Ultrastructural Changes in the Cell Wall of *Candida albicans* Following Cessation of Growth and their Possible Relationship to the Development of Polyene Resistance

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The ultrastructure of the wall of *Candida albicans* strain 6406 was examined in polyene-resistant organisms obtained by continued incubation after the cessation of growth. The walls of organisms harvested either during the exponential phase of growth or after 24 h starvation, when examined *in situ*, showed the typical layered appearance. After 72 h starvation, when the resistance to amphotericin B methyl ester (AME) was 60 times greater than that of exponentially growing organisms, both the periplasmic material and the distinct electron-dense layers were absent from the wall. At this stage there was no increase in the thickness of the wall. After 144 h starvation the thickness of the wall had increased from 143 ± 22 nm (exponential phase organisms) to 211 ± 58 nm. If after 144 h starvation the organisms were incubated for 1 h in fresh nutrient medium they regained their sensitivity to AME and the wall regained the periplasmic material and its characteristic multi-layered appearance. During the first 24 h starvation there was a considerable fall in the soluble glucan fraction, but on continued incubation there was little change in the relative proportions of the major carbohydrate constituents of the cell. Thin sections of purified walls isolated from organisms harvested either during exponential growth or after 144 h starvation were identical in appearance and characterized by the absence of the electron-dense layers observed in sections of intact cells and by a reduction in thickness to 100 ± 20 nm.

INTRODUCTION

Candida albicans strain 6406 shows a marked phenotypic variation in its sensitivity to the polyene antibiotics (Gale *et al.*, 1975; Johnson *et al.*, 1978). These changes in antibiotic sensitivity are particularly pronounced in the organism after the cessation of growth when the minimum inhibitory concentration (determined by following K^+ release) may be two orders of magnitude greater than that of exponentially growing organisms (Gale *et al.*, 1975; Gale *et al.*, 1977). This change in antibiotic sensitivity is apparently associated with modifications of the wall since protoplasts prepared from resistant cells harvested during the stationary phase of growth have a sensitivity to amphotericin B methyl ester (AME) comparable to that of organisms harvested during the exponential phase of growth (Gale *et al.*, 1975). The wall of *C. albicans* is a complex structure both chemically and structurally (Chattaway *et al.*, 1968; Cassone *et al.*, 1973; Poulain *et al.*, 1978) and these studies were undertaken to examine the structure of the wall after the cessation of growth and to attempt to correlate such changes with the increased resistance to AME.

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METHODS

Organism. The organism used throughout this study was *Candida albicans* strain 6406 obtained from Dr D. W. R. McKenzie, Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine. It was maintained by weekly subculture in a liquid medium (YNBG) containing Difco Bacto yeast nitrogen base (6.7 g l^{-1}) supplemented with 1 % (w/v) glucose.

Growth and incubation conditions. Organisms were grown in a 1 litre continuous flow fermenter (FL101, AB Biotec, Stockholm, Sweden) as described by Gale *et al.* (1977). The rate of stirring, supply of medium, aeration and temperature were controlled and monitored by the appropriate accessories (LP100 process instrumentation panel, AB Biotec). YNBG medium (1 litre) was inoculated with *C. albicans* and incubated aerobically (air flow 2.7 l min^{-1}) at 37°C with the medium stirred at $1000 \text{ rev. min}^{-1}$. After 16 h, continuous flow of medium was started ($D = 0.15 \text{ h}^{-1}$) and continued for 36 h; at this time the flow of medium was stopped but incubation was continued under the same conditions of temperature, aeration and stirring. Samples were withdrawn at 24 h intervals for determination of AME-induced K^+ release, examination by electron microscopy, cell fractionation and carbohydrate estimation.

Estimation of K^+ release and AME sensitivity. Organisms were suspended at a final density of $1 \text{ mg dry wt ml}^{-1}$ in 0.03 M-Tris/HCl buffer pH 7.5 at 20°C and the rate of K^+ release induced by AME was estimated using a K^+ -sensitive electrode (Gale, 1974). The concentration of AME required to induce a rate of K^+ release equal to $1 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$ above that in the control without AME in 8 min at 20°C was determined; this concentration is termed the standard release concentration (s.r.c.).

Cell breakage and fractionation. Washed organisms (25 mg dry wt) were resuspended in 1 ml ice-cold distilled water and disrupted by vigorous vortex mixing (Whirlimixer; Scientific Industries International) for 2 min in the presence of glass beads (0.45 to 0.5 mm diam.). The walls were harvested by centrifuging and washed 10 times with ice-cold distilled water prior to carbohydrate assay and examination by electron microscopy.

Intact organisms or walls from an equivalent quantity of cells were fractionated into their major carbohydrate constituents by the method of Trevelyan & Harrison (1956) as described by Herbert *et al.* (1971). Organisms (25 mg dry wt), or walls from an equivalent amount of organisms, were resuspended in $0.5 \text{ M-trichloroacetic acid}$ and incubated at 0°C for 1 h. The cells were separated by centrifuging and the supernatant fluid containing the low molecular weight carbohydrates was retained. After extraction of the RNA from the pellet with $0.5 \text{ M-perchloric acid}$ for 90 min at 38°C , the mannan and alkali-soluble glucan were extracted with $0.25 \text{ M-Na}_2\text{CO}_3$ for 45 min at 100°C . The alkali-soluble fraction was divided into two parts: one was used for determination of the total alkali-soluble carbohydrate and the other for determination of the mannan after precipitation as the insoluble mannan-copper complex following addition of KOH (final concentration 1 M) and Fehlings solution to the extract. The pellet after alkali extraction was further extracted with $0.5 \text{ M-perchloric acid}$ for 30 min at 100°C ; this extract contained the acid-soluble glucan. The final residue was resuspended in 2 M-NaOH prior to carbohydrate assay.

The carbohydrate content of each fraction was assayed using the anthrone reagent as described by Herbert *et al.* (1971). Glucose standards were always included. The value for the mannan fraction was corrected for the lower absorbance (55 %) that this fraction gives relative to the glucose standard.

Electron microscopy. The standard procedure used to prepare samples for electron microscopy was as follows. Pelleted washed organisms or walls were fixed with a mixture of 0.5% (v/v) glutaraldehyde and 1.0% (v/v) acrolein in $0.075 \text{ M-phosphate buffer}$ pH 7.2 for 30 min at 4°C . This was followed by postfixation with 4% (w/v) osmium tetroxide in water for 12 h at 4°C . The samples were stained 'en bloc' with 0.5% (w/v) uranyl acetate in $0.5 \text{ M-veronal acetate buffer}$ pH 5.4 for 3 h at 4°C . All samples were dehydrated through a graded ethanol series, infiltrated with propylene oxide and embedded in Spurr's low-viscosity resin (Spurr, 1969). Sections were cut using a LKB Ultratome III equipped with a glass knife and, unless otherwise stated, were stained with lead citrate (Reynolds, 1963) for 30 min prior to examination in either an AEI 801 or a Siemens 1A electron microscope operating at 60 to 80 kV . Where other fixatives were used, details are given in the text.

Polysaccharides in isolated walls were detected by the cytochemical test of Thiéry (1967) as applied to intact *Candida* cells by Cassone *et al.* (1972). The samples were fixed with either glutaraldehyde or osmium tetroxide since preliminary experiments had shown that the standard fixation procedure introduced a sufficient number of 'artefactual' aldehyde groups into control preparations to elicit a positive Thiéry reaction. The thin sections were oxidized with 1% (w/v) periodic acid for 30 min, washed with water and transferred to a solution of 0.5% (w/v) thiosemicarbazide in 20% (v/v) acetic acid for 60 min. After thorough washing in a descending acetic acid series ($20, 10, 5, 1 \%$, v/v) and finally water, the sections were mounted on uncoated grids and stained with 1% (w/v) silver proteinate for 30 min. In the control preparations either periodic acid or thiosemicarbazide was omitted.

The thickness of the wall was obtained from measurements on $3\times$ enlarged prints. For the measurements on intact cells, care was taken to measure only walls in nearly median section as judged by the appearance of the plasma membrane profile. This was not possible with the preparations of purified walls, and the values of the wall thickness in these preparations are an overestimate due to the presence of oblique sections. In some sections the thickness of the wall profile was different in different places; nevertheless a comparison of the relative mean values of different samples is justified. Approximately 100 wall profiles taken from different sections for each sample were measured and the results are expressed as the mean value $\pm$ the standard deviation.

RESULTS

Ultrastructure of the wall during exponential growth and subsequent starvation

Organisms harvested from a culture of *C. albicans* growing exponentially in continuous culture, fixed with glutaraldehyde and osmium tetroxide and stained with lead citrate showed the usual multilayered appearance (Cassone *et al.*, 1973), with outer electron-dense components and inner electron-transparent layers together with some partially electron-dense granules within the wall (Fig. 1*a*). Scattered highly electron-opaque granules were present on the surface of the wall; these were absent from the cells harvested after 24 h starvation (Fig. 1*b*) and could be artefactual. There was a prominent periplasmic layer in both exponentially growing organisms and organisms which had been starved for 24 h and this closely followed the invaginations of the plasma membrane. The periplasmic layer was always detected in exponentially growing organisms, regardless of the nature of the fixative and the osmotic strength of the fixation mixture. The thickness of the wall in *C. albicans* harvested during exponential growth was 143 ± 22 nm and after 24 h starvation, 144 ± 26 nm. The wall thickness was not significantly different after 72 h starvation (Fig. 1*a, b, c*).

Major changes in the ultrastructure of the wall were observed in organisms which had been starved for prolonged periods (Fig. 1*c, d*). Most electron-dense outer components were absent, the overall structure appearing essentially electron-transparent with only a faint electron-dense line at the extreme periphery of the wall. The inner periplasmic layer was first greatly reduced and after prolonged starvation was absent. After prolonged starvation (144 h) the wall had a uniform electron-transparent structure with no layering of components and was considerably thicker than that of exponentially growing organisms, being 211 ± 58 nm.

There were no marked changes in the wall during the first 24 h starvation, during which time the s.r.c. [concentration of AME required to give a K^+ release of $1 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$ above the control rate] increased from 0.2 to $1.5 \mu\text{g AME ml}^{-1}$. After 72 h starvation, when there were appreciable changes in the wall ultrastructure, the s.r.c. for AME had risen to $12 \mu\text{g ml}^{-1}$.

Other fixatives and stains, including permanganate and uranyl acetate, were used to detect changes in the organization of the wall during starvation. Despite the differences in the primary visualization observed using different fixatives and stains, the pattern of changes during starvation was comparable to those shown in Figs 1 and 2. When the organisms were intensely stained, either 'en bloc' or in section with uranyl acetate, the walls of stationary phase organisms showed a variable amount of electron-dense components; but in contrast to the exponential phase organisms, these components were not layered but uniformly distributed throughout the wall. The over-staining effect of uranyl acetate was enhanced in organisms previously subjected to ultrasonic treatment for 10 min in a Dawe Sonicleaner (type 6441A Dawe Instruments, London) (Fig. 2 and see below).

If after prolonged incubation in the stationary phase the organisms were resuspended in fresh nutrient medium (YNBG) and aerated at 37°C , they quickly regained the wall configuration typical of exponentially growing organisms with electron-dense layers, an inner electron-transparent region and a dense periplasmic layer. Recovery began before the first cell division occurred and was clearly distinguishable after regrowth for 1 h (Fig. 1*e*).

Fig. 1. Walls (CW) of *Candida albicans* in organisms harvested (a) from an exponentially growing culture, (b) after 24 h incubation in the stationary phase, (c) after 72 h incubation in the stationary phase, (d) after 144 h incubation in the stationary phase, (e) after incubation of organisms previously starved for 144 h in fresh nutrient medium for 1 h. Note the significant increase in wall thickness from (c) to (d) and the recovery of the layered configuration of the oldest wall (d) after regrowth (e). Also the progressive thinning of the periplasmic layer and its absence from (d); thin arrows indicate the plasma membrane. In (d), the wall is delimited by the broad arrows. Sections stained with lead citrate for 30 min. Bar markers represent 100 nm.

Fig. 2. Effect of ultrasonic treatment on exponential phase (*a, b*) and stationary phase (*c*) organisms. Discrete areas of wall breakage are arrowed in (*a*) and (*b*). Note that the 'isolated' wall in (*b*) maintains its layered organization. Sections heavily stained with lead citrate and uranyl acetate for 45 min (*a*) or 30 min (*b, c*). Bar markers represent 100 nm (*a*) or 500 nm (*b, c*).

The walls of organisms undergoing budding appeared to be identical to those shown in Fig. 1 (*a, b*).

Effect of ultrasonic treatment on wall structure

Walls from exponentially growing and stationary phase organisms differed markedly in their response to ultrasonic treatment. A relatively mild ultrasonic treatment for 10 min in a Dawe Sonicleaner (type 6441A) was sufficient to break a small proportion of exponential phase organisms but stationary phase organisms were unaffected (Fig. 2). Breakage occurred at discrete, definite regions of the wall, presumably at the weakest points corresponding to the budding zones (Fig. 2*a*). If sufficient of the wall was broken, the cytoplasm still enclosed within the plasma membrane was seen to emerge leaving a 'clean' wall (Fig.

2b). The latter appeared to be similar to the wall of untreated organisms showing electron-dense layered components. There was, however, a slight reduction in thickness to an average value of approximately 115 nm. The walls of both exponential phase and stationary phase organisms were more easily stained with conventional heavy metal stains after ultrasonic treatment suggesting that even in the absence of cell breakage there were changes in wall polymers which exposed stainable end-groups. Apart from a direct effect of sonication on some molecular bonds, this could have resulted from a stimulation of lytic enzymes present in the wall with consequent partial degradation of the wall constituents.

Ultrastructure of isolated walls

A comparative ultrastructural analysis of isolated walls was undertaken to confirm the differences between exponential and stationary phase organisms and to correlate them with the chemical composition of the wall. Purified walls from organisms harvested during the exponential and stationary phases of growth had a uniformly electron-transparent appearance under the electron microscope regardless of the methods of fixation and staining employed (Fig. 3). The appearance was the same even when cytoplasmic remnants remained attached to the wall or the wall itself was not completely purified. Only in a few instances were thin, inconspicuous low-density components seen outlining the outer wall profile (Fig. 3). Layered electron-dense components could be preserved, to a limited extent, by fixing the organisms before breakage, but the purity of such preparations was very low, there being massive cytoplasmic contamination. Isolated walls from organisms harvested from both exponentially growing and stationary phase cultures were consistently thinner than their counterparts in intact cells. The measured thicknesses were: exponential phase cells, 101 ± 20 nm; stationary phase cells, 100 ± 19 nm. These values are, however, only approximate due to the sectioning effect and the observation that in the same wall profile some parts were thicker than others.

Cytochemical detection of polysaccharides in isolated walls

The organization of the constituents of the walls of both exponentially growing and stationary phase organisms was examined by the method devised by Thiéry (1967) for the cytological detection of polysaccharides. By this staining procedure mannan and 1,6- β -glucan, but not 1,3- β -glucan or chitin, are detected. The applications of this technique to intact cells have been described by Cassone *et al.* (1972) and, recently, by Poulain *et al.* (1978) who confirmed the complex nature of wall layering in *C. albicans*. Isolated walls from both exponential phase and stationary phase organisms reacted intensely with the Thiéry stain giving a reproducible pattern of silver grains (Figs 4, 5). In both wall preparations the reaction was strongest at the outer surface, but the outermost material was irregular and discontinuous and there was no precise separation into layers. In walls from exponentially growing organisms the staining reaction was less intense in the inner regions of the wall, the innermost part being almost unreactive (Fig. 5a). This finding is consistent with the postulated abundance of 1,3- β -glucan and chitin in this region (Chattaway *et al.*, 1976; Cassone *et al.*, 1978; Poulain *et al.*, 1978). In walls from organisms harvested from stationary phase cultures the staining reaction was strong and uniform across the wall, indicating a more uniform distribution of reactive and unreactive polysaccharides consistent with a greater intermixing of wall constituents in these organisms (Fig. 5b, c).

Changes in the main carbohydrate fractions during growth and starvation

Since electron microscopic examination had demonstrated considerable differences in the organization and structure of the wall of *C. albicans* following the onset of starvation, both when examined *in situ* and in isolated purified preparations, an attempt was made to

Fig. 3. Isolated and purified walls from exponential phase (a), stationary phase (b) and late-stationary phase (c) cultures. Note the absence, or the very scanty appearance, of electron-dense components at the outer wall surface (arrows in c). Very few membrane profiles are seen in (a) and (b). Sections were stained with lead citrate, uranyl acetate and lead citrate again for 15 min (a), 45 min (b) or 30 min (c); due to this overstaining, the embedding plastic is electron-dense. Bar markers represent 500 nm (a, b) or 100 nm (c).

Fig. 4. Isolated walls from an exponential phase culture stained for polysaccharides by the method of Thiéry. Note the intense reaction of the outer wall region (arrows). The inset shows a control preparation in which the periodate oxidation step was omitted; walls had a similar appearance when the thiosemicarbazide treatment was omitted. Bar markers represent 500 nm and 100 nm (inset).

Fig. 5. Polysaccharide detection in isolated walls from exponential phase (a), early-stationary phase (b) and late-stationary phase (c) organisms. Note the more uniform reactivity in the inner region of the wall (arrows) as the cells progressed towards the late-stationary phase of growth (compare a with c). Bar markers represent 100 nm.

Table 1. Carbohydrate content of intact cells of *Candida albicans*

The organisms were grown in YNMG at 37 °C and after cessation of growth incubation was continued under the same conditions. The organisms were fractionated and carbohydrate was assayed as described in Methods. The figures in parentheses indicate the percentage composition.

	Carbohydrate content [μg (mg dry wt) $^{-1}$]				
	Mannan	Alkali-soluble glucan	Acid-soluble glucan	Insoluble glucan	Total
Exponentially growing organisms	53 (22)	11 (5)	123 (52)	48 (20)	238
After 24 h starvation	56 (29)	4 (2)	74 (39)	57 (30)	191
After 96 h starvation*	51 (29)	7 (4)	68 (37)	58 (31)	184

* Similar values were recorded after 144 h starvation.

correlate these changes with the carbohydrate composition of the various preparations. The one major change which occurred following the onset of starvation was a fall in the 'soluble glucan' fraction during the first 24 h after the cessation of growth. Thereafter the relative proportions of the carbohydrate constituents remained approximately constant up to 144 h starvation, although there was a slight decrease in the total carbohydrate on continued incubation (Table 1). Analysis of the carbohydrate composition of isolated purified walls from both exponentially growing and starved organisms demonstrated that, although the recovery of total carbohydrate was somewhat low (67% for the exponential phase organisms and 75% for the stationary phase organisms), the relative ratios of the major constituents were approximately the same as in the intact cells and there was apparently no preferential loss of particular carbohydrates during the isolation and purification of the wall which might account for the different appearance of the wall *in situ* and in purified preparations.

DISCUSSION

The studies reported in this paper have clearly demonstrated that the wall of *Candida albicans* undergoes significant ultrastructural changes during continued incubation after the cessation of growth. Therefore there is a structural basis for the finding by Gale *et al.* (1975) that stationary phase organisms are more resistant to the polyene antibiotics than exponentially growing organisms and that this resistance is associated with the wall since protoplasts prepared from resistant cultures are sensitive. Hammond & Kliger (1974) reported an increase in the thickness of the wall of *C. albicans* during ageing and this has been confirmed. There is, however, no direct relationship between the increase in thickness of the wall and the acquisition of resistance, since there was a 10-fold increase in resistance during the first 24 h and a 60-fold increase in resistance after 72 h starvation during which time there was no appreciable change in the thickness of the wall. Only when organisms are resistant to AME at concentrations in excess of 20 $\mu\text{g ml}^{-1}$ are the walls substantially thicker. There appears to be a better correlation between the development of resistance to polyene antibiotics and the changes in the ultrastructure of the wall, as exemplified by the loss of the layered appearance of the wall on continued incubation after the cessation of growth in a period corresponding to an increase in resistance from 10-fold to more than 100-fold. It should also be stressed that the recovery of antibiotic sensitivity following resuspension in a fresh nutrient medium is associated with the reappearance of the layered structure of the wall. Poulain *et al.* (1978) also reported a loss of some layered wall components, probably mannan-proteins, from the wall of *C. albicans* starved for 2 months.

There were no significant differences in appearance between the walls isolated from exponentially growing cultures and stationary phase cultures comparable to those observed in walls examined *in situ*. The profiles were significantly thinner than those observed *in situ* and had a rather dull, uniformly electron-transparent structure lacking any significant

electron-dense components and layering. Some electron-dense material could be detected on the outermost surface of the wall after fixation and intense overstaining of the sections, but the slight differences that could be observed in some preparations could not be correlated with those observed in intact organisms. These ultrastructural observations are in accord with chemical analyses of isolated wall material which failed to demonstrate any significant difference in the gross chemical composition of the walls prepared from organisms harvested from exponential and stationary phase cultures (Kerridge *et al.*, 1976).

One explanation of the apparent differences between the ultrastructure of walls *in situ* and walls purified by extensive washing after the organisms have been broken mechanically by shaking with glass beads is that certain of the wall constituents which are less firmly bound to the rigid glucan-chitin network are lost during the preparative procedure. Since the wall profiles remaining after ultrasonic disintegration of exponential phase organisms did retain some layered organization it would appear that it is not cell breakage *per se* which is responsible for the changed appearance but the technique employed for this purpose. Indeed, there is much circumstantial evidence for the loose binding of several 'plastic' components to the rigid glucan-chitin network of the wall (Kessler & Nickerson, 1959; Chattaway *et al.*, 1968; Mason, 1978). Recently, Nickerson & Swaminathan (1977) emphasized the role of non-covalent, weak bonds in the overall wall organization of *C. albicans*. The electron-dense components responsible for the layering in the walls of *Candida* (and possibly other yeasts) have been shown to be alkali-soluble glucans and mannan-proteins which are present in the periplasmic layer and also secreted through the wall (Cassone *et al.*, 1978). These stainable constituents may represent a relatively small proportion of wall material, for instance the proteins which expose ionized groups reactive to ordinary fixatives and stains. Thus, loss of a few wall components may greatly change the ultrastructural pattern of the wall. In this connection it is of interest to note that the periplasmic layer was either greatly reduced or absent from the walls of organisms which had been subjected to prolonged starvation. If these constituents are important in the penetration of the polyene antibiotics through the wall of *C. albicans* it would not have been possible to detect them in wall preparations.

The reduction in both alkali- and acid-soluble glucans of intact organisms associated with the onset of starvation favours the idea that fewer 'plastic' wall components are present in the walls of stationary phase organisms. If this is so then the overall structure of the wall may be more tightly cross-linked in a network which includes all the major wall constituents without any significant spatial separation. This concept is supported by the more uniform reaction to the Thiéry cytochemical test exhibited by walls from stationary phase organisms which would indicate that the reactive 1,6- β -glucan and mannan and unreactive 1,3- β -glucan and chitin are intermixed, and it must be emphasized that an increased cross-linking of the wall polysaccharide matrix can be achieved without gross changes in the chemical composition of the wall. It has not been possible in these studies to identify the precise changes in the structure and organization of the wall of *C. albicans* which are responsible for the phenotypic development of resistance to the polyene antibiotics. However they do demonstrate a structural basis for this phenomenon since the organization of the wall is significantly changed on prolonged incubation. The other important finding is that extreme caution must be observed in attempting to extrapolate results obtained with isolated purified walls to the structure and organization of the wall in intact organisms.

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