Sterols and the Sensitivity of *Pythium* Species to Filipin¹

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ABSTRACT

SCHLOSSER, ECKART (University of Illinois, Urbana), AND DAVID GOTTLIEB. Sterols and the sensitivity of *Pythium* species to filipin. *J. Bacteriol.* **91**:1080-1084. 1966.—The growth of several *Pythium* species was not affected by filipin. No leakage of inorganic phosphate was observed after treatment with the antibiotic. No sterol could be detected in 1 g (dry weight) of mycelium. Thus, the insensitivity of these fungi to the antibiotic may be explained by the lack of sterols, the postulated reaction site for filipin in the cell membrane. Though not capable of synthesizing sterols, *Pythium* species can incorporate exogenous sterols, which renders them sensitive to filipin; such treatment causes a lag in growth and leakage of inorganic phosphate. The leakage after filipin treatment is indirect evidence that the sterols have been incorporated into the cell membrane. Induced sensitivity to filipin was reversible; it was lost when the sterols were diluted out by one transfer through a medium free from sterols. The hypothesis that the primary site of interaction of filipin is the sterol located in the cell membrane was strengthened by these studies. The experiments further demonstrated a change in sensitivity of a fungus to a toxic agent due to nutritional conditions.

Filipin inhibited growth and respiration of fungi (7, 8). This inhibition was attributed to an alteration of the selective permeability of the cell membrane, which resulted in leakage of cell constituents (2, 7). Bovine erythrocytes were rapidly hemolyzed by filipin, again indicating a change in selective permeability. The hemolysis was accompanied by an increased release of cholesterol into the medium (Schlosser and Gottlieb, *unpublished data*). Furthermore, filipin formed a specific complex with sterols, such as ergosterol and cholesterol (6, 7, 8). Based on these results, we postulate that the principal site of action of filipin is the sterol located in the cell membrane. This postulate implies that only those organisms which contain sterols in their cell membranes would be sensitive to filipin.

Most fungi are very sensitive to filipin and other antifungal polyenes. Species belonging to the *Pythiaceae* have been reported insensitive to one of the polyenes, pimarinin (4, 12). Since we were interested in the mode of action of filipin, we investigated its effect on several *Pythium* species

and the role which sterols play in the sensitivity of these organisms to the antibiotic.

MATERIALS AND METHODS

All *Pythium* species were obtained from the collection of A. L. Hooker (University of Illinois). The *Rhizoctonia solani* isolate was the one used by Van Etten (Ph.D. Thesis, University of Illinois, Urbana, 1965).

The basic medium contained (per liter): glucose, 10 g; Difco yeast extract, 2 g; and a trace element solution, 2 ml. The trace element solution was composed of (milligrams per liter): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 500; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 4.4; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.75; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 1.8; CaCl_2 , 4.5; NaCl , 2.6. The pH of the basic medium was 6.3. When solid medium was desired, 17 g of agar was added per liter.

The fungi were grown on agar plates containing the basic medium. Mycelial discs (8.5-mm, diameter) were cut with a cork-borer from 48-hr cultures. These were then incubated in 50 ml of a 1% glucose solution containing various concentrations of filipin. After incubation at 26 C on a reciprocal shaker (80 strokes per minute), the discs were transferred to agar plates free from the antibiotic. Growth was determined as diameter (millimeters) of a colony after 20 hr at 26 C.

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For leakage experiments, the fungi were grown in 500 ml of liquid medium in 1-liter flasks. Mycelium from the phase of active growth from each flask was collected by filtration through a milk filter (Kendall Co., Walpole, Mass.) and was washed twice with 100 ml of 1% glucose solution. A 1.5-g amount (wet weight) of mycelium was placed in 50 ml of 1% glucose and was incubated on a reciprocal shaker at 26 C for 45 min to make a uniform suspension. Filipin, dissolved in dimethylformamide, was added at a concentration of 200 $\mu\text{g}/\text{ml}$. After 2 hr of incubation on the shaker, the mycelium was collected by filtration through a Whatman no. 41 filter, and 1.5 ml of the filtrate was used for the determination of inorganic phosphate by a modified Fiske-SubbaRow method (3).

To determine the sterol content, 1 g of dried mycelium was saponified in the absence of light with 50 ml of 20% KOH in 70% methanol for 3 hr. The mixture was extracted once with 100 ml of petroleum ether (30 to 60 C). The solvent was evaporated, and the residue was redissolved in chloroform. Sterol concentration was determined by the Liebermann-Burchard method (13).

To determine the uptake of cholesterol by *P. paroecandrum*, the fungus was grown on the basic medium to which cholesterol had been added at a concentration of 200 $\mu\text{g}/\text{ml}$. The mycelia were collected on milk filters after 48 hr of incubation at 26 C on a rotary shaker. Excess cholesterol was eliminated by thoroughly washing the mycelium with 3 liters of distilled water. The cholesterol content per gram (dry weight) of mycelium was then determined by the Liebermann-Burchard method.

RESULTS

Differences in inhibition by filipin were striking when mycelial discs of *R. solani* and *P. ultimum* were incubated in different concentrations of filipin for 30 min and then transferred to agar plates which were free from the antibiotic. After 20 hr, *R. solani* was inhibited about 50% by 2.5 μg of filipin per ml, and completely by 10 to 25 $\mu\text{g}/\text{ml}$. On the other hand, *P. ultimum* was not inhibited by concentrations as high as 100 $\mu\text{g}/\text{ml}$ (Fig. 1). In a similar experiment, but with 1 to 5 hr of incubation and 200 μg of filipin per ml, all the *Pythium* species remained unaffected (Table 1). The insensitivity of *Pythium* species to filipin is further demonstrated by the fact that filipin treatment did not cause any increased leakage of inorganic phosphate, suggesting that no cell membrane alteration had occurred (Table 2).

Pythium species apparently cannot synthesize sterols in glucose-yeast extract medium in shake culture. No sterol was detected in 1-g samples of dried log-phase mycelium. This is in sharp contrast to *R. solani*, which had 0.1% of its dry weight (1,300 μg) as sterols, a value which is in good agreement with that reported by Van Etten

FIG. 1. Inhibition of radial growth of *Rhizoctonia solani* (○) and *Pythium ultimum* (●) by filipin.

(Ph.D. Thesis, University of Illinois, Urbana, 1965) for stationary culture.

If the presence of sterols was responsible for the inhibition of fungi by filipin, then an incorporation of sterols into *Pythium* species might render these sensitive to the antibiotic, resulting in inhibition of growth and leakage of cell constituents. Mycelial discs of *P. ultimum* which were grown in the presence of cholesterol and then incubated with filipin showed a distinct lag period of 4 hr before growth began (Fig. 2). After this period, the fungus resumed growth at the same rate as the control. The same phenomenon was observed for *P. graminicolum* and *P. paroecandrum* when they were grown on a cholesterol-containing medium. *P. irregulare*, when grown on a medium containing cholesterol, ergosterol, stigmasterol, or sitosterol, gave a similar response after filipin treatment. This growth retardation is an indication that the fungi had become sensitive to filipin. The change in sensitivity was confirmed in leakage experiments, where a considerable release of inorganic phosphate occurred after treatment with the antibiotic (Table 2). There is

TABLE 1. Inhibition* of radial growth of *Pythium* species and *Rhizoctonia solani* by filipin

Organism	Growth after filipin treatment/growth of control†				
	1 hr‡	2 hr	3 hr	4 hr	5 hr
<i>P. graminicolum</i>	37/40	40/38	37/38	40/39	37/38
<i>P. paroecandrum</i>	36/37	39/37	40/39	39/39	40/40
<i>P. ultimum</i>	41/41	41/41	44/44	44/45	44/44
<i>R. solani</i>	0/33	0/37	0/36	0/33	0/36

* Averages of three replicates in each of two experiments.

† Growth expressed as diameter (in millimeters) of colony.

‡ Mycelial discs were incubated in 200 µg of filipin per ml for 1 to 5 hr, then transferred to agar plates free from the antibiotic. Growth was determined after 20 hr.

TABLE 2. Release of inorganic phosphate from mycelium of *Pythium* species after filipin treatment

Organism	Cholesterol in the growth medium	Filipin in the suspension medium	PO ₄ * released	Ratio of treated to untreated
	µg/ml	µg/ml	µ/moles	
<i>P. graminicolum</i>	0	0	0.040	1.63
	0	200	0.065	
	200	0	0.150	
<i>P. irregulare</i>	200	200	8.500	56.7
	0	0	1.080	
	0	200	1.305	
<i>P. paroecandrum</i>	200	0	0.700	11.4
	200	200	8.000	
	0	0	0.370	
<i>P. ultimum</i>	0	200	0.330	0
	200	0	0.125	
	200	200	11.200	
<i>Rhizoctonia solani</i>	0	0	0.650	29.1
	0	200	0.535	
	200	0	0.210	
<i>Rhizoctonia solani</i>	200	200	6.100	40.0
	0	0	0.080	
	0	200	3.200	

* Values refer to 1.5 g (wet weight) of mycelium and are averages of three replicates.

apparently no marked specificity in the ability of the four major naturally occurring sterols to alter the resistance of *Pythium* to filipin (Table 3).

These results suggested that exogenous sterols were taken up by *Pythium* species and presumably incorporated into their membrane. Sterol analysis showed that cholesterol was taken up and incorporated into *P. paroecandrum*. Control cultures contained no detectable sterol, whereas the quantity in the mycelium grown on cholesterol was 163 to 188 µg/g (dry weight). This value is in the lower range of the normal sterol content of fungi (1).

The induced sensitivity of *Pythium* species to filipin because of sterols was reversible. After one

FIG. 2. Change in sensitivity of *Pythium ultimum* to filipin when grown in the presence of cholesterol. Mycelial discs (diameter, 8.5 mm), obtained from cultures grown on a basic medium in the absence or presence of cholesterol (100 µg/ml), were incubated in 200 µg of filipin per ml for 5 hr. They were then transferred to agar plates free from antibiotic and cholesterol. Growth was determined at 1-hr intervals.

transfer through a medium free from sterols, *P. irregulare* was again resistant. Probably the sterol was diluted out.

DISCUSSION

The hypothesis for the action of filipin is that it complexes with cell membrane sterols to alter the selective permeability (7, 8). That *Pythium* species are different from other fungi in their reaction to filipin was at first puzzling; we presumed that all fungi contain sterols. The failure of the antibiotic

TABLE 3. Release of inorganic phosphate after filipin treatment from *Pythium irregulare* grown on different sterols

Exogenous sterol	Fil pin	PO ₄ released*
		<i>μmoles</i>
—	—	1.08
—	+	1.17
Cholesterol	—	0
Cholesterol	+	7.50
Stigmasterol	—	0
Stigmasterol	+	8.16
Sitosterol	—	0
Sitosterol	+	8.32
Ergosterol	—	0
Ergosterol	+	9.15

* Values refer to 1.5 g (wet weight) of mycelium and are averages of three replicates.

to inhibit the growth of *Pythium* species was shown also by the inability of filipin to alter cell permeability. The absence of sterols in the membranes of these fungi now make the entire phenomenon understandable. *Pythium* neither synthesizes nor requires sterols for its normal cell membrane activities; thus, there is no vital sterol mechanism with which filipin could interfere.

The concept of the role of sterols in inhibition by filipin is further strengthened by the data showing that when *Pythium* species were grown on sterols they incorporated them into the cell, presumably into their cell membranes. After transfer through a normal medium, this sterol is probably diluted out by the synthesis of other cell materials, since sterols cannot be synthesized by these fungi. The organism then naturally returns to its original insensitivity to the antibiotic. The conversion to sensitivity was, however, never complete, and involved only a 4-hr lag period, after which all species resumed growth at the same rate as the controls. The present knowledge on the structure and the function of the lipid part of the cell membrane does not offer a basis for a discussion on how the filipin effect is overcome.

Although *Pythium* species do not require sterols to grow, these compounds do play a role in the life cycle of these organisms. Haskins et al. (9) and Hendrix (10, 11) found that sexual reproduction of *Pythium* species could only be achieved by addition of sterols to the growth medium.

Results similar to those on *Pythium* species have been obtained with *Mycoplasma* by Weber and Kinsky (14) and Feingold (5), who found that the bacterium *M. laidlawii*, which cannot synthesize sterols, is insensitive to antifungal polyenes. When this microorganism was grown on

a sterol-containing medium, it incorporated the exogenous sterol and became sensitive to polyene antibiotics.

That the specific site for filipin action in the cell membrane is on its sterol is supported by experiments with bovine erythrocytes, in which filipin treatment caused the release of cholesterol from the cell membrane (Schlosser and Gottlieb, unpublished data).

The results also show that the sensitivity of a fungus to a toxic agent could be changed by nutritional conditions, so that resistance can be converted to susceptibility and vice versa.

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