Mode of Action of the Polyene Antibiotic Candididin: Binding Factors in the Wall of *Candida albicans*

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The polyene antibiotic candididin produces a rapid efflux of K+ ions from a suspension of *Candida albicans*. Onset of K+ leakage depends on the culture age, stationary-phase yeasts leaking K+ more slowly than exponential-phase yeasts. The time taken for potassium leakage to begin represents the time taken by the antibiotic to cross the cell wall and produce membrane damage. It was shown that there were factors in the cell wall of *C. albicans* that increased their total binding capacity and their affinity for candididin during growth. An attempt was made to relate changes in the lipid content of the yeast cell with the increased time taken to produce membrane damage.

Polyene antibiotics, e.g., nystatin, amphotericin B, and candididin, display a selective toxicity towards organisms whose membranes contain sterols. This group of antibiotics destroys the selective permeability properties of a wide variety of fungi and other eukaryotic organisms (membranes that contain sterols) but has no effect on prokaryotic organisms (membranes that do not contain sterol) (22). In common with other membrane-active antimicrobial agents, treatment of yeasts with polyenes produces leakage of small molecules. Losses from the yeast of potassium, inorganic phosphate, carboxylic and amino acids, and phosphate esters have all been reported from yeasts treated with growth-inhibitory concentrations of polyenes (22). Yeasts assimilate potassium from the environment against the concentration gradient (25). Changes in the selective permeability of the yeast cell membrane, induced by polyene antibiotics, lead to a rapid efflux of these ions into the suspending medium. Specific ion electrodes have been used to study the effects of polyenes on membrane permeability (10, 14, 21). It has been shown that the age of the *Candida albicans* culture affects the time taken for the polyene-induced potassium efflux to begin. Older yeasts leak potassium more slowly than younger yeasts (14, 21). It is thought that the time taken for the efflux of potassium to begin after polyene application represents the time taken for the antibiotic to penetrate the cell wall and affect the cell membrane. It has been reported that the bulk of the bound candididin is associated with an unknown component in the yeast cell wall in addition to the active site in the cell membrane (13). Addition of fatty acids to yeast suspensions prevents polyene antifungal activity (11, 15, 26). The lipid composition of the cell wall of *C. albicans* was investigated to see whether this could provide a possible explanation of the observed facts.

**MATERIALS AND METHODS**

Antibiotic. Candididin (lot 681 NOP2) with a potency of 218% (manufacturer's bioassay, in which original candididin made by S. B. Penick Ltd. was given an activity of 100%) was supplied by Pharmax Ltd., Beasley, Kent, and stored desiccated at −20 C. Aqueous solutions were prepared by diluting candididin dissolved in dimethyl sulfoxide (Me2SO) with 0.025 M sodium dimethyl glutarate buffer (DMG) to give a final Me2SO concentration of less than 0.05% (vol/vol). [14C]Candididin was prepared as described previously (12), dissolved in Me2SO and diluted with 0.025 M DMG buffer.

Fresh antibiotic solutions were prepared daily. The potency of the antibiotic, as measured by microbiological assay (28), did not change during the experimental period. Aqueous solutions of the antibiotic were protected from light, to which they are sensitive, wherever practicable.

Organism and method of culture. Approximately 10⁶ cells from a maintenance slope of *C. albicans* NCTC 713 grown on Sabouraud dextrose agar (Oxoid Ltd., London) for 24 h at 37 C, and stored at 4 C, were transferred to 100 ml of prewarmed sterile Sabouraud dextrose broth (SDB) (Oxoid Ltd.) in a 300-ml Erlenmeyer flask fitted with a loose cotton-wool plug and incubated at 37 C in shake culture to ensure aerobic growth. At set time intervals the yeast was harvested by centrifugation (MSE 18, Croydon; 10,000 × g for 10 min) and washed once with distilled water and once with 0.025 M DMG (pH 6.0).

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Preparation of standard yeast suspension. Enough washed yeast was suspended in 0.025 M DMG, pH 6.0, to give $E_26_{6}_{0} = 1.0$ (Cecil 304 spectrophotometer, Cecil Instruments Ltd., Cambridge). Such suspensions were found to contain $3.7 \times 10^7$ yeast/ml (total hemocytometer count), and when dried to constant weight at 105°C they contained 0.92 mg (dry weight)/ml.

Preparation of protoplasts of C. albicans. Yeasts grown in SDB for 18 h at 37°C were harvested by centrifugation (MSE 18; 10,000 × g for 10 min) and washed with 0.025 M DMG, pH 8.0. Protoplasts were prepared according to the method of Schibeci et al. (27).

Protoplast formation was followed using (i) phase-contrast microscopy, (ii) lysis of protoplasts viewed under the microscope on addition of 0.1 M MgCl$_2$, and (iii) the decreasing extinction of samples at 660 nm on addition of distilled water. After 3 h of Helicase treatment it was found that the yield was 95 to 100%. Protoplasts formed by this method were viable, and, on plating out on corn meal agar supplemented with 15% manitol after serial dilution, the viable count was 85% of the total hemocytometer count. Protoplasts grown on corn meal agar produced cell walls and chlamydoospores within 3 days at 37°C.

Preparation of a wall fraction from C. albicans. Approximately 20 g of C. albicans (wet weight) was plasmolyzed in 1 M sodium chloride for 1 h at 20°C. The plasmolyzed yeasts were rapidly washed in distilled water and resuspended in 25 ml of distilled water before freeze-pressing (X press, AB Biotech, Croydon) according to the method of Edebo (8). The press was operated at −25°C, and the yeast was passed through the orifice five times at a pressure of approximately 2,000 kg/cm$^2$. It was found by determining the viable count that this method disrupted 98 to 99% of the yeast cells.

The freeze-pressed cells were then differentially centrifuged using an MSE 18 8 by 50 rotor. A wall fraction was obtained by centrifugation of the freeze-pressed yeast for 5 min at 10,000 × g. The wall fraction was a white creamy precipitate, and this was examined by phase-contrast and electron microscopy. It was found to contain exclusively large wall fragments, which were almost complete. The fraction was freeze dried (Genevac, model CFD-1, Redcliffe, Lancs.) and stored at −20°C. The wall fraction constituted 16 to 19% of the dry weight of the yeast. The wall fraction was examined for membrane contamination by assaying the fraction for sterol (3). Negligible amounts of this known membrane component were present.

Determination of bound $[^{14}C]$candididin. Bound $[^{14}C]$candididin was determined by the method of Hammond and Kliger (12).

Detection of potassium leakage. Candididin-induced potassium fluxes from C. albicans in the standard yeast suspension were monitored in situ with a Phillips IS 560K specific ion electrode as described previously (14).

Determination of yeast ergosterol. The ergosterol level of C. albicans was determined by the method of Breivik and Owades (3). Determination of triglycerides in yeast cell walls. Preweighed freeze-dried wall fractions of C. albicans were suspended in 0.5 M KOH in 90% (vol/vol) aqueous spectroscopic ethanol using an ultrasonic probe (MSE 100-W ultrasonic disintegrator, 1.8-cm probe, 20-μm amplitude, at 20 kHz for 15 min at 5°C). A 1-ml amount of 0.1 M MgCl$_2$ was added, and the mixture was incubated for 45 min at 55°C. The mixture was centrifuged (MSE 18, 20,000 × g for 15 min) and assayed for triglycerides according to the method of Eggstein (9).

Extraction, purification, and gas-liquid chromatography of lipids from cells of C. albicans. A 50-ml amount of freshly prepared yeast cell walls was treated with 80% (vol/vol) aqueous ethanol for 15 min at 80°C to inactivate enzymes, particularly lipase, and lipids were extracted with 10 ml of chloroform-methanol (1:1, vol/vol). The material was stirred continuously during three successive 3-h extraction periods. The extracts were pooled, evaporated, dissolved in chloroform-methanol (2:1, vol/vol), and purified by a modification of the method proposed by Pederson (24). A 25-ml beaker was submerged in a 100-ml beaker containing 90 ml of distilled water. The crude lipid extract was delivered very slowly into the submerged small beaker, allowing the extract to fall through the entire water depth. The contents of the small beaker were stirred magnetically overnight at room temperature, when 1 ml of methanol was added to dissolve the cloudy interface. The lipid sample was removed with the chloroform-methanol layer, evaporated, dissolved in 0.5 ml of chloroform, and stored at −20°C. Methyl esters of the fatty acids present in the extracted lipids were prepared by direct methanolysis using the method of Stoffel et al. (29) as modified by Kates (19). The purified lipid extract was evaporated and dissolved in 2 ml of methanolic hydrogen chloride (2.5%, made by saturation of methanol with hydrogen chloride gas) and refluxed for 1 h. The methylated fatty acids were then extracted using successive quantities of petroleum ether (bp 30 to 60°C). The petroleum ether fractions were combined and evaporated to dryness.

The methyl esters were separated by gas-liquid chromatography using a Pye 104 apparatus (Pye-Unicam, Cambridge) with a glass column (0.125 inch by 5 feet [ca. 0.2 by 152.4 cm]) packed with 10% (wt/wt) polyethylene glycol adipate on Chromosorb W. The column temperature was 105°C, and the rate of carrier gas (nitrogen) flow was 60 ml/min. Methyl esters were identified by comparing retention times with known standards (Sigma Ltd., Kingston, Surrey).

RESULTS

Potassium leakage from C. albicans treated with candididin. Standard yeast suspensions made of C. albicans cells known to be in the logarithmic growth phase (yeasts harvested 6 h after inoculation into SDB) and a similar suspension of yeasts known to be in the stationary phase (harvested 18 h after inoculation into SDB) were prepared in 0.025 M DMG, pH 6.0.
The potassium efflux on treatment with 25 µg of candididin per ml at 20°C was followed with a potassium-sensitive electrode. The experiment was repeated with an equivalent suspension of C. albicans protoplasts. Results appear in Fig. 1. The age of the yeast culture from which the protoplasts were prepared was found to have no effect on the leakage pattern observed. Me₂SO (0.1%) alone produced negligible K⁺ leakage from C. albicans protoplasts. Low levels of candididin, sublethal for the standard C. albicans suspension, produced almost immediate K⁺ leakage in equivalent suspensions of C. albicans protoplasts (Fig. 1). The absence of a cell wall to absorb candididin raises the effective antibiotic concentration and also makes the sites for candididin binding in the cell membrane more accessible. C. albicans cells known to be in the exponential growth phase, when treated with lethal levels of candididin, begin to leak potassium 90 s after antibiotic application, leakage being complete within 15 min. Equivalent suspensions of yeasts from the stationary growth phase treated with candididin begin to leak potassium 4 min after antibiotic treatment, and leakage is completed within 20 min. Hence the time taken for antibiotic to penetrate the cell wall of C. albicans and damage the cell membrane is of the order of 90 s after antibiotic application for exponential-phase yeasts and 4 min for stationary-phase yeasts (as protoplasts undergo almost instantaneous leakage).

Consistent potassium leakage curves were produced as a result of candididin action. By taking the midpoint of such curves, i.e., the time taken for 50% of the total potassium leakage, a convenient index permitting comparison between different conditions was obtained. Figure 2 shows the effect of culture age upon the time taken for 50% of the potassium to leak from the standard suspension of C. albicans in 0.025 M DMG, pH 6.0, treated with 25 µg of candididin per ml.

C. albicans cells from an 18-h culture grown in SDB at 37°C were made into a standard suspension, and 2,000 U per ml of a lipase known to be free of amylase and protease (type VII purified from Candida cylindracea; Sigma Ltd.) was added. The mixture was incubated for 1 h at 37°C, washed in 0.025 M DMG, pH 6.0, and made up to the original volume with fresh buffer. Figure 3 shows the potassium leakage induced by candididin from lipase-treated and untreated C. albicans cells. Candididin elicits a more rapid K⁺ efflux from stationary-phase yeasts after lipase treatment.

Binding of [¹⁴C]candididin to cell walls derived from C. albicans cells of differing ages. C. albicans was grown in SDB at 37°C and harvested by centrifugation at 12 and 24 h. Cell wall fractions were prepared from the yeasts in both growth phases. A 30-mg portion of freeze-dried, 12- and 24-h walls was suspended in 6 ml of 0.1 M DMG (pH 6.0) and 4 ml of [¹⁴C]candididin to make a final antibiotic concentration of 40 µg/ml. The mixture was incubated at 25°C for 1 h, and the walls were centrifuged (MSE 18, 10,000 × g for 10 min) and washed with 0.1 M DMG (pH 6.0) until the supernatant gave no extinction at 380 nm. The washing procedure was kept uniform (using a Gallenkamp bench shaker, setting 10 for 10 min). The...


[14C]candidin levels were determined for the bound candidin, the nonbound candidin, and the washings. From the amount of nonbound candidin it was estimated that the 12-h cell walls bound 48.8% of the antibiotic applied and the 24-h cell walls bound 61.4% of the antibiotic applied. After six washings, 12-h cell walls retained 3% of the [14C]candidin applied, whereas after six washings 24-h walls retained 13.7% of the applied [14C]candidin. Table 1 shows how this [14C]candidin was released with washing. Candidin appears to be bound much more loosely by 12-h cell walls than by older walls; i.e., candidin can be washed more easily from younger cell walls. Chemical analysis showed that there was negligible sterol in either of these wall fractions, suggesting that a nonsteroid, candidin-binding component is present in the cell wall of C. albicans, which with time increases in total binding capacity and degree of binding affinity.

Effects of fatty acids on [14C]candidin uptake by C. albicans. The effects of fatty acids on [14C]candidin uptake by C. albicans were studied by adding sufficient acetic, caprylic, capric, lauric, palmitic, stearic, oleic, linoleic, or α-linolenic acids (Sigma Ltd.) (dissolved in 1 ml of absolute ethanol) to the standard C. albicans suspension to give a final fatty acid concentration of 0.4 mM, and the pH was adjusted to 4.5. The suspensions were treated with 20 μg of [14C]candidin per ml, and the amount of [14C]candidin bound was determined after 1 h (when binding to C. albicans is complete; 12). The results appear in Table 2. When the standard C. albicans suspension is treated with 20 μg of [14C]candidin at pH 4.5, 4.3 μg of candidin becomes bound per mg (dry weight) of yeast. The addition of fatty acids having less than 12 carbon atoms has little or no effect on candidin uptake, but addition of longer-chain fatty acids can prevent candidin uptake by intact yeasts (Table 2). The effect on antibiotic uptake increases with fatty acid chain length. However, more significant are changes in the degree of saturation upon candidin uptake. In the C18 series, stearic acid (saturated) prevents 20%, oleic acid (one double bond) 25%, lineoeic

![Fig. 2. Effect of culture age on the time taken for 50% K⁺ leakage from C. albicans after treatment with 25 μg of candidin per ml (20°C, pH 6.0).](image)

![Fig. 3. Potassium leakage from 18-h C. albicans cells treated with 25 μg of candidin per ml (■); leakage induced by 25 μg of candidin per ml from 18-h C. albicans cells pretreated with 2,000 U of lipase per ml (■); leakage from C. albicans treated with 1% Me₂SO (○); and leakage from lipase-treated yeasts + 1% Me₂SO (□) (pH 6.0, 20°C).](image)

<table>
<thead>
<tr>
<th>Condition</th>
<th>mg of candidin bound/g of cell wall</th>
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<tr>
<td>12-h walls</td>
<td>24-h walls</td>
</tr>
<tr>
<td>Initially bound</td>
<td>6.45</td>
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<tr>
<td>After 1 washing</td>
<td>1.65</td>
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<tr>
<td>After 2 washings</td>
<td>1.05</td>
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<tr>
<td>After 3 washings</td>
<td>0.80</td>
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<tr>
<td>After 4 washings</td>
<td>0.60</td>
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<tr>
<td>After 5 washings</td>
<td>0.40</td>
</tr>
<tr>
<td>After 6 washings</td>
<td>0.20</td>
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</table>
TABLE 2. Effect of the addition of 0.4 mM fatty acids on the uptake of [14C]candididin by C. albicans treated with 20 µg of antibiotic per ml

<table>
<thead>
<tr>
<th>Aliphatic fatty acid</th>
<th>Systematic name</th>
<th>No. of carbon atoms</th>
<th>Candididin bound (µg/mg of yeast, dry wt)</th>
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</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>n-Hexadecanoic acid</td>
<td>16</td>
<td>3.61</td>
</tr>
<tr>
<td>Lauric</td>
<td>n-Decanoic acid</td>
<td>18</td>
<td>3.49</td>
</tr>
<tr>
<td>Oleic</td>
<td>cis-9-octadecenoic acid</td>
<td>18 (1=)</td>
<td>3.01</td>
</tr>
<tr>
<td>Linoleic</td>
<td>cis,cis-9,12-octadecadienoic acid</td>
<td>18 (2=)</td>
<td>2.20</td>
</tr>
<tr>
<td>α-Linolenic</td>
<td>All-cis-9.12.15-octadecatrienoic acid</td>
<td>18 (3=)</td>
<td>1.60</td>
</tr>
</tbody>
</table>

Acid (two double bonds) 49%, and α-linolenic acid (three double bonds) 64% of antibiotic uptake.

Changes in ergosterol content of C. albicans during growth. Approximately 10⁶ yeasts from an overnight culture were inoculated into 100 ml of prewarmed SDB in a 300-ml Erlenmeyer flask and sampled at 6-h intervals, and total ergosterol was determined by the method of Breivik and Owades (3). Figure 4 shows that the total ergosterol content of C. albicans increases with the age of culture.

Changes in the triglyceride content of the cell wall of C. albicans during growth. Cell wall fractions of C. albicans were prepared every 3 h after inoculation into SDB at 37 C. The triglyceride level of each of these fractions was determined by the method of Eggstein (9). Figure 5 shows that wall triglycerides increase with culture age.

A purified lipid extract was prepared from these wall fractions, which were methylated and separated by gas-liquid chromatography. The components in the extracted lipids were identified by measuring retention times (Rt) and comparing these values to the Rt of standards and by the addition of known amounts of lipid standards to the lipid extract and examining the trace for coincidence. Table 3 shows the distribution of fatty acids in cell walls of C. albicans of increasing culture age. There is an increase in the total amount of fatty acids present, an increase in the chain length, and an increase in the degree of unsaturation with age of culture.

DISCUSSION

The susceptibility of C. albicans to polyene antibiotics, as measured by K⁺ leakage patterns, decreases progressively throughout the yeast growth cycle, the loss in susceptibility
being greatest when the culture reaches the stationary phase (10, 14). Polysene uptake also alters with culture age, reflecting changes in the permeability properties of the yeast (23). Electron microscopy has shown that the cell wall of C. albicans increased by 6% during growth (13). Such small increases in cell wall thickness cannot account for the reduction in polysene susceptibility or the doubling of the lag phase before the onset of candididin-induced K⁺ leakage (Fig. 1). If the yeast cell wall acts only as a simple diffusion barrier, the time taken for candididin to cross the wall (0.15 to 0.2 μm in diameter) and damage the cell membrane would be much shorter than was observed.

An alternative explanation would be the suggestion of qualitative and/or quantitative changes in cell wall components as the culture ages and that these changes prevent the antibiotic penetrating the cell wall and reaching the active site in the membrane. If there is a binding site for polysene antibiotics in the yeast cell wall, it cannot be to sterols, since they are not present in the yeast cell wall (16).

Kinsky et al. (20) stated that interaction could occur between polyenes and alcohol groups other than sterols, particularly to cetyl alcohols. These authors also reported that interaction could occur to a lesser extent with cerebrosides. The absorption and antifungal activity of polysene antibiotics are inhibited by the presence of fatty acids. Inhibition increases with fatty acid chain length and unsaturation (11, 15, 26). The presence of long-chain unsaturated fatty acids in the medium prevented a large proportion of added candididin binding to the yeast. The fatty acids either combined with the yeast surface, masking sterol sites in the cell membranes, or, alternatively, the antibiotic may have combined with fatty acid micelles in the medium.

Treatment of C. albicans with lipase reduces the wall triglyceride level of 24-h yeasts without any apparent effect on yeast morphology or viability. Lipase-treated, 24-h yeasts showed a reduction in the time lag before the onset of K⁺ leakage; i.e., this treatment reduces the effect of the candididin binding factor in the cell wall. It was thought that if the fatty acid component of the cell wall increased in concentration or changed in the degree of saturation during yeast growth, then fatty acids or fatty acid esters could conceivably be the factors in the wall of C. albicans protecting membrane sterol sites from damage. The bulk of lipids in yeasts is located in membranes of one sort or another. Reports on the lipid content of cell walls of yeasts range from 1 to 12% of the wall dry weight (16).

Dyke (7) demonstrated that the lipid found in the cell wall of yeasts was a bona fide component and not a cytoplasmic contaminant. Hurst (17) suggests that lipids contribute to the stiffness of the wall. The lipid is firmly bound to the cell wall (2). The lipid composition of yeasts is very susceptible to variations in growth conditions (17). Several workers have observed changes in the lipid composition, notably in the total amount of lipid, the degree of lipid saturation, and the relative proportions of different phospholipids during growth (4, 6, 16, 18). Total yeast lipid and degree of unsaturation are affected by deficiency of nitrogen, phosphate, or carbon source and changes in O₂ tension or temperature.

In the experiments described in this paper C. albicans was grown aerobically in batch culture at 37 C, and the growth conditions were not fully defined. The limiting factors for growth would be nitrogen, carbon, and possibly phosphate sources. Figure 5 shows that the total triglyceride content of the cell wall of C. albicans increases markedly with culture age. Figure 6 shows this increase superimposed upon changes in the potassium leakage pattern of C. albicans with age of culture. It will be seen that the two phenomena follow closely.

Gas-liquid chromatography analysis of lipid extracts from cell wall fractions of C. albicans of increasing culture age revealed that the fatty acids in the wall triglycerides also change in composition (Table 3). The proportion of fatty acids increases in total amount, chain length, and degree of unsaturation during growth in batch culture.

The sterol content of C. albicans increases

<table>
<thead>
<tr>
<th>Table 3. Fatty acid content of cell walls of C. albicans (mg/100 mg, dry wt) determined by gas-liquid chromatography</th>
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<tbody>
<tr>
<td>Fatty acid</td>
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</tr>
<tr>
<td>Caprylic</td>
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<td>Capric</td>
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<td>Lauric</td>
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<td>Palmitoleic</td>
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<td>Linoleic</td>
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<td>Linolenic</td>
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* +, Trace; -, none found.*
satisfied before candidicidin can bring about its lethal effect by binding to sterol in the cell membrane.

ACKNOWLEDGMENTS

We thank D. Trigger (Pharmax Ltd., Bexley, Kent) for helpful discussions.

LITERATURE CITED