Protection of *Pythium* Species Against Antibacterial Antibiotics by Cholesterol

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ABSTRACT

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Cholesterol (10 μg/ml) decreased the growth inhibitory action of tetracycline, erythromycin, and chloramphenicol against *Pythium ultimum* and *P. debaryanum* on a defined glucose-amino acid agar medium. For example, at 10 μg/ml of tetracycline inhibited radial growth almost completely in the absence of cholesterol but only 30–35% in the presence of cholesterol. Similarly, 50 μg/ml of erythromycin and 100 μg/ml of chloramphenicol reduced growth 70–75% in the absence of the sterol but only 20–35% in the presence of cholesterol. Cholesterol also significantly reduced the growth inhibitory effects of the antibiotics in stationary liquid culture, with growth measured as dry weight increase. About four to five times as much antibiotic was needed to give the same degree of growth inhibition in the presence of cholesterol. When tested against tetracycline, which impaired growth of both species even at 1 μg/ml in the absence of cholesterol, the effect of cholesterol in reversing inhibition was detectable at 0.03 μg/ml (about 10^{-7} M) and above but not at 0.01 μg/ml. The very small stimulation of growth by cholesterol in mycelia not treated with antibiotics was too small to account for the protective effect of cholesterol.

Members of the genera *Pythium* and *Phytophthora* differ from most other fungi in their inability to synthesize sterols (2,9), apparently because of inability to epoxidize squalene (31). Although these fungi can grow without sterols, exogenous sterols exert several striking effects in them. Sterol is required for sexual reproduction and for the production of functional zoosporangia (9). Sterols stimulate hyphal growth of many species and isolates (2,3,10,17,32) and have been reported to improve tolerance of temperature extremes (5,26). Sterol reduces leakiness of cells (1,27) and increases glucose utilization in some species (25). Further, these fungi, which typically are not sensitive to polyene antifungal antibiotics in the absence of sterol, become sensitive to a degree on media containing sterol (1,24,27). This polyene:sterol connection presumably reflects a role of sterols in cell membrane structure and function (2,14,20). The means by which other sterol effects are exerted are not known but may also involve changes in permeability. The mode of action of sterols in inducing sexual reproduction probably differs from that involved in alterations of vegetative physiology (2,9).

Pythiaceous fungi also differ from most other fungi in being quite sensitive to 70S ribosome-active antibiotics such as tetracyclines, chloramphenicol, streptomycin, and erythromycin (15,16,21,28). It is likely that any of these would inhibit growth by interfering with mitochondrial protein synthesis if it gained access to the cell interior. Unlike the cytoplasmic ribosomes in eukaryotic cells, mitochondrial ribosomes are of the prokaryotic type and, therefore, are impaired by such antibiotics (23). However, the matter of access to the cell interior has received little attention

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(21,30), despite the fact that all fungal groups, not just Pythiaceae (assorted exceptions aside), would be subject to growth inhibition via antibiotic action at the mitochondrial ribosome unless the antibiotics were excluded from the cell or rendered inactive. Some of these antibiotics may have more than one mode of action in pythiaceous fungi (21).

In view of the many and diverse ways in which sterols seem to affect these fungi, we investigated the possibility that sterol could modify the sensitivity of *Pythium* species to these ribosome-active antibacterial antibiotics. Cholesterol was chosen as test sterol because it has been used frequently as a representative sterol in studies of these fungi.

MATERIALS AND METHODS

The isolate of *Pythium ultimum* Trow is the one studied in earlier work (21,22). The isolate of *Pythium debaryanum* Hesse was obtained from Carolina Biological Supply Co. (Burlington, NC). The identification as *P. debaryanum* was made by the supplier; we made no attempt to resolve an apparent uncertainty about the taxonomic status of the specific epithet (6,7).

The fungi were grown in a defined medium similar to one used previously (22). It contained, per liter: 10 g of glucose, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 0.2 ml of Vogel trace element solution (29), 0.24 g of arginine, 0.175 g of aspartic acid, 0.33 g of glutamic acid, 0.69 g of glycine, 0.13 g of lysine, 0.095 g of valine, 0.030 g of histidine, 0.060 g of isoleucine, 0.027 g of methionine, 0.048 g of threonine, 0.015 g of tryptophan, 0.069 g of tyrosine, 0.010 g of $CaCl_2 \cdot 2H_2O$, 1.36 g of KH_2PO_4, and 1.74 g of K_2HPO_4. This medium provided, per liter, 4 g of carbon as glucose, about 0.77 g of carbon as amino acid, and 0.32 g of nitrogen as amino acid.

All components of the medium, except the two phosphates, were dissolved in distilled water at 90% of final volume; the two phosphate salts were dissolved together in distilled water at 10% of final volume. These two solutions were adjusted to pH 6.4, autoclaved in separate flasks (10 min, 121 C), and combined after cooling to yield the complete medium. To solidify the medium, bacto agar (Difco Laboratories, Inc., Detroit, MI) was added to the glucose-amino acid portion (solution) of the medium to a final agar concentration of 1.5%. On removal from the autoclave, the 500-ml Erlenmeyer flasks containing 225 ml of the glucose-amino acid portion of the medium were swirled to distribute the dissolved agar and then cooled to 45 C; the phosphate solution, 25 ml at 45 C, was then added to the agar solution.

Cholesterol was dissolved in *N,N*-dimethylformamide (DMF) and added to the complete, sterile medium at 45 C at the rate of 0.25 ml of DMF per 250 ml of medium. Controls received the same amount of DMF without cholesterol. Antibiotics were dissolved in the sterile, complete medium at 45 C. Agar media were put into 9-cm-diameter petri dishes, 30–35 ml per dish. In tests using stationary liquid culture, the complete medium without agar was dispensed into 125-ml Erlenmeyer flasks, 20 ml per flask. Each flask or petri dish received one 5-mm-diameter agar disk taken from the edge of petri dishes containing 4-day-old colonies, which covered the entire agar surface at the time of disk removal, with a cork borer. These stock agar cultures had the same medium described above, without cholesterol, except that the concentrations of glucose and amino acids were reduced by two-thirds to decrease development of aerial mycelium. In most tests, the agar disk was placed at the edge of the petri dish, in others, at the center of the dish. In all tests on agar medium, the agar disk of mycelium was placed such that the fungus on the disk surface was in contact with the surface of the fresh medium.

Test cultures were kept at 22–25 C. No precautions were taken to exclude light or to regulate the amount of light to which cultures were exposed. In general, cultures received about 14 hr of fluorescent light daily from lab ceiling fixtures.

Mycelial dry weights were determined in stationary liquid culture tests by blotting the mycelial mats on paper towels and oven-drying them (90 C) in tared containers. These values were corrected for the weight of the agar disk of mycelium. Values for radial growth on agar reflect new growth from the edge of the agar disk; four measurements were taken for each colony, and the average was rounded to the nearest millimeter. Results shown in the tables are representative of those obtained in at least three tests.

Tetracycline (free base), chloramphenicol, erythromycin (Grade I: approximately 98%), and cholesterol (99+%) were obtained from Sigma Chemical Co., St. Louis, MO.

RESULTS

In preliminary experiments that used the highest concentrations of antibiotics shown in Table 1, cholesterol at 10–40 μg/ml gave similar results, of the kind shown in Table 1, regardless of whether cholesterol was added to the medium before or after autoclaving, as a solid (with vigorous stirring or not), or as a dimethylformamide solution. Cholesterol was not completely soluble in the medium at 10 μg/ml and above. All three antibacterial antibiotics tested inhibited radial growth of both *Pythium* spp. on agar medium without added sterol (Table 1). With each antibiotic the degree of growth inhibition was concentration dependent. Tetracycline was especially inhibitory. Addition of cholesterol to the medium significantly improved radial growth in both species in every case in which growth had been inhibited in the absence of added sterol (Table 1). This effect was striking in many instances; for example, the virtual abolition of growth by 10 μg/ml of tetracycline was replaced by substantial growth, 65–70% of control. In contrast to these large growth improvements, radial growth of colonies on agar without antibiotic increased only slightly, 7–15% among several tests, in response to added cholesterol. Each antibiotic caused visible reductions in colony density. Antibiotic-treated colonies appeared to have less aerial mycelium and less hyphal branching on the agar than did colonies not treated with antibiotic. Cholesterol also relieved this aspect of growth inhibition. Antibiotic-treated colonies were visibly denser in the presence of cholesterol.

TABLE 1. Effects of cholesterol (10 μg/ml) and 70S ribosome-active antibiotics on radial growth of *Pythium* on a defined glucose-amino acid agar medium

		P. debaryanum				*P. ultimum*			
		No sterol		Plus sterol		No sterol		Plus sterol	
Antibiotic	μg/ml	Growth[a] (mm)	Control (%)	Growth[a] (mm)	Control (%)	Growth[a] (mm)	Control (%)	Growth[a] (mm)	Control (%)
None	···	63 ± 0.7	100	68 ± 0.6	100	46 ± 0.3	100	51 ± 1.0	100
Tetracycline	1	52 ± 0.3	83	68 ± 0.3	100	35 ± 0.9	76	52 ± 1.2	102
	5	20 ± 1.5	32	57 ± 0.7	84	12 ± 1.0	26	44 ± 0.9	86
	10	5 ± 0.9	8	47 ± 0.7	69	1 ± 0.0	2	33 ± 0.7	65
Erythromycin	10	39 ± 0.9	62	61 ± 0.9	90	25 ± 0.9	54	48 ± 0.9	94
	25	26 ± 1.5	41	57 ± 0.7	84	15 ± 1.2	33	40 ± 0.3	78
	50	17 ± 0.7	27	50 ± 1.2	74	12 ± 0.9	26	32 ± 0.6	63
Chloramphenicol	25	41 ± 0.7	65	59 ± 0.3	87	37 ± 0.6	80	48 ± 0.3	94
	50	29 ± 0.6	46	51 ± 0.3	75	28 ± 0.9	61	45 ± 0.9	88
	100	15 ± 0.9	24	45 ± 0.3	66	15 ± 0.9	33	40 ± 0.3	78

[a] Mean colony radii (mm) and standard errors of triplicate colonies after 63 hr of incubation.

TABLE 2. Effects of cholesterol (10 μg/ml) and 70S ribosome-active antibiotics on dry weight increase of *Pythium* in stationary cultures on a defined glucose-amino acid liquid medium

		P. debaryanum				*P. ultimum*			
		No sterol		Plus sterol		No sterol		Plus sterol	
Antibiotic	μg/ml	Growth[a] (mg)	Control (%)	Growth[a] (mg)	Control (%)	Growth[a] (mg)	Control (%)	Growth[a] (mg)	Control (%)
Tetracycline	0	115 ± 2.0	100	116 ± 1.5	100	106 ± 4.3	100	118 ± 3.7	100
(Expt. 1)	1	40 ± 2.0	35	105 ± 5.0	91	35 ± 1.5	33	92 ± 3.1	78
	5	4 ± 0.7	3	38 ± 2.9	33	5 ± 0.6	5	42 ± 1.5	36
	10	ND		30 ± 2.4	26	ND		36 ± 0.6	31
	25	ND		14 ± 0.9	12	ND		25 ± 2.0	21
Erythromycin	0	121 ± 1.5	100	124 ± 2.6	100	89 ± 2.5	100	96 ± 3.3	100
(Expt. 2)	25	18 ± 0.7	15	62 ± 0.7	50	10 ± 0.3	11	32 ± 0.6	33
	50	6 ± 0.3	5	48 ± 1.7	39	5 ± 1.0	6	29 ± 2.1	30
	100	ND		31 ± 2.2	25	ND		11 ± 1.0	11
	250	ND		3 ± 0.0	2	ND		2 ± 0.3	2

[a] Mean dry weights (mg) and standard errors of triplicate 4-day-old cultures. ND: not determined.

TABLE 3. Effect of cholesterol concentration on growth of *Pythium* on a defined glucose-amino acid agar medium containing tetracycline (TET, 5 μg/ml)

	Colony radius (mm)[a]			
	P. debaryanum		*P. ultimum*	
Cholesterol (μg/ml)	No TET	Plus TET	No TET	Plus TET
0	51 ± 0.9 (73 ± 2.1)	18 ± 0.7 (34 ± 0.3)	35 ± 0.0 (60 ± 1.2)	12 ± 0.6 (17 ± 0.3)
0.01	52 ± 0.9 (71 ± 1.5)	19 ± 0.9 (34 ± 0.7)	37 ± 0.0 (62 ± 0.3)	13 ± 0.9 (18 ± 0.7)
0.03	52 ± 0.6 (74 ± 1.5)	24 ± 1.2 (43 ± 1.5)	35 ± 0.7 (60 ± 1.5)	16 ± 1.5 (27 ± 2.1)
0.10	52 ± 0.0 (74 ± 2.7)	31 ± 0.7 (52 ± 0.3)	37 ± 0.3 (65 ± 2.3)	20 ± 1.7 (39 ± 1.8)
0.30	53 ± 0.3 (78 ± 2.3)	36 ± 0.7 (58 ± 0.6)	37 ± 0.3 (67 ± 1.2)	29 ± 0.6 (48 ± 1.2)
1.00	54 ± 0.3 (ND)	38 ± 0.3 (ND)	38 ± 0.0 (ND)	32 ± 1.2 (ND)

[a] Mean colony radius (mm) and standard error of triplicate colonies after 50 and 75 hr (in parentheses) incubation. ND: not determined.

TABLE 4. Effect of cholesterol concentration on growth of *Pythium* in a defined glucose-amino acid liquid medium containing tetracycline (TET, 1 μg/ml)

	Mycelium dry weight (mg)[a]			
	P. debaryanum		*P. ultimum*	
Cholesterol (μg/ml)	No TET	Plus TET	No TET	Plus TET
0	124 ± 2.8	23 ± 0.9	97 ± 5.8	13 ± 0.3
0.01	121 ± 1.8	23 ± 1.8	105 ± 4.6	14 ± 1.2
0.03	121 ± 1.8	32 ± 2.2	102 ± 3.3	27 ± 3.1
0.10	118 ± 1.9	50 ± 0.9	108 ± 1.7	45 ± 2.6
0.30	121 ± 1.5	64 ± 2.3	105 ± 1.5	74 ± 2.3
1.00	123 ± 1.5	72 ± 1.9	101 ± 2.6	97 ± 1.7

[a] Mean dry weight (mg) and standard error of triplicate stationary liquid cultures of *P. debaryanum* after 96 hr or *P. ultimum* after 144 hr.

Estimates of the extent to which cholesterol relieved antibiotic reductions in total mycelial mass were obtained by assaying the antibiotics against the fungi in stationary liquid culture. The results of these tests confirm those of the agar tests. In the absence of cholesterol, tetracycline and erythromycin strongly inhibited growth, measured as dry weight, of both *Pythium* species (Table 2). Inhibition of *Pythium* in liquid culture at 1 μg/ml of tetracycline and 25 μg/ml of erythromycin was greater than observed in the radial growth experiments. This is consistent with the observation of antibiotic-induced reductions in colony density on agar. Cholesterol significantly improved the growth of cultures exposed to tetracycline or erythromycin. However, in cultures that were not exposed to antibiotic, cholesterol did not significantly affect dry weight (Table 2). In both agar and stationary liquid tests, about four to five times as much antibiotic was required to produce the same degree of growth inhibition when 10 μg/ml of cholesterol was present.

In subsequent tests, the sterol concentration was reduced in an effort to find the minimum protective dosage. In agar assays, 5 μg/ml was selected as the tetracycline dosage on the basis of previous results (Table 1). The protective effect of cholesterol at 0.03–0.1 μg/ml against tetracycline could be seen consistently as an increase in radial growth (Table 3); increased density of the tetracycline-treated colonies also was evident at the low cholesterol levels. Colonies not grown on tetracycline-amended medium showed no increase in radial growth in response to this level of cholesterol. The degree of growth improvement in cultures of *Pythium* grown on tetracycline-amended medium increased as the cholesterol concentration was raised above 0.03 μg/ml. The 2-day-old cultures that gave these results were examined again one day later, at 75 hr of growth (Table 3, parenthetic values); still the sterol effect was not detected at 0.01 μg/ml, but it remained evident at 0.03 μg/ml and above. Therefore, under the conditions of this study the capacity of cholesterol for diminishing the growth inhibitory action of tetracycline in two species of *Pythium* had a threshold near 0.03 μg/ml, about 10^{-7} M.

The same sterol threshold (0.03 μg/ml) was found when growth was measured as dry weight in stationary liquid cultures (Table 4). In liquid culture, a lower concentration of tetracycline (1 μg/ml) was used because the incubation period was about twice that used for agar cultures. Cholesterol gave no apparent relief of the tetracycline inhibition of growth at 0.01 μg/ml in either species. At 0.03 μg/ml and above cholesterol did reduce the growth inhibition by tetracycline in both species. However, in the absence of tetracycline, increasing the concentration of cholesterol did not significantly increase dry weight of either species of *Pythium*.

DISCUSSION

The mechanism by which cholesterol reverses the inhibitory action of these antibacterial antibiotics is unknown. However, the phenomenon was demonstrated in two different experimental systems. Both *Pythium* spp. had the same response in liquid and solid media. With both culture methods, cholesterol caused very little, if any, increase in growth of colonies that received no antibiotic. This was the case even with cholesterol present at a saturation level in the media (Tables 1 and 2). Therefore, the protective effect of cholesterol is not simply a matter of sterol stimulating growth of an otherwise unimpaired fungus.

Although the possibility cannot be ruled out, three points weigh against a cholesterol:antibiotic binding in the culture medium as the reason for the protective effect of cholesterol. First, it seems unlikely that cholesterol, whose structure offers limited binding opportunities, would effectively bind to three molecules as

structurally diverse as tetracycline, chloramphenicol, and erythromycin. Second, cholesterol had no detectable effect on the ultraviolet (UV) absorption spectra of tetracycline or chloramphenicol in the growth medium (Rawn and Schwarz, *unpublished*); the aromatic amino acids were deleted from the medium for these determinations. In contrast, in aqueous solution cholesterol does alter the UV absorption spectrum of filipin, one of the sterol-binding polyene antifungal antibiotics (4). Erythromycin could not be evaluated in this regard as it does not absorb appreciably in the wavelength range 250–400 nm. Third, the dosage response data from the agar assays seem inconsistent with substantial sterol: tetracycline binding. For example, tetracycline at 5 μg/ml is clearly less inhibitory in the presence of 0.1 μg/ml of cholesterol, a molar ratio of about 43 tetracycline: 1 cholesterol. If each sterol molecule bound only one tetracycline molecule, then the effective tetracycline dosage in that test would have been 4.88 μg/ml instead of 5 μg/ml, a difference much too small to be detected by this sort of assay. Therefore, each cholesterol molecule would have to bind more than one tetracycline molecule in order to effect the visible growth improvement. However, an excess of cholesterol, 10 μg/ml (25.9 nmole/ml), was inadequate to reverse the effect of tetracycline at a concentration of 5 μg/ml (11.2 nmole/ml).

Cholesterol did not serve as a carbon source for *Pythium* at the concentrations used in this study. Even at 10 μg/ml cholesterol adds only about 0.008 g of carbon per liter to the 4.77 g of carbon per liter already provided by the glucose and amino acids in the medium. Preference of *Pythium* for cholesterol as a carbon source is unlikely, because pythiaceous fungi metabolize sterols to a limited extent (10–12,18,26) and seem to use them for structural purposes rather than as respiratory substrates (2,26,27).

Hendrix (9), Elliott (2), and Nes et al (18) have summarized the evidence that the mode of action of sterols in induction of reproduction differs from that (those) by which vegetative changes are brought about in these fungi. Reported sterol effects on vegetative physiology seem to involve changes in hyphal permeability. The protective effect of cholesterol against these antibacterial antibiotics clearly is a vegetative phenomenon, but the mechanism by which the sterol acts remains to be shown.

Many studies in which sterols have been reported to stimulate growth or elicit various changes in metabolism or permeability in *Pythium* or *Phytophthora* spp. have used sterol concentrations of 10 μg/ml or greater (8,13,15,17,18,27,32). Though few have sought the sterol concentrations sufficient for incipient activity in such vegetative respects, it seems that induction of reproduction occurs at lower sterol dosages than do the various vegetative changes. The reproductive response to sterol is extremely sensitive, occurring at as little as 0.1–0.5 μg/ml, about 10^{-6} M (9). Undoubtedly sterol thresholds will vary among species, but the mitigating effect of cholesterol in our study, with threshold at 0.03–0.1 μg/ml, is about as sensitive an indicator of sterol presence as any that we have seen reported.

The low effective concentrations of cholesterol that reverse antibiotic inhibition may contribute to understanding why the sensitivity of pythiaceous fungi to antibiotics or fungicides may vary substantially from one growth medium to another. For example, Leary et al (15) found that some isolates of *Phytophthora cinnamomi* were less sensitive to streptomycin on a V-8 juice agar than on a minimal medium agar. A similar observation with *Pythium ultimum* exposed to various antibiotics on several undefined media, including one containing V-8 juice, was made several years ago by one of us (Rawn, *unpublished*). Hendrix (10) noted that a member of Pythiaceae was much less sensitive to the fungicide Dexon, sodium [4-(dimethylamino) phenyl] diazenesulfonate, on a peptone medium than on a glutamate medium. Natural products or their processed derivatives may contribute enough sterol to culture media to affect vegetative physiology. Indeed, the agar that is used to solidify media may be sterol-contaminated to some extent (19). We took no precaution to ensure that our agar was absolutely sterol-free, but the similarity of results we found with agar medium and liquid medium argues that the cholesterol threshold that we report here is not artificially low.

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