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Interaction of *Streptomyces felleus* with Bromoxynil during Growth on Laboratory Media*

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ABSTRACT. *Streptomyces felleus* resistant to the herbicide bromoxynil (BX) took up 95 % of the initial amount of BX from the solid or liquid medium containing 100 μg of the herbicide per mL during a 5-d incubation. 50 % of the amount taken up was degraded and 45 % deposited in the cell (90 % in the cytoplasm, 10 % in the cell wall). A prolonged incubation time did not result in any further decrease of BX concentration. The addition of KCl (the effect of NaCl was less pronounced) increased the affinity of BX for the cell wall and slowed down both the uptake and degradation of BX. Though P-14 was capable of growing at 5— to 10 times higher concentrations of BX in comparison with sensitive *Streptomyces* strains, the herbicide caused its physiological (growth rate decrease, antibacterial antibiotic production, pigmentation, dehydrogenase activities), morphological and ultrastructural changes.

About 10 ppm of the total bacterial population present in nonsterile soil participate in the biological degradation of bromoxynil (3,5-dibromo-4-hydroxybenzonitrile; BX) (Cullimore and Kohout 1974). Only two pure cultures of microorganisms degrading BX have been described so far, a strain of *Flexibacterium* marked BR4 (Cullimore and Kohout 1974; Smith and Cullimore 1974) that was not characterized in detail, and *Streptomyces* P-14 (Krištůfek and Blumauerová 1983*a*), later classified as *S. felleus* (Krištůfek *et al.* 1987). In contrast to the strain BR4 transforming BX in two major products, 3,5-dibromo-4-hydroxybenzamide and 3,5-dibromo-4-hydroxybenzoic acid (Smith and Cullimore 1974), P-14 degraded BX by direct splitting of the aromatic ring catalyzed by an intracellular Fe^{2+}-dependent decyclizing dioxygenase (Neužil — to be published). Physicochemical analysis of the extracts of the P-14 cultures grown on solid media that were incubated 10 d in the presence of BX showed a nearly two-fold decrease of the herbicide concentration in the medium (Krištůfek *et al.* 1987).

This paper presents detailed results on the time course of the degradation, fate of the nondegraded remainder of BX in the P-14 cultures on solid and liquid media, and the effect of BX on the physiological activity and morphological properties of the strain P-14.

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* Second paper of a series on *Streptomyces felleus* resistance to bromoxynil.
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MATERIALS AND METHODS

Microorganism and cultivation. The origin and the characteristics of *Streptomyces felleus* P-14 were described in previous papers (Krištůfek and Blumauerová 1983*a*; Krištůfek *et al.* 1987). All cultivations were carried out using the malt extract-yeast extract medium at 28 °C. The cultures on solid media were grown for 10 d on cellophane discs (diameter of 90 mm) placed on the surface of the agar medium in Petri dishes. The discs were inoculated with 0.1 mL of an aqueous spore suspension. Submerged cultures in 300-mL Erlenmeyer flasks containing 50 mL of the medium were incubated on a reciprocal shaker (1.6 Hz) for 5 d. A solution of BX in methanol (0.5 mL) was added to the medium, the final concentration being 100 μg/mL. 0.5 mL of ethanol was added instead to the control cultures.

Analyses. Intact mycelium was used to determine the dry mass and for the extraction procedures. The mycelium was separated from the solid medium by removing the cellophane and from the liquid medium by centrifugation. The content of BX in the extracts was estimated by using TLC, UV-spectrometry and HPLC (cf. Krištůfek *et al.* 1987). The antibiotic production in the submerged cultures was determined by using a plate diffusion method with *Bacillus subtilis* as the indicator. The total dehydrogenase activity of the submerged cultures was estimated by using a modified method of Novikova and Makarevich (1984): methylene blue (0.01 % in distilled water) was added to the mycelium separated by centrifugation in calibrated tubes (0.5 mL of the methylene blue solution per mL of the mycelial sediment), the samples were stirred properly and the time necessary for complete bleaching was measured.

Intracellular localization of BX. A mycelium collected from eight cellophane discs was suspended in 30 mL of distilled water and disintegrated by sonication (MSE Ultrasonic Disintegrator, 20 MHz, 3 × 1 min with 1-min cooling intervals). The cell-free extract and cell-wall fraction, separated by centrifugation (Sorwall, 10 000 ***g***, 15 min) were extracted and analyzed as described above. 1 and 10 % water solutions of NaCl or KCl were used for washing BX from the intact cells (distilled water was used as the control). The liquid phase was then extracted and analyzed as other samples.

Microscopic observations. Preparations for optical microscopy were stained with carbolfuchsin. Transmission electron microscopy was done in a JEM 100B microscope at an accelerating voltage of 60 kV. Morphological properties of the aerial mycelium were observed in preparations obtained by a direct impression of the cultures. The samples for ultrathin sections were fixed with 1 % OsO_4 and embedded in Vestopal (Kellenberger *et al.* 1958). After polymerization the blocks were sliced using a LKB 4800A Ultrotom (glass knives). The sections were contrasted with 5 % aqueous solution of uranyl acetate and stained with lead citrate (Reynolds 1963).

RESULTS

Effect of BX on growth, morphology and physiological activity. When cultivated on a solid medium without BX, the strain P-14 grew and sporulated very well. The substrate mycelium appeared after 1 d, the aerial mycelium after 2 d, spores and a brown pigment in the mycelium 4 d after inoculation. The maximum yield of dry biomass was obtained after 5 d (Fig. 1). If a

TABLE I. Effect of BX on some physiological characteristics of submerged cultures of the strain P-14[a]

Concentration of BX µg/mL	Pigmentation[b]	Antibiotic inhibition halos (mm)[c] throughout cultivation (d)					Total dehydrogenase activity (min)[d] throughout cultivation (d)				
		1	2	3	4	5	1	2	3	4	5
0	yellow-brown	0	12.3	13.5	15.6	15.0	>60	31	5	2	>60
100	cream	0	0	0	traces	traces	>60	>60	35	13	>60

[a] Cultivation conditions and growth curves, cf. Fig. 2.
[b] After 4–5 d.
[c] Determined by the diffusion plate method using *Bacillus subtilis* as a test organism. According to Lindenbein (1952), *S. felleus* produces the macrolide antibiotic picromycin effective against Gram-positive bacteria.
[d] Determined by the method of Novikova and Makarevich (1984) based on the decolourization of a methylene blue solution.

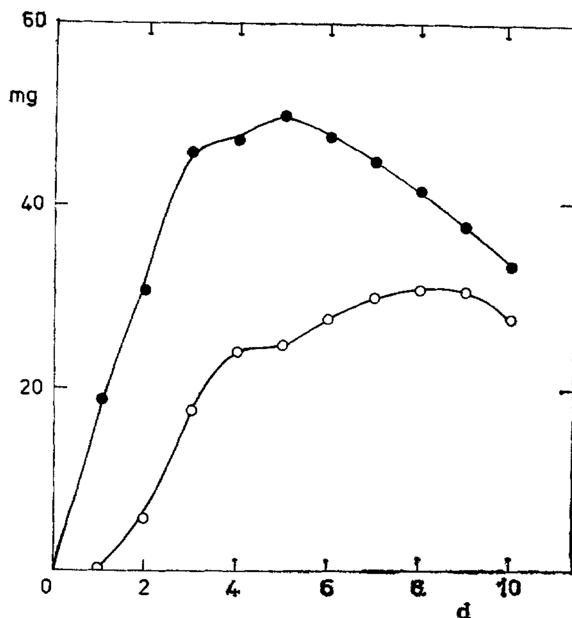

FIG. 1. Dry mass (mg) of *S. felleus* cultures grown on yeast extract-malt extract agar non-supplemented (*closed symbols*) and supplemented (*open symbols*) with BX (100 μg/mL) at 28 °C. Sterile cellophane discs, previously dried and weighed, were placed on the medium and inoculated with spore suspension (10^8 of cells in 0.1 mL). After incubation, the whole cultures (cellophane plus mycelium) were taken off, dried and weighed. Individual data represent a mean of five parallel values.

medium containing BX was used, the growth phase was delayed by 1 d, less aerial mycelium was formed, sporulation (Plate 1) and pigmentation were suppressed. Maximum dry mass yields (after 8 d) were reduced by 50 % in comparison with the 5-d control culture (Fig. 1). The macroscopic appearance of these cultures after 10 d was similar to that of a 3-d control culture. The electron-microscopic observations revealed in the cells grown in the presence of BX more frequent occurrence of electron transparent vesicles (Plate 2), irregular septation, and also a great number of electron-dense microbodies that were not observed in the control cells (Plate 3).

BX inhibited germination and growth of a spore inoculum in submerged cultures (the mycelium did not grow after as many as 4 d). Therefore, in the other experiments, the media were inoculated with a 1-d vegetative inoculum grown in a medium without BX. Under these conditions, maximum growth yields were reached both with the control without BX and with the culture growing in the presence of BX after 4 d, the latter being decreased by 20 % only (Fig. 2). The control cultures grew in the form of oval, compact pellets, whereas in the presence of BX, large, irregular forms exhibiting less compact structure with long protruding bundles of filaments were observed (Plate 4). Comparison of the physiological activities of both cultures showed that BX suppressed the production of the antibiotic and brown pigment and also

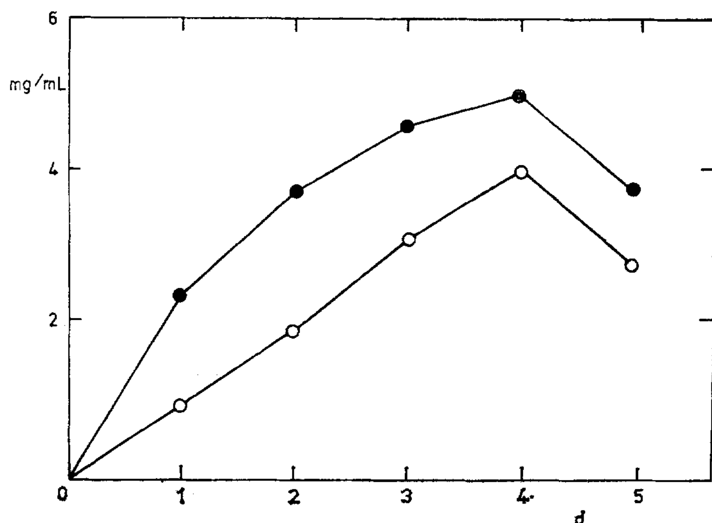

FIG. 2. Dry mass (mg/mL) of submerged cultures of *S. felleus* grown in liquid yeast extract-malt extract medium (50 mL) non-supplemented (*closed symbols*) and supplemented (*open symbols*) with BX (100 μg/mL) on a reciprocal shaker (1.6 Hz) at 28 °C. Media were inoculated with a 1-d vegetative inoculum (2.5 mL) grown in the absence of BX. After incubation, mycelium of each culture was filtered (paper filter), washed three times with distilled water and its dry mass determined. Individual data represent a mean of five parallel values.

TABLE II. Effect of washing of intact mycelium on the content of BX in cell fractions of the strain P-14[a]

		BX (%)[b] in		
Washing agent	Concen-tration	Washing liquid[c]	Cell walls	Cytoplasm
None	—	—	10	90
NaCl	1	55	15	30
	10	61	18	21
KCl	1	45	41	14
	10	30	58	12
Water	—	38	20	42

[a]Mycelia of 8 cultures grown on a BX-containing solid medium for 10 d (see Fig. 1) were combined, suspended in 30 mL of the washing agent and centrifuged (2000 *g*, 10 min). After repeating the procedure six times, the mycelium was resuspended in distilled water and disrupted by sonication (3 × 1 min). Cytoplasmic and cell-wall fractions separated by centrifugation (10 000 *g*, 15 min) as well as the corresponding washing volumes were extracted and analyzed as described previously (Krištůfek *et al.* 1987); in the last washing volume traces of BX were only found.
[b]Percentage of the total amount of BX extracted from unwashed intact cells.
[c]The sum of 6 values obtained in the individual steps of washing procedures.

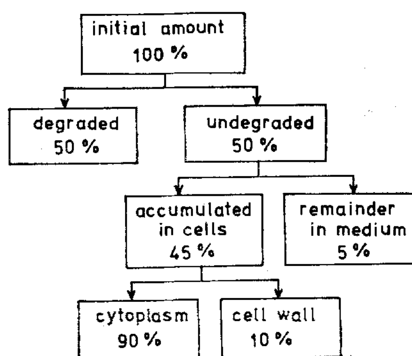

FIG. 3. Fate of BX in cultures of *S. felleus* grown on solid medium. Cultivation conditions, see Fig. 1. After a 5-d incubation, the whole cultures (unwashed intact mycelium on cellophane discs) and the agar medium were extracted and analyzed (cf. Krištůfek *et al.* 1987). The results are expressed as the percentage of the total amount of BX extracted from the non-inoculated media at zero time. The BX content in cell fractions (separated from sonicated mycelium) was calculated as the percentage of the total amount of BX extracted from the intact cells. When analyzing the substrate *vs.* aerial mycelium, BX was found evenly distributed in both cell types.

negatively affected the dehydrogenase activity that was decreased by as much as 7-fold (Table I). No significant ultrastructural differences, however, were found between the cells in the submerged cultures growing in the presence or absence of BX.

Uptake and degradation of BX during cultivation. By analyzing the extracts from the medium, unwashed mycelium and its fractions we found that the agar P-14 cultures took up about 95 % of the initial amount of BX during 5 d. Half of the amount taken up was decomposed, while the remainder accumulated intact, prevalently in the cytoplasm (Fig. 3). Similar experiments with washed mycelium showed that 30 to 60 % of the total amount of the accumulated BX could be washed out (depending on the solution used and on its concentration), which was parallelled by an increase of the BX content in the cell-wall fraction. The affinity of BX for the cell wall increased especially after the cells were washed with 10 % KCl. If NaCl was used, the content of the herbicide in the washing solution was raised (Table II).

The supernatant of the submerged cultures did not contain any BX after a 4-d incubation. Analysis of the mycelial extracts showed that, as in the

TABLE III. Effect of NaCl and KCl supplements to solid media on the uptake and degradation of BX in cultures of the strain P-14[a]

Salt added[b]	Amount of BX (%)[c]			
	Degraded	Nondegraded		
		Total	Accumulated in cells	Remainder in medium
None	50	50	45	5
NaCl	42	58	36	22
KCl	9	91	67	24

[a]After a 5-d cultivation under the conditions given in Fig. 1. After 10 d similar results were obtained.
[b]1 % final concentration (0.5 % NaCl or KCl were present in the medium as the regular components).
[c]Expressed as the percentage of the total amount of BX extracted from non-inoculated media at zero time of incubation (cf. Fig. 3).

agar cultures, only half of the original amount of the herbicide was decomposed. If BX was added to the control cultures after a 5-d cultivation, as much as 60 % of the herbicide was bound to the mycelium during 30 min. The BX, however, was not degraded.

A prolonged cultivation, either in the solid or liquid medium, did not result in a further decrease of the BX concentration or in a change of the proportion between the BX present in the mycelium and in the medium. If the solid medium cultures (preincubated without BX) were transferred on a herbicide-containing medium as late as during the exponential phase of growth (cf. Fig. 1), a more rapid uptake of BX could be seen. The final result, however, was the same as in Fig. 3.

No degradation products of BX were detected in the submerged cultures by chromatography.

Effects of KCl and NaCl on growth of P-14 and degradation of BX. The inhibition effect of BX decreased after 1 % KCl was added to the medium (growth yield and antibiotic activity increased by 20 to 50 %, sporulation efficiency also increased, *etc.*). At the same time, the uptake and degradation of BX slowed down. After 5 d about 90 % of BX, which was not decomposed, remained in the cultures, a part of it in the medium (24 %). The addition of 1 % NaCl affected, in a positive way, mainly growth and sporulation of the control cultures but its effect on the degradation of BX was less pronounced than that of KCl. The uptake of BX from the medium slowed down but the degradation was reduced by only 8 % (Table III). In the presence of KCl most of the intact BX in the mycelium accumulated in the cell wall but no changes in ultrastructure of the cell wall were observed.

DISCUSSION

The results of this work not only confirmed a previous finding that cultures of *S. felleus* P-14, if grown on a solid medium containing BX, were able to decompose as much as 50 % of the total amount of the herbicide but they also showed that (1) the degradation process was confined to the phase of exponential growth, (2) the degradation was preceded by the uptake (adsorption, absorption) of the herbicide by the cells, (3) the uptake of BX was always greater than the degradation of the compound, (4) imbalance between the rates of the two processes was increased in the presence of potassium or sodium ions, (5) the degradation of BX occurred even in the submerged culture.

A more rapid uptake of BX in submerged cultures might result from a more efficient adsorption by the mycelial pellets caused by a direct contact of their relatively large surface with the environment. This capability, however, did not influence the final degradation.

A simple cultivation technique on cellophane discs provided general information on the uptake of BX and the localization of the nondegraded remainder of the compound in the solid medium cultures. Though the morphological differentiation of these cultures excluded all the possibilities of a direct interaction of the aerial mycelium with the herbicide in the medium, the intact BX accumulated both in the primary and secondary mycelium. We can hypothesize that the cells of the lower layer of the substrate mycelium served as a pump taking up the herbicide across cellophane from the medium. BX was then translocated across the cell septum to the

later formed aerial hyphae in the same way as, generally, herbicides were supposed to penetrate through plant tissues (Ashton and Crafts 1973). If this is true (*i.e.* if BX is not excreted later from the cells and adsorbed on its dry outer surface), it follows from Table I that the intact BX accumulated mostly in the cytoplasm where it was not firmly bound and could be partly washed out of the intact cells. With respect to the fact that BX exhibits a typical "salting-in" effect (Edsall and Wyman 1958), *i.e.* its weak solubility in water is increased in the presence of salts (Carpenter *et al.* 1964; Smith 1971), we chose KCl and NaCl solutions as the washing agents (in comparison with water). As compared with NaCl acting as expected, KCl also helped us to wash out BX from the cytoplasm but, on the other hand, significantly increased the binding of the herbicide to the cell wall. The BX content in the cell wall also increased during growth in the medium to which KCl was added. At the same time, however, the rate of BX degradation decreased (Table III). Though the total concentration of the herbicide in the mycelium under these conditions was higher by 20 % than in the cultures grown in the medium without KCl, the inhibitory effect on the morphological properties and physiological activity of the cultures was decreased. These results suggest that (1) the intracellular dioxygenase known to decompose BX in P-14 (Neužil, to be published) can attack only free molecules of the herbicide in the cytoplasm and not those bound in cell wall; (2) the cytoplasmic portion of BX is primarily responsible for BX toxicity; (3) growth improves if the inhibitory effect of BX is weak; consequently, the number of cells capable of binding BX increases; (4) since a 4-fold concentration of BX remains in the soil in comparison with the medium without KCl, a limited (possibly by the number of the BX receptor sites) capacity of the cell wall for the binding of BX is indicated.

The mechanism of binding the herbicide to the cell wall cannot be explained on the basis of our results.

The preceding papers (Krištůfek and Blumauerová 1983*a,b*) showed that *Streptomycetes* (both fresh soil isolates and collection strains) were very sensitive to BX and did not grow at as low concentrations as 80 μg/mL (and even lower). *S. felleus* P-14 isolated from soil, the only strain of the total of about 100 tested, tolerated as high a concentration as 400 μg BX per mL. A four times lower concentration of BX, however, already slowed down growth, affected morphological properties and decreased the physiological activity of the strain P-14, in the same way as the sublethal herbicide concentrations did in the case of the sensitive strains (Krištůfek and Blumauerová 1983*a*). Recent studies of the other microorganism capable of decomposing BX, a *Flexibacterium* designated BR4 (Cullimore and Kohout 1974; Smith and Cullimore 1974), focused on the degradation process itself, has not yet yielded any data on the effect of BX that would enable us to compare the results. It cannot be excluded that during the interaction between BX and the microbial cultures, mechanisms similar to those of the plant—herbicide interaction can be involved, *e.g.* the inhibition of oxidative phosphorylation and electron transport in the respiratory chain (cf. Ashton and Crafts 1973). Some phenomena observed in this work (a higher sensitiviy of spores to BX in the liquid medium, suppressed dehydrogenase activity, *etc.*) suggest an effect of BX on oxidative metabolism. However, precise biochemical techniques will be necessary to explain these observation.

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The Plates will be found at the end of the issue.

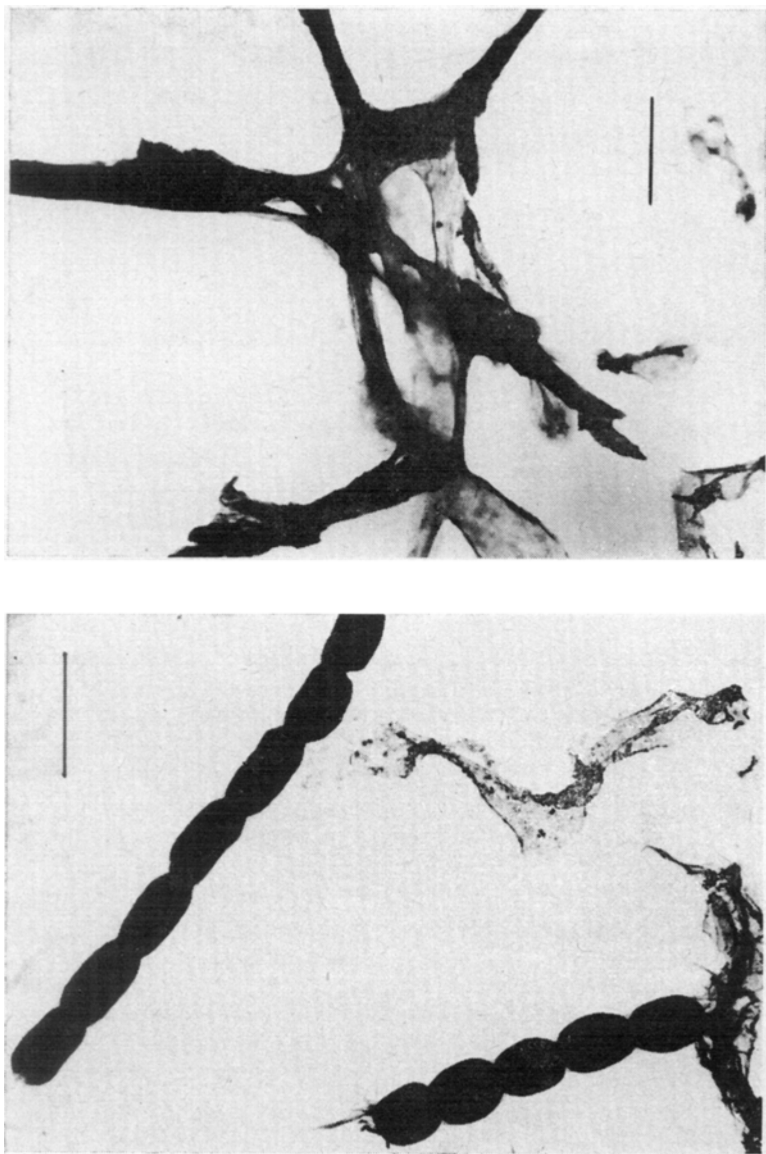


PLATE 1. Transmission electron microscopy of aerial hyphae of *S. felleus* grown 10 d on solid media nonsupplemented (*left*) and supplemented (*right*) with BX (100 μg/mL). Cultivation conditions, see Fig. 1. Bars represent 1 μm.

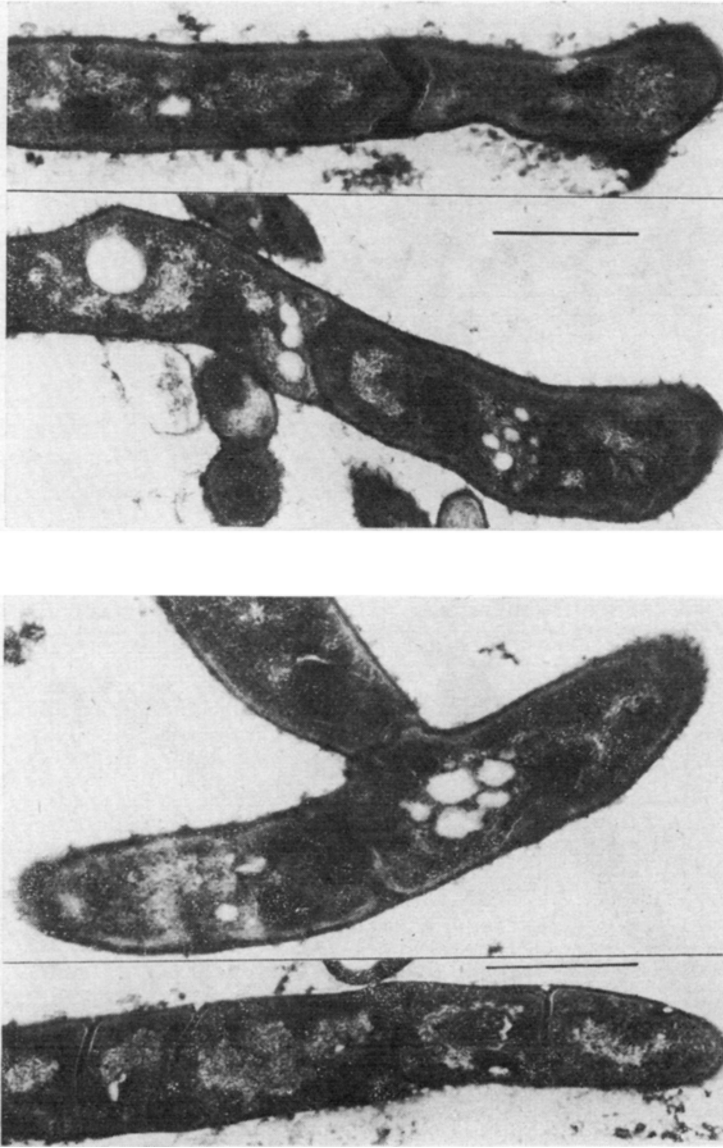


PLATE 2. Cell ultrastructure of *S. felleus* grown 5 d on solid media non-supplemented (*top*) and supplemented (*bottom*) with BX (100 μg/mL). Cultivation conditions, see Fig. 1. Bars represent 0.5 μm. Note the increased occurrence of electron-transparent vesicles in cells grown on BX-containing media.

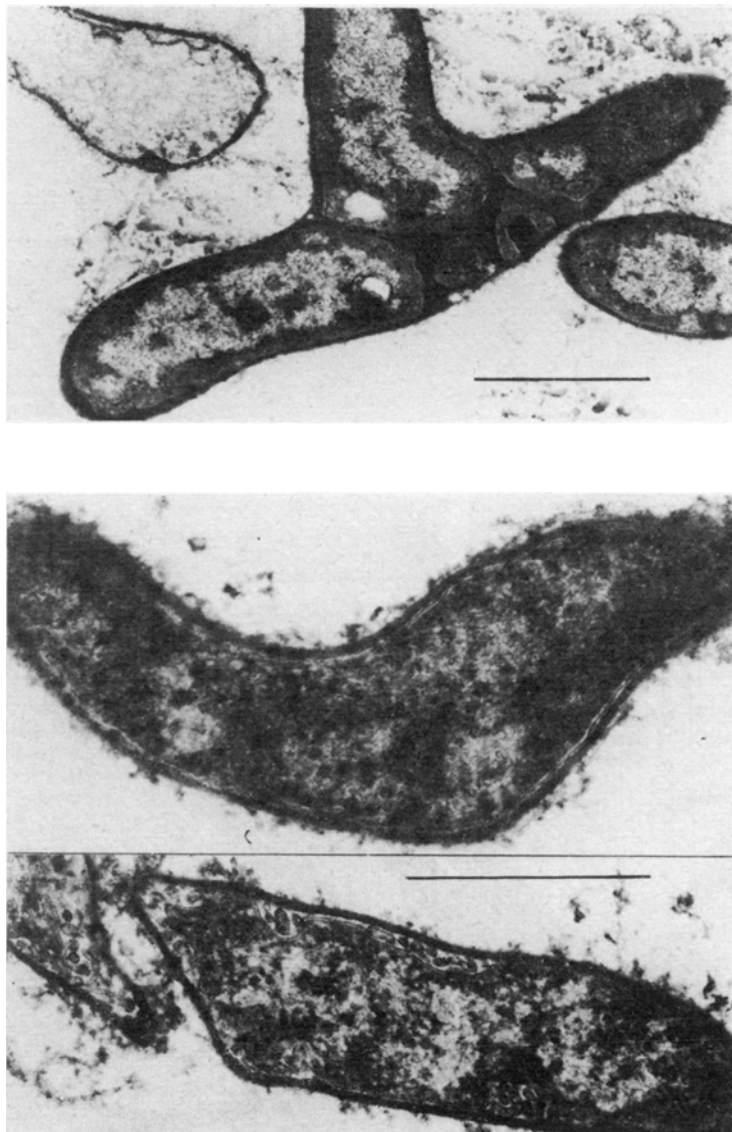
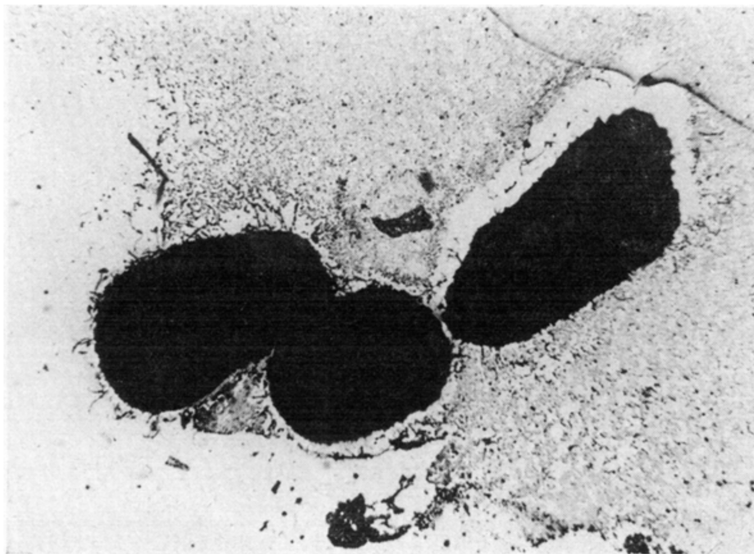


PLATE 3. Cell ultrastructure of *S. felleus* grown 5 d on solid media supplemented with BX (100 μg/mL). Cultivation conditions, see Fig. 1. Bars represent 0.5 μm. Note the irregular septation (*top*) and a high number of electron-dense bodies (*bottom*) in comparison with the control cells (Plate 2, *top*).



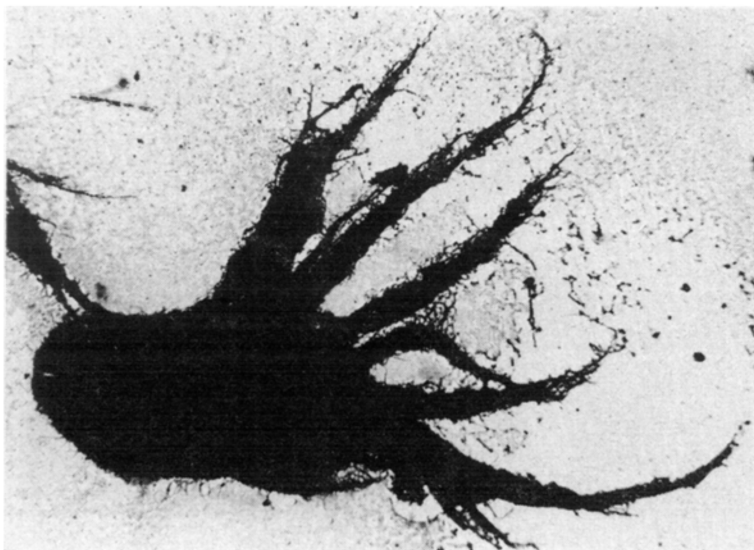


PLATE 4. Morphology of submerged mycelium of a 5-d culture of *S. felleus* grown in media non-supplemented (*top*) and supplemented (*bottom*) with BX (100 μg/mL). Cultivation conditions, see Fig. 2. The pellet length was about 1 to 2 mm in the control medium, and 4 to 6 mm in the BX-containing medium. The preparations for optical microscopy were stained with carbolfuchsin.