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Lipid Composition and Sensitivity of *Prototheca wickerhamii* to Membrane-Active Antimicrobial Agents

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The lipid composition of *Prototheca wickerhamii* ATCC 16529 is presented and discussed in relation to the unique susceptibility of the organism to drugs of three membrane-active antimicrobial classes: the polyenes, the polymyxins, and the imidazoles. The presence of ergosterol in the neutral lipid fraction of the membrane is likely responsible for the exquisite susceptibility to amphotericin B. The presence of a large quantity of free fatty acids in the membrane appears responsible for imidazole susceptibility. The membrane determinants of polymyxin B susceptibility are less well defined.

Biochemical study of organisms with unusual susceptibility patterns to antimicrobial agents may offer insight into their mechanisms of action. *Prototheca wickerhamii* ATCC 16529 has a very unusual pattern. It is extremely susceptible to antimicrobial agents from three classes of membrane-active agents: the polyenes (amphotericin B), the polymyxins (polymyxin B), and the imidazoles (clotrimazole and miconazole). We know of no other cells as susceptible to all these agents. Fungal and mammalian cell membranes are usually resistant to the polymyxins, although high concentrations may effect some fungal membranes (5, 20). All bacteria are resistant to the polyenes. The imidazoles are active against fungi and some gram-positive bacteria.

P. wickerhamii is an occasional pathogen in numans. Its classification is not secure. Some authorities consider *Prototheca* species to be achlorhidric algae (4), others think that they are fungi (1), and others place them in between (8). In this presentation we report the lipid composition of the organism and discuss the unique antimicrobial susceptibilities in light of the lipid chemistry. Qualitative lipid composition studies of *Prototheca* species have been reported previously (8).

MATERIALS AND METHODS

Organism cultivation. *P. wickerhamii* strain YB 4330 ATCC 16529 was grown in Sabouraud dextrose broth (Difco) and was maintained on slants of the same medium solidified with 2% agar. The pH of the medium was adjusted to 6.8 to 7.0 with 1 N NaOH before sterilization.

MICs. Serial twofold dilutions of drugs in broth were employed to determine the minimum inhibitory concentrations (MICs). The drugs examined were amSons, assayed as 927 μg/mg), polymyxin B sulfate (Aerosporin, Burroughs Wellcome Co.), miconazole nitrate (powder supplied by Johnson and Johnson), and clotrimazole (Powder supplied by Delbay Pharmaceuticals, Inc.). Standard solutions of the drugs were made in water (polymyxin B) or dimethylsulfoxide (amphotericin B and imidazoles); the latter solvent did not interfere with growth at the concentration used. A 24-h culture was diluted to give 10^7 cells per ml as determined by direct count in a Petroff-Hausser particle counting chamber, and 50 μl of this suspension was added to 2 ml of broth containing the various drug concentrations. There were 2.5×10^5 *P. wickerhamii* per ml. The tubes were incubated at 30°C and read for visible turbidity after 48 h. The MIC was the lowest drug concentration inhibiting visible growth.

Lipid extraction. Six-liter flasks containing 2 liters of broth were inoculated and shaken in a rotary shaker for 4 to 5 days at room temperature. The cells were harvested by centrifugation, washed three times with distilled water, and lyophilized. The yield of dry cells was 1.2 g/liter. A 5-g amount of lyophilized cells was suspended in 100 ml of methanol and stirred for 1 h at 80°C. A 200-ml quantity of chloroform was then added to the cooled suspension, and the mixture was stirred for 1 h at room temperature. The cells were removed by filtration and were reextracted. The combined extracts were dried in a rotary evaporator, and the residue was taken up in 100 ml of chloroform. The insoluble material was removed by centrifugation, and the supernatant was washed once with 20 ml of 0.9% NaCl solution. The chloroform phase was separated and dried under a stream of nitrogen. The final residue was dried under vacuum over NaOH pellets and weighed. The lipids were then dissolved in chloroform and stored under nitrogen at −5°C.

Fractionation of lipids. The total lipids (75 to 100 mg) were applied to a column (2.5 by 10 cm) of activated silicic acid (Unisil; 100 to 200 mesh) which was prewashed with chloroform. The column was successively eluted with 2 bed volumes of chloroform, 3 bed volumes of acetone, and finally 4 bed volumes of

methanol. Each fraction was dried under vacuum, its weight was determined, and the fraction was then dissolved in chloroform and stored under nitrogen at −5°C.

TLC. For thin-layer chromatography (TLC), lipids were chromatographed on 0.25-mm-thick layers of Silica Gel G (E.M. Laboratories, Inc.). For polar lipids, the developing systems were (i) chloroform–methanol–water (65:25:4) or (ii) chloroform–methanol–28% ammonia (65:35:5). For analysis of nonpolar lipids, the solvents used were benzene–ethyl acetate (5:1) or 1,2-dichloroethane–methanol (49:1). Phospholipids were identified by comparison with standards of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidylinositol (PI) purchased from Applied Sciences, Inc. For neutral lipids a standard containing monoglycerides, 1,2- and 1,3-diglycerides and triglycerides were used in addition to standard solutions of ergosterol and oleic acid. Lipid spots were detected by use of the following reagents: (i) iodine vapors, (ii) ninhydrin 0.2% in acetone, (iii) Hanes and Isherwood reagent (9), and (iv) charring with sulfuric acid.

Paper chromatography. Phospholipids were hydrolyzed in dilute alkali by the method of Tarlov and Kennedy (25), and the water-soluble deacylated products were chromatographed on Whatmann no. 1 filter paper in a solvent system of water-saturated phenol–ethanol–acetic acid (50:6:5). After development, the paper was thoroughly dried in a stream of air and then passed through ether to remove traces of phenol. Detection reagents used were ninhydrin, Hanes and Isherwood reagent, and Dragondorff reagent (22).

Acid hydrolysis was done with 6 N HCl at 100°C for 4 h in sealed ampoules. The cooled hydrolysates were extracted twice with petroleum ether, and the aqueous phase was concentrated by evaporation. Products were examined by paper chromatography with the solvent phenol–*n*-butanol–80% formic acid–water (50:50:3:10) saturated with solid KCl. The paper used had previously been dipped in 1 N KCl solution and dried. Other solvents used were: isopropanol–water (4:1) and *n*-butanol–acetic acid–water (60:15:25). Alkaline silver nitrate and reagents previously mentioned were used for detection purposes. In all hydrolytic procedures, appropriate lipid standards were subjected to the same treatment, and the products were chromatographed along with the samples.

Quantitation of phospholipids. Two-dimensional TLC was employed for estimating individual phospholipids. The lipids were deposited on one corner of the plate (20 by 20 cm) and developed in one direction with chloroform–methanol–water (65:25:4). The plate was then dried in air for 30 min and developed in the second direction with *n*-butanol–acetic acid–water (30:10:10). A second plate carrying a mixture of standard phospholipids was developed in a similar fashion. The plates were briefly exposed to iodine vapor, and the various spots were outlined. The plate was lightly sprayed with water, and the silica gel from spots was scrapped onto glassine paper. All scrappings, including a silica gel blank, were transferred to glass tubes and digested with 0.6 ml of perchloric acid for 2 h at 220°C. Phosphorus was estimated by the method of Bartlett (2); after the development of color, the tubes were centrifuged and the absorbance of the clear supernatants at 815 nm was determined in a spectrophotometer. Preliminary experiments showed that 90 to 92% of the phospholipid phosphorus was recovered after TLC.

Fatty acid analysis. Total cellular fatty acids were analyzed after saponification of lyophilized cells as previously described (18). Free fatty acids present in neutral lipids were isolated by extraction of silica gel with hexane–ether (1:1) after TLC of neutral lipids. The free fatty acids were methylated by borontrifluoride–methanol reagent (18). In the case of phospholipids, methyl esters of fatty acids were prepared by the direct action of the methylating reagent on the phospholipids. Methyl esters were analyzed by gas-liquid chromatography.

Sterol analysis. Total cellular sterols were isolated from lyophilized cells as described elsewhere (14) and then identified by TLC and gas-liquid chromatography. Sterols in the neutral lipid fraction were identified by similar techniques.

Gas-liquid chromatography. Gas-liquid chromatography was performed in a Packard gas-liquid chromatograph model 804 with a 6-foot (ca. 183-cm) glass column and a flame ionization detector. For fatty acid analysis, 10% diethylene-glycol-succinate coated on 80/100-mesh Chromosorb W-AW (Chemical Research Services, Inc.) was used at 180°C; 3% SE-30 on 80/100-mesh Chromosorb W HP and 3% OV-17 on 80/100-mesh Supelcoport (Supelco, Inc.) at 250°C were used for sterol analysis. The carrier gas was N_2 with a flow rate of 40 ml/min.

The fatty acid methyl esters were identified by comparison of retention times with those of methyl ester standards. The identity of unsaturated fatty acid esters was further confirmed after hydrogenation of the esters with hydrogen in the presence of platinum oxide. Peak areas were calculated by triangulation, and the percentage of each acid was determined from the ratio of the area under its curve to the total area under all curves. Identification of sterols was based on comparison with sterol standards. Amount of sterols was estimated by comparison of areas with those obtained from a standard solution.

Liposome preparation and testing. Liposomes were prepared from *P. wickerhamii* phospholipids with added *Prototheca* free fatty acid fraction and standard free fatty acid, where indicated, by methods previously described (10). The liposomes assayed contained 10 mol% of dicetyl phosphate. Fatty acids were incorporated into liposomes at 5% of the total lipid by weight. Glucose was used as marker molecule to ascertain drug-induced membrane damage. The glucose-loaded liposomes were incubated with the antimicrobial agents for 1 h at room temperature; the glucose freed into the medium was measured enzymatically. The amount of glucose released after boiling for 1 min in 10% Triton was considered as 100% release (10).

RESULTS

MICs. Table 1 records the MICs obtained for different drugs. The organism was susceptible to polymyxin B, amphotericin B, and the imidazole antibiotics miconazole and clotrimazole. Several

TABLE 1. *MICs of polymyxin B, amphotericin B, and the imidazole drugs for P. wickerhamii*

Drug	MIC (μg/ml)
Amphotericin B	0.31
Polymyxin B	0.39
Miconazole	3.13
Clotrimazole	5.0

P. wickerhamii previously examined are resistant to imidazoles (17, 21). Comparison of the lipids of some of these imidazole-resistant organisms to the susceptible one is in progress.

Lipid content. Total extractable lipid was 5 to 6% of the dry weight of the cells. On silicic acid column chromatography, 94.6% of the lipid applied was recovered, and the following composition of the recovered lipid was obtained: chloroform fraction (neutral lipids), 29.2% by weight; acetone fraction, 16.6%; and methanol fraction (total phospholipids), 54.2%. The acetone fraction was not further examined.

Phospholipids. TLC of phospholipids revealed four iodine-staining spots, three of which were identified as PE, PG, and PC by comparison with standards and by their staining characteristics. The fourth iodine-positive spot also gave a positive ninhydrin reaction and was identified as PS; this spot, however, also contained PI and was designated as PS-PI fraction. Solvent systems employed for TLC failed to separate PS and PI.

The identity of phospholipid components was confirmed by paper chromatography of products of alkaline and acid hydrolysis. Chromatograms of glyceryl phosphate esters gave spots which were identified by comparison with published R_f values (6) and with our own standards. Table 2 shows the identification of various components based on R_f and staining characteristics. The acid hydrolysate of the phospholipids showed two ninhydrin-positive spots and one Dragondorff reagent-positive spot corresponding to serine, ethanolamine, and choline, respectively.

Table 3 shows the phospholipid composition of *P. wickerhamii* based on phosphorus estimations after two-dimensional TLC of phospholipids. PC was the major component comprising 45.7% of the total phospholipid. PS and PI were estimated together because the TLC procedure failed to separate them adequately.

Neutral lipids. The major components of this lipid fraction were found to be free fatty acids and triglycerides. Trace amounts of 1,3-diglycerides were also detectable on TLC plates. Ergosterol, 4% by weight, was the major sterol in the neutral lipid fraction, comprising greater than 90% of the total sterol.

Fatty acid composition. Table 4 shows the total cellular fatty acids, free fatty acids in neutral lipids, and the fatty acids present in phospholipids. The fatty acid composition from these three sources was similar; fatty acids with 18 carbon atoms were predominant, and the proportion of unsaturated fatty acids was higher than the saturated ones. $C_{18:1}$ was the major fatty acid present, comprising almost half of the free fatty acids present in neutral lipid.

Imidazole sensitivity of liposomes. Figure 1 depicts a dose-response curve of glucose release from liposomes caused by the imidazoles, miconazole (Fig. 1A), and clotrimazole (Fig. 1B). The imidazole-induced release from *Prototheca* phospholipids is dramatically increased by addition of 5% of the neutral lipid fraction of the

TABLE 2. *Characteristics and identification of phospholipid components after alkaline hydrolysis*

R_f	Staining characteristics			
	Ninhydrin	Molybdenum[a]	Dragondorff	Parent phospholipid
0.21	−	+	−	PI
0.38	+	+	−	PS
0.54	−	+	−	PG
0.62	+	+	−	PE
0.88	−	+	+	PC

[a] Hanes and Isherwood reagent.

TABLE 3. *Phospholipid composition of P. wickerhamii*

Phospholipid	% of total phospholipid
PC	45.7
PE	27.3
PS-PI	15.5
PG	11.4

TABLE 4. *Fatty acids of P. wickerhamii*

Fatty acid	% of total fatty acids in:		
	Cellular fatty acid	Free fatty acid	Phospholipid fatty acids
14:0	3.9	2.1	2.8
14:1	1.0	1.1	
16:0	24.4	21.6	24.1
16:1	2.3	2.0	1.3
16:2	1.0		
18:0	5.5	1.8	4.5
18:1	35.2	49.0	37.8
18:2	23.9	18.3	23.6
18:3	2.7	4.0	5.8
Total saturated acids	33.8	25.6	31.4
Total unsaturated acids	66.1	74.4	68.5

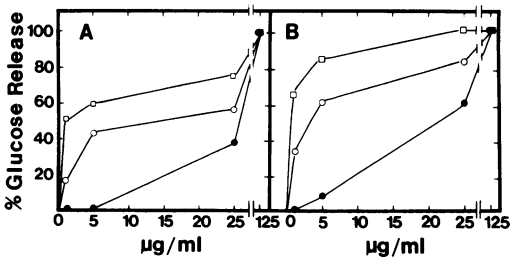

FIG. 1. *Effect of various concentrations of miconazole (A) and clotrimazole (B) on glucose release from liposomes without added fatty acid (●); with 5% added free fatty acid from the neutral lipid fraction of P. wickerhamii (○); and with 5% added $C_{18:1}$ (□).*

organism (date not shown). When the individual components of neutral lipid were examined, only the free fatty acid fraction caused the potentiation. Addition of standard free fatty acid ($C_{18:1}$) to the phospholipid liposomes had an even greater potentiating action (Fig. 1). At high imidazole concentrations, both imidazoles caused damage of liposomes without fatty acids. At low imidazole concentrations, concentrations near the MICs, substantial glucose release was only obtained when fatty acids were incorporated into the liposomes.

DISCUSSION

Cells which contain membrane sterols that have planar A and B rings and a free 3-hydroxy group are usually susceptible to the polyene antibiotics such as amphotericin B (11, 12). Several studies confirm that membrane ergosterol, the major fungal sterol, is much more effective than cholesterol, the major mammalian sterol, in conferring polyene susceptibility (3, 15). Hence, polyenes are more effective against fungi than mammalian cells and, although toxic for humans, have adequate selective toxicity to be useful in treating severe fungal infections. The exquisite susceptibility of *P. wickerhamii* to amphotericin B can be explained by the presence of 4% ergosterol in the lipid. Infection with this organism has been treated with amphotericin B (17).

The membrane determinants of polymyxin susceptibility are not as well defined. Previous work from our laboratory (7) suggests that high PC concentrations in membranes inhibit polymyxin action, that PE may be a central polymyxin target, and that the mechanism of polymyxin action involves disruption of cohesive ionic forces in the membrane by the cationic antibiotic. Work by Teuber and Bader indicates that PG, and possibly other anionic phospholipid polar head groups, may be targets for polymyxin action (26). When one looks at the phospholipid composition of *P. wickerhamii*, it is not easy to attribute polymyxin susceptibility to the presence of a specific phospholipid or phospholipid combinations, although both PE and PG are present. The phospholipid composition of *P. wickerhamii* is quite similar to that of *Candida albicans* (unpublished data), and the latter organism is much more resistant to polymyxin B. The dramatic difference in polymyxin susceptibility of *P. wickerhamii* and *C. albicans* in the face of similar phospholipid composition may stem from altered distribution of the phospholipids in the membrane bilayer (27), from the effect of other membrane components, or from differences in access of the agent to the membrane.

Conclusions from most reported studies of imidazole action suggest that imidazoles damage membranes of susceptible organisms (13, 24). Unsaturated fatty acids and phospholipids with unsaturated fatty acids when added to the medium can reverse the action of imidazoles (16, 28). Our data with liposomes prepared from *P. wickerhamii* lipids as a model membrane system suggest that free fatty acids in membranes are most important in determining imidazole susceptibility. Extensive marker release from phospholipid liposomes occurred only at very high concentrations of imidazoles. Addition of the neutral lipid fraction dramatically sensitized liposomes to the imidazoles, yielding major marker release at drug concentrations similar to the MICs of the antimicrobial agents. In fractionating the neutral lipids, it became clear that free fatty acids were responsible for the increase in imidazole susceptibility.

Prototheca species show extreme variability in imidazole susceptibility (21). Most species of *P. wickerhamii* examined have miconazole MICs of more than 50 µg/ml (17, 21). The organism that we examined, *P. wickerhamii* YB4330 (ATCC 16529), on the other hand, was susceptible, having imidazole susceptibilities comparable to those of *C. albicans*. We are presently examining imidazole-resistant *P. wickerhamii* and seeking an explanation for the different antimicrobial susceptibility. Imidazoles are also effective antimicrobials against most fungi. Fungal membranes are rich in free fatty acids (unpublished data). Mammalian cells and gram-negative bacilli, which are quite resistant to imidazoles, have negligible membrane free fatty acids. A detailed study of the imidazole susceptibility of liposomes with various lipid composition is in progress.

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