treatment; (C) 400-ppm treatment. These spore silhouettes show the reduction in hair production and a change to a more spherically shaped spore as a result of the 400-ppm DFB treatment. H, Hair. Bars, 1 μm.

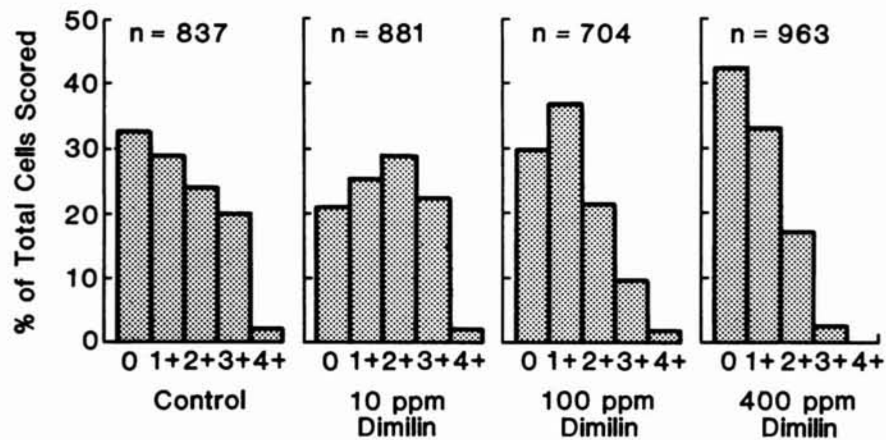

FIG. 2. Influence of DFB on *S. bambergiensis* degree of spore ornamentation. Zero indicates that no spore hairs are visible, and 4+ indicates the most pronounced ornamentation, as determined by subjective categorization. Other values reflect intermediate classifications.

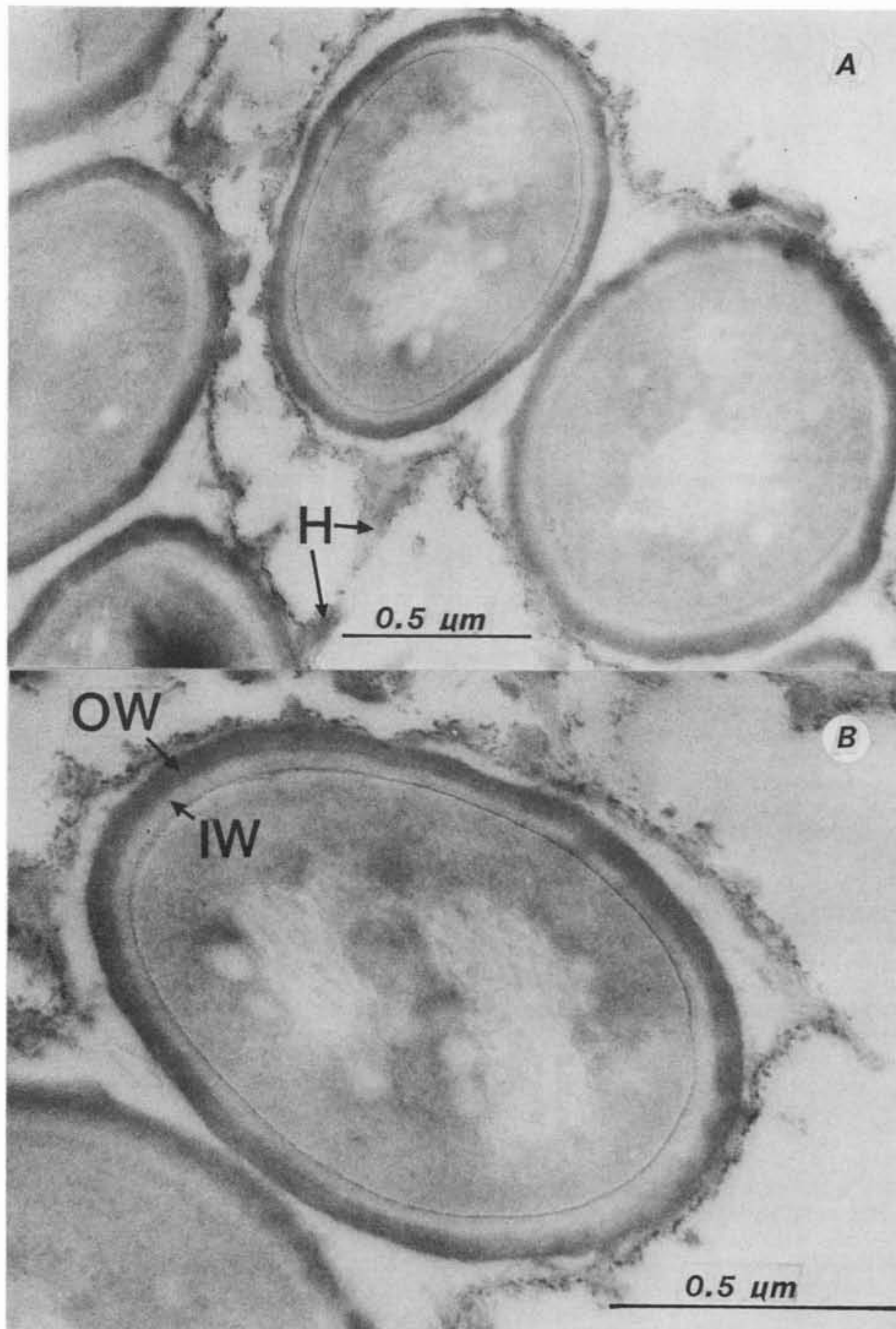

FIG. 3. Ultrastructure of normal *S. bambergiensis* spores. (A) Note the robust hair-like surface protrusions and the uniform width of the electron-lucent inner and outer spore walls. (B) Higher magnification, showing spore envelope more clearly. H, Hair; OW, spore outer wall; IW, spore inner wall.

Six or more fields of each of four grids from each treatment were scored for hair production. The degree of ornamentation was categorized from none to 4+ (the highest degree of ornamentation). Measurements of hair length were not successful because of hair interlooping after drying and because of the difficulty in determining sites of attachment.

RESULTS AND DISCUSSION

S. bambergiensis colonial growth rate, pigment production, and sporulation appeared to proceed normally in the presence of DFB but resulted in changes in the morphology and ultrastructure of the spores. Spores of *S. bambergiensis*

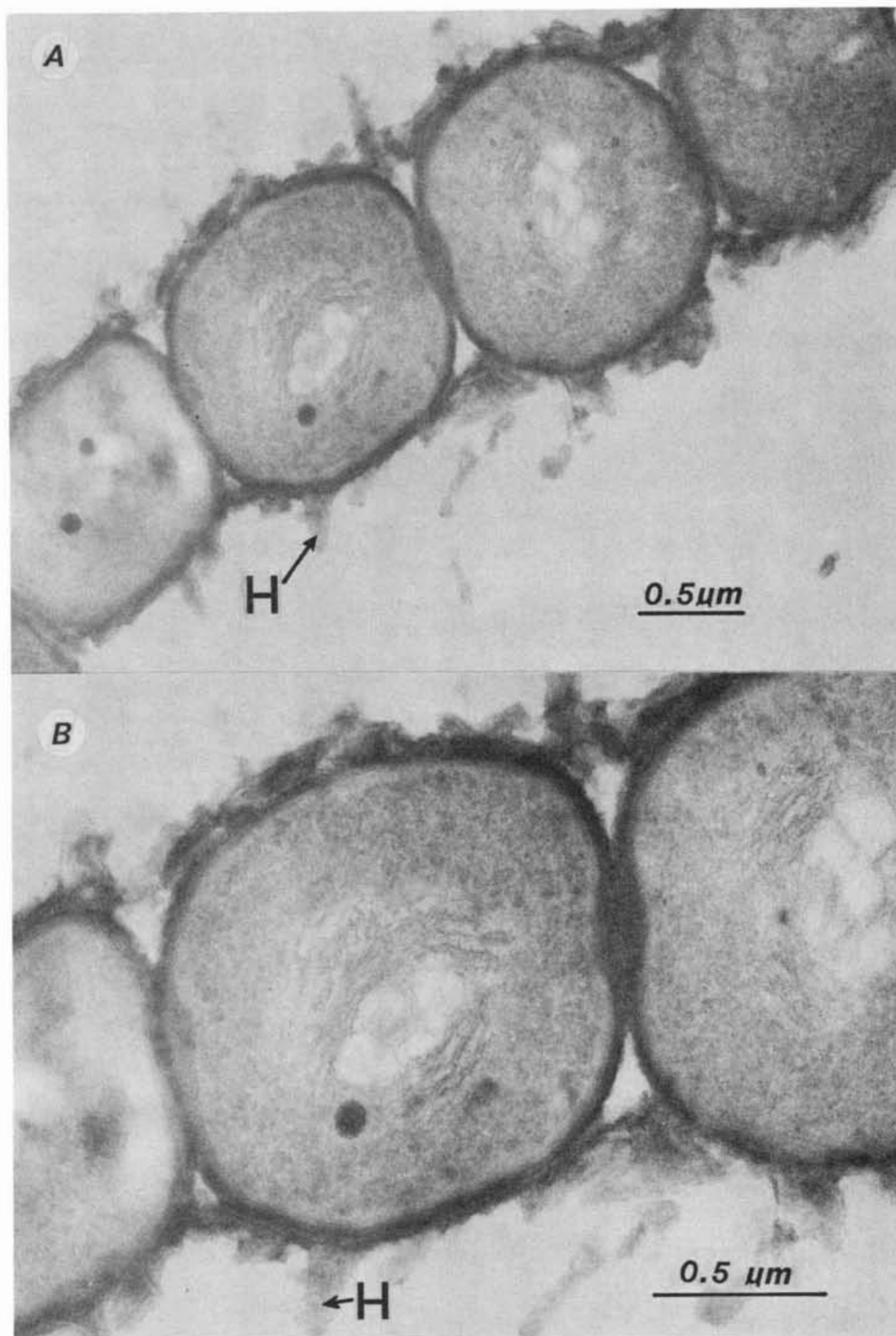

FIG. 4. (A) Ultrastructure of *S. bambergiensis* spores after treatment with 400 ppm DFB. The outer wall is invaginated (arrow) and generally has less well organized hairs (H) compared with controls (Fig. 3). (B) Higher magnification of panel A. The inner spore wall is either absent or represented only by a thin layer.

are normally elongated and highly ornamented (Fig. 1). Spore envelope extensions, commonly referred to as hairs, were still produced at DFB concentrations as high as 400 ppm (μg/ml), but they were thinner and considerably reduced in apparent length compared with those of the controls. Spores produced during treatment at a concentration of 10 ppm had what appeared to be thicker hairs, or at least more electron-dense hairs (Fig. 1). Spores produced on media containing 25 to 200 ppm DFB were affected similarly to those from media containing 400 ppm DFB, but to a lesser degree. The 25-, 50-, 100-, 200-, and 400-ppm treatments produced increasingly less pronounced hairs. In Fig. 2 the degree of hair ornamentation in populations exposed to three pesticide levels is shown. In contrast to other treatments, growth on 10 ppm DFB-containing agar appeared to have more predominant hairs than did controls. Electron micros-

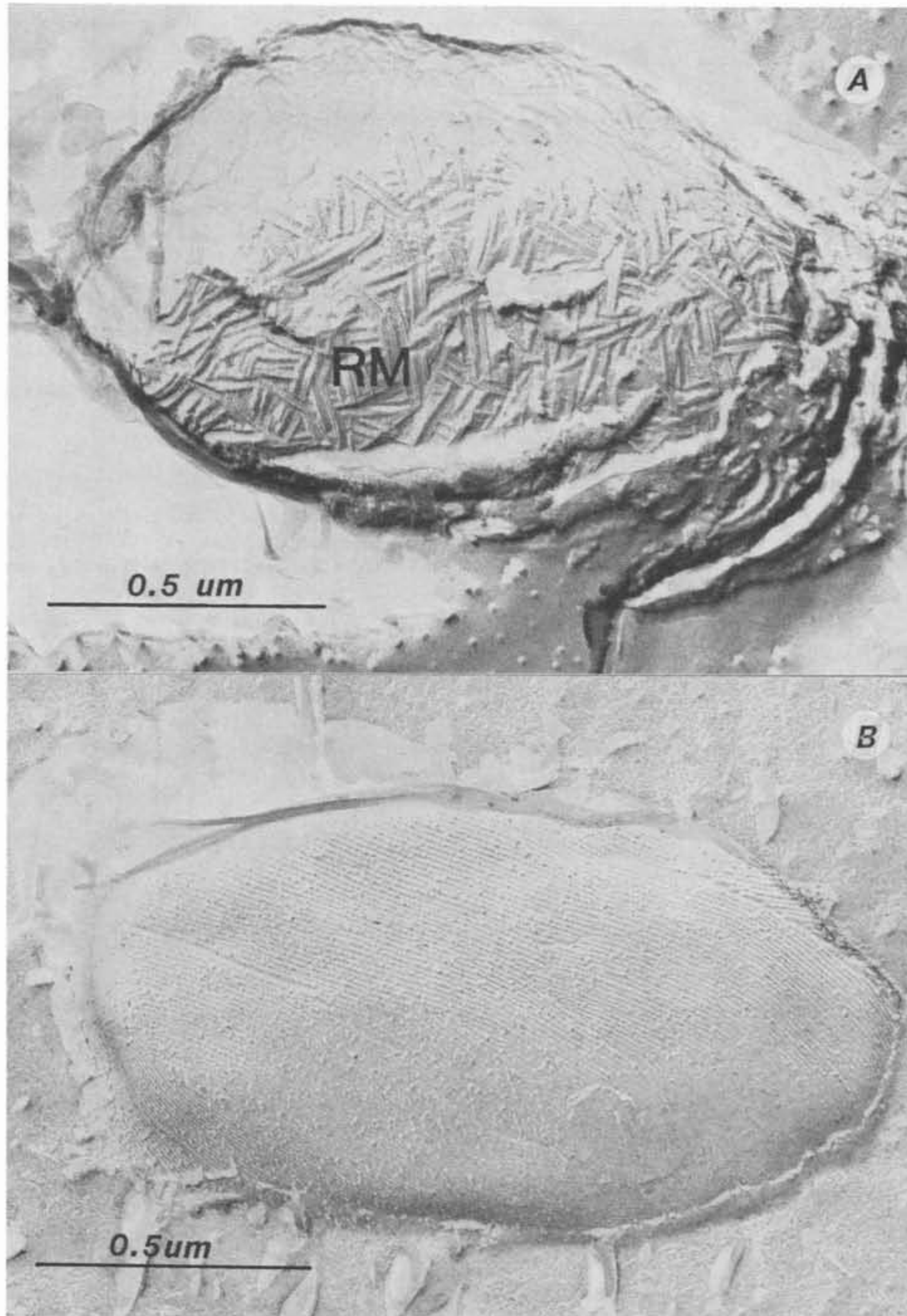

FIG. 5. Electron micrographs of freeze-etch replicas of *S. coelicolor*. (A) Control; (B) treatment with 1,600 ppm DFB. The rodlet mosaic of the control spores (A) is lacking in the DFB-treated cells (B), which show parallel arrangements of fibers. RM, Rodlet mosaic.

copy of control cultures (acetone only) showed normal aerial spore envelope structure (Fig. 3). The presence of inner and outer layers of the spore wall and the spore hairs with associated sheath material is consistent with the current *S. bambergiensis* spore envelope model, as described previously (18). Spores from the treatment with 400 ppm DFB had distorted outer walls (Fig. 4). The outer wall was reduced in width, while the inner wall was absent, except for a narrow dense layer of material adjacent to the outer wall. Some of these cells had outer wall depressions extending almost to the inner wall layer. The presence of DFB affected the synthesis of the externally associated envelope components and outer wall layer and prevented synthesis of the inner spore wall.

Embedded DFB-treated cells were more easily sectioned than the controls, and the cytoplasm of spores treated with 400 ppm DFB generally had a coagulated appearance. It may be inferred that dehydrant interaction with DFB-treated spores was facilitated by altered wall permeability due to loss of much of the material composing the spore wall, and

TABLE 1. DFB effect on *Streptomyces* spp. cell-free chitinase production and anitibotic pigment production

Organism and culture condition	Amt of chitinase production (nmol of GlcNAc h^{-1} ml^{-1})[a]	Amt of protein (mg/ml)[b]	Optical density of antibiotic pigment at 350 nm[c]	Sp. act. of chitinase (nmol of GlcNAc mg $protein^{-1}$ h^{-1}
S. coelicolor				
DMSO control	40.4 ± 1.0 (4)	28.1 ± 0.40 (4)	0.490, 0.425	1,438
DFB (420 ppm)	48.4 ± 1.6 (4)	31.1 ± 0.56 (4)	0.604, 0.605	1,556
S. bambergiensis				
DMSO control	52.5 ± 0.99 (4)	15.7 ± 0.69 (4)		3,344
DFB (420 ppm)	64.5 ± 1.6 (4)	16.2 ± 0.64 (4)		3,981

[a] Chitinase levels in cell-free supernatants expressed as the capacity to release nanomoles of *N*- acetylglucosamine (GlcNAc) equivalents per milliliter per hour at 30°C. Details in text (121.5 mg of chitin per 0.5 ml). Results are expressed as the mean ± standard error. Numbers in parentheses are sample sites.

[b] Protein in the culture supernatants determined by the assay described by Bradford (1). Values are the means ± standard error. Numbers in parentheses are sample sizes.

[c] *S. coelicolor* A3(2) produces an antibiotic-pigment complex which is purple at pH 7.6. Blanks for these readings were uninoculated Tris-salts basal medium. Values for each flask are shown.

thus, subsequent ethanolic nucleoid denaturation occurred. Uranyl acetate stabilization of the nuclear apparatus was not employed in these experiments.

High concentrations of DFB (final concentration, 1,600 ppm) spread over the surface of glycerol-asparagine agar blocked normal cross-hatching of the *S. coelicolor* fibrils (Fig. 5). This treatment resulted in a fibrillar pattern similar to that of the normal appearance of freeze-etched *Streptomyces spadicus* spores (22) which have a ribbed appearance of parallel fibers. There may be a significant difference(s) in the fibril polymer chemistry in DFB-treated spores versus that in the controls. One difference may be a developmental block at a stage after the fibers are formed. The mosaic layer of the wall has been described in an earlier report as chitin fibrils (17).

DFB is relatively insoluble in water (maximum solubility, 0.3 ppm) (5). Since the cultures were grown as agar lawns, the aerial sporophores and associated spores actually may have been exposed to a small fraction of the pesticide carried in the agar. Increased DFB concentrations may have increased the probability of a given aerial sporophore to be exposed to DFB microparticles. Other cell systems also show increased sensitivity to high DFB concentrations. The mechanism for this effect is not understood (J. O. Norman, U.S. Department of Agriculture, College Station, Tex., personal communication).

DFB uncouples the normal synthetic pattern of spore walls and the spore ornamentation in *S. bambergiensis*. The spherical nature of DFB-exposed *S. bambergiensis* spores may be explained by the loss of integrity of inner and outer wall components. In many cases, in the 400 ppm DFB treatment, the inner wall layer was considerably reduced or lost. Furthermore, DFB treatment resulted in lower resistance to UV radiation (unpublished data).

In *S. bambergiensis*, DFB alters normal development of the inner spore wall, the outer spore wall, and the spore hairs. The hairs are composed, in part, of chitin (16) and would be an expected target of this pesticide. DFB has no effect on fungal chitin synthesis in vitro or in vivo while it is very inhibitory to insect chitin synthesis in vivo (6). This is consistent with the results seen in this study with *Streptomyces* spp., in which chitin fibrils were produced even in the presence of 1,600 ppm DFB.

S. coelicolor A3(2) and *S. bambergiensis* expressed higher chitinase activity in the cell-free state when exposed to 420 ppm DFB versus that in the controls (Table 1). *S. coelicolor* A3(2) also exported higher levels of pigment-antibiotic when grown in the presence of 420 ppm DFB. Alterations in envelope synthesis could conceivably affect export rates of macromolecules. One explanation of this phenomenon would be fewer binding sites available in DFB-treated cell walls (compare Fig. 3 and 4). The 400 ppm DFB-treated *S. bambergiensis* spores (Fig. 4) were devoid of the conspicuous inner electron-lucent wall layer seen in controls (Fig. 3).

Leighton et al. (6) reviewed chitin synthesis inhibitors and noted the highly effective capacity of DFB to block cuticle formation. The mode of DFB action typically has been ascribed to inhibition of chitin synthesis (5). However, in vitro assays with insect (2) and fungal (6) chitin synthase extracts do not support the theory of chitin synthesis inhibition. In separate studies with *Thalassiosira* spp. (diatoms), DFB had no significant inhibitory effect on chitin fiber production (L. G. Morin, R. A. Smucker, and W. Herth, unpublished data).

Several investigators have demonstrated inhibition of cellular phenomena by DFB at regulatory levels. Norman and Meola (12) reported DFB inhibition of mouse melanosome synthesis and release. DeLoach et al. (2) reported DFB inhibition of DNA synthesis in stable fly pupae. In view of these reports it is not surprising to observe that DFB did not block *Streptomyces* fibril synthesis but modified other cell features. DFB may prove to function as a useful chemical probe in unraveling some mysteries of *Streptomyces* developmental processes.

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