Growth Characteristics and Polyene Sensitivity of a Fatty Acid Auxotroph of *Candida albicans*

By T. Y. KOH,* M. S. MARRIOTT,† J. TAYLOR AND E. F. GALE

*Sub-Department of Chemical Microbiology, Department of Biochemistry,
Tennis Court Road, Cambridge CB2 1QW*

(Received 11 March 1977)

A fatty acid auxotroph of *Candida albicans* 6406, designated A'44 and originally isolated as an oleic acid requiring strain, has been shown to be a Δ^9 desaturase mutant. Although lacking this step in fatty acid biosynthesis, it appears to retain the ability to desaturate mono-unsaturated fatty acids. The polyene sensitivity of the organism grown on different fatty acid supplements varied between 0.08 ± 0.02 and $1.20 \pm 0.30 \mu\text{g}$ amphotericin B methyl ester ml^{-1} for exponentially growing cells. In spite of this variation, the sterol composition remained fairly constant, the major differences lying in fatty acid composition. Stationary-phase cells were more resistant to amphotericin B methyl ester, although again this change was not associated with changes in sterol content. The organism was most resistant when grown in the presence of oleic or linoleic acid. Protoplasts derived from resistant organisms grown on these two fatty acids were also resistant, indicating that the structure of the cell wall was less important than that of the plasma membrane in determining polyene sensitivity under these conditions.

INTRODUCTION

Recent work indicates that the resistance of *Candida albicans* to polyene antibiotics during growth in batch culture increases as the cells enter the stationary phase (Gale, 1974). This phenotypic variation results from changes in the cell wall, since enzymic (protoplast formation) or chemical modification of the wall greatly reduces this resistance (Gale *et al.*, 1975). The chemical procedures used to modify the polyene sensitivity would all be expected to interact with protein components of the wall. The changes in cell wall organization which render a cell resistant are as yet unknown.

Douglas *et al.* (1975) found that modifying the fatty acid content of the plasma membrane of *Saccharomyces cerevisiae* resulted in cells with walls which had various degrees of sensitivity to enzymic hydrolysis. These changes in susceptibility could be attributed to altered patterns of glucan and mannan (mannan-protein) biosynthesis. Marriott (1977) has also found that the plasma membrane-bound mannan synthetase of *C. albicans* is influenced by its lipid environment.

We have therefore investigated the relative roles of plasma membrane and cell wall structure in determining polyene sensitivity. For this we have studied the characteristics of polyene-induced K^+ release from a fatty acid auxotroph of *C. albicans* grown under a variety of conditions. This paper describes the results obtained and also presents information on the mechanism of fatty acid biosynthesis in this organism.

* Present address: Department of Biochemistry, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia.

† Present address: Fachbereich Biologie und Vorklinische Medizin, Universität Regensburg, 8400 Regensburg, West Germany.

METHODS

Mutagenesis. The parent organism was *Candida albicans* 6406, obtained from the Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine, London. Cells from a culture growing exponentially in a synthetic yeast (SY) medium (Davies, 1953) were harvested and resuspended in fresh medium containing 200 µg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine ml⁻¹ (Koh, 1975). After 30 min incubation at 37 °C, the cells were harvested, washed six times and resuspended in sterile water. Samples of different dilutions were plated out on a complex agar medium [containing (% w/v): Difco yeast extract, 1; Difco Bactopeptone, 1; glucose, 2; agar, 2] plus supplements [oleic acid, 0.2 mg ml⁻¹; tergitol, 0.1 % (w/v)] to determine the number of survivors. Subsequently, master plates were prepared with approximately 100 colonies per plate. Replicas from these master plates were transferred, using sterile velvet pads, to complex agar medium and synthetic agar medium [SY medium containing 2 % (w/v) glucose and 2 % (w/v) agar], with and without supplements. From the replica plates, a number of strains were selected which appeared to be fatty acid auxotrophs. Only one of these, designated A'44, proved to be a strict auxotroph, although in a subsequent experiment a temperature-sensitive mutant was isolated.

Growth requirements. Although tergitol was originally used to solubilize the fatty acid in the growth medium, it was later found that the sodium salt would substitute satisfactorily. The abilities of several fatty acids, including dodecanoic (C_{12:0}), tetradecanoic (C_{14:0}), hexadecanoic (C_{16:0}), octadecanoic (C_{18:0}), Δ⁹-hexadecenoic (C_{16:1}), Δ⁹-octadecenoic (C_{18:1}), Δ^{9,12}-octadecadienoic (C_{18:2}) and Δ^{9,12,15}-octadecatrienoic (C_{18:3}), to support the growth of strain A'44 were tested at a range of concentrations.

Fatty acid and sterol composition. Cells were harvested after either 24 h (exponential phase) or 48 h (stationary phase) growth and lyophilized. The freeze-dried cells were extracted three times with chloroform/methanol (2:1, v/v), at least one extraction being carried out overnight (Marriott, 1975). The lipid extracts were washed according to the method of Folch, Lees & Sloane-Stanley (1957). Samples of the lipid extract were transmethylated for gas-liquid chromatography using methanol/benzene/sulphuric acid (20:10:1, by vol.) (Nichols & Moorhouse, 1969). Sterols were extracted, after saponification (Breivik & Owades, 1957), into heptane and analysed by gas-liquid chromatography without further modification. Fatty acid methyl esters were separated on a Pye series 104 gas chromatograph using a polyethylene glycol adipate column. For the separation of sterols, a JXR (polydimethylsiloxane) column was used (Vandenheuvel & Court, 1968). Sterols were identified as described by Marriott (1975).

Polyene sensitivity. The sensitivity of cells grown on various fatty acid supplements to the methyl ester of amphotericin B (AME) was tested by measuring the release of potassium ions as described previously (Gale, 1974). The sensitivity of protoplasts derived from these cells was also tested. The standard release concentration (s.r.c.), defined as that concentration of antibiotic which would elicit a release rate of 1 nmol K⁺ min⁻¹ within 8 min (Gale, 1974), was used as a comparative measure of polyene sensitivity.

Materials. All materials used were analytical reagent grade where available. C_{12:0}, C_{14:0}, C_{16:0}, C_{16:1} and C_{18:0} fatty acids were obtained from Calbiochem. C_{18:1}, C_{18:2} and C_{18:3} fatty acids were from Sigma. The purity of each fatty acid was tested by gas-liquid chromatography of its methyl ester, with the following results: C_{16:1} contained 99 % C_{16:1} and a trace of C_{18:1}; C_{18:1} contained 6 % C_{16:0}, 10 % C_{16:1}, 76 % C_{18:1} and 4 % C_{18:2}; C_{18:2} contained 2 % C_{16:1}, 8 % C_{18:1} and 89 % C_{18:2}; C_{18:3} contained 5 % C_{18:1}, 19 % C_{18:2} and 76 % C_{18:3}.

AME was the kind gift of Professor C. P. Schaffner of Rutgers University, U.S.A.

RESULTS

Growth requirements

The auxotrophic strain of *Candida albicans* designated A'44 was originally isolated as an oleic acid (C_{18:1}) requiring mutant. The ability of this strain to grow on other fatty acids is shown in Table 1. Whilst other unsaturated fatty acids could substitute for oleic acid, saturated ones could not. The mean generation times of the mutant grown on the various fatty acids were considerably longer than that of the parent strain growing in unsupplemented SY medium (approximately 90 min).

Strain A'44, when grown in the presence of 5 % (v/v) horse serum, produced germ tubes. This is a standard diagnostic test for *Candida albicans*.

Table 1. Growth characteristics of *Candida albicans* strain A'44 in SY medium supplemented with various fatty acids, present as their sodium salts

Fatty acid supplement (mg ml ⁻¹)	Mean generation time (min)	Growth yield (mg ml ⁻¹)
C _{12:0} , C _{14:0} (each 0.1)	—	—
C _{16:0} , C _{18:0} (each 0.1)	—	—
C _{16:1} (0.1)	190 ± 10	1.70
C _{18:1} (0.2)	220 ± 15	2.10
(0.1)	220 ± 10	2.00
(0.05)	220 ± 15	1.60
(0.025)	220 ± 10	0.69
C _{18:2} (0.1)	270 ± 20	1.40
C _{18:3} (0.1)	290 ± 20	2.00

Table 2. Percentage fatty acid and lipid composition of whole cells of *Candida albicans* strain A'44 grown with various fatty acid supplements

Strain A'44 was grown with fatty acids at 0.1 mg ml⁻¹ unless otherwise stated. The results are the mean of at least three determinations and standard deviations are given. Results for both exponential- and stationary-phase cells were within these limits. The fatty acid composition of the parent strain 6406 is included for comparison.

Fatty acid	Strain 6406	Strain A'44, grown with:				
		C _{16:1}	C _{18:1}	C _{18:1} *	C _{18:2}	C _{18:3}
C _{14:0}	2	—	—	1.2 ± 0.2	—	0.6 ± 0.2
C _{15:0}	1	0.8 ± 0.1	3.3 ± 0.3	4.8 ± 0.4	2.6 ± 0.3	1.6 ± 0.2
C _{16:0}	15	16.5 ± 1.3	18.7 ± 2.1	36.8 ± 1.4	39.3 ± 0.7	31.6 ± 3.3
C _{16:1}	20	59.4 ± 0.4	6.9 ± 0.6	—	3.6 ± 0.4	—
C _{18:0}	2	16.1 ± 0.7	15.4 ± 1.4	32.6 ± 2.1	33.8 ± 0.9	27.5 ± 2.6
C _{18:1}	31	—	32.7 ± 3.5	6.3 ± 0.2	—	—
C _{18:2}	23†	—	22.4 ± 2.2	15.5 ± 1.3	14.2 ± 1.6	9.0 ± 1.1
C _{18:3}	4	—	0.9 ± 0.1	2.6 ± 0.4	6.2 ± 0.8	30.0 ± 2.9
Lipid content (%)	—	4.6 ± 1.2	4.8 ± 1.1	3.1 ± 0.5	4.3 ± 0.6	3.7 ± 0.9

* Present at 0.025 mg ml⁻¹.

† Resolved into two components of approximately equal proportions. These may represent $\Delta^{9,12}$ and $\Delta^{9,15}$ isomers of C_{18:2}.

Fatty acid and sterol composition

Table 2 gives the fatty acid composition of whole-cell lipid extracts, after growth on various unsaturated fatty acids. The fatty acid composition of the parent strain grown in SY medium has been included for comparison. When strain A'44 was grown on C₁₈ fatty acids, extracts contained little or no C_{16:1}. The small amounts detected could have been due to impurities in the fatty acids used. However, when grown with C_{16:1} as supplement, this was the major fatty acid and no unsaturated C₁₈ fatty acids were present. C_{16:1} was also found in significant amounts in lipid extracts from the parent organism. As the fatty acids at 0.1 mg ml⁻¹ were not limiting growth, the fatty acid compositions of exponential- and stationary-phase cells were not significantly different.

The sterol composition of strain A'44 grown in SY medium with different fatty acid supplements is given in Table 3. Small but significant differences were observed between exponential- and stationary-phase cells. However, the overall patterns of sterol composition were essentially similar under the various growth conditions. The sterols present were also the major sterols found in the parent organism.

Table 3. *Percentage sterol composition of whole cells of Candida albicans* 6406 grown in SY medium and the fatty acid auxotroph A'44 grown with various fatty acid supplements

Fatty acids were present at 0.1 mg ml⁻¹ unless otherwise stated. Cells were harvested after 24 h (exponential phase) or 48 h (stationary phase). The results are the mean of three determinations; maximum variations were within $\pm 10\%$ of these values.

Sterol	Exponential phase					Stationary phase				
	Strain 6406	Strain A'44, grown with:				Strain 6406	Strain A'44, grown with:			
		C _{16:1}	C _{18:1}	C _{18:2}	C _{18:3}		C _{16:1}	C _{18:1} *	C _{18:2}	C _{18:3}
Total (% dry wt)	0.66	0.75	0.59	0.58	0.69	0.90	1.17	0.74	0.71	0.96
Zymosterol	15	41	34	26	26	29	36	34	35	33
Ergosterol	66	35	45	36	38	36	30	44	27	49
24,28-Dehydroergosterol	4	9	9	16	14	13	12	9	14	7
4-Methylzymosterol	5	5	6	12	9	6	10	7	10	6
4,4'-Dimethylzymosterol	2	10	6	10	13	15	12	6	14	5

* Present at 0.025 mg ml⁻¹.

Table 4. *Polyene sensitivity of Candida albicans* strain A'44 grown with various fatty acid supplements

The fatty acids were present in the growth medium at 0.1 mg ml⁻¹ unless otherwise stated. Results are expressed as s.r.c. of AME (see Methods) under the different growth conditions. They are the mean of at least three determinations and standard deviations are given. Results of Gale *et al.* (1975) for the parent strain 6406 are included for comparison.

Fatty acid supplement	Polyene sensitivity (s.r.c. in $\mu\text{g AME ml}^{-1}$)			
	Exponential-phase cells	Protoplasts from exponential-phase cells	Stationary-phase cells	Protoplasts from stationary-phase cells
C _{16:1}	0.08 $\pm$ 0.02	—	0.65 $\pm$ 0.15	0.90 $\pm$ 0.30
C _{18:1}	1.20 $\pm$ 0.30	0.80 $\pm$ 0.30	2.50 $\pm$ 0.20	—
C _{18:1} *	—	—	0.70 $\pm$ 0.20	0.90 $\pm$ 0.40
C _{18:2}	0.75 $\pm$ 0.25	0.85 $\pm$ 0.15	3.80 $\pm$ 1.00	2.80 $\pm$ 0.30
C _{18:3}	0.08 $\pm$ 0.02	—	1.60 $\pm$ 0.30	0.70 $\pm$ 0.30
Strain 6406	0.12	0.07	5.00	0.15

* Present at 0.025 mg ml⁻¹.

Polyene sensitivity

Values of the s.r.c. of AME for strain A'44 grown in the presence of various unsaturated fatty acids are given in Table 4. This table also compares the sensitivity of exponential- and stationary-phase cells and protoplasts derived from them. When grown in the presence of C_{16:1} and C_{18:3}, exponential-phase cells were as sensitive as the parent strain and, hence, protoplasts from these cells were not tested. Under all growth conditions, an increase in resistance to AME-induced K⁺ leakage was observed as the cells entered stationary phase.

DISCUSSION

The fatty acid auxotroph *Candida albicans* A'44 has a growth requirement for an unsaturated fatty acid. Although not tested rigorously, it clearly resembles the Δ^9 desaturase mutants of *Saccharomyces cerevisiae* isolated by Resnick & Mortimer (1966). The formation of Δ^9 unsaturated fatty acids from the saturated acid was first shown in *S. cerevisiae* by Bloomfield & Block (1960). Keith, Resnick & Haley (1969) identified a number of independent genetic loci in this organism, which control this desaturation step. However, little

information is available on the enzymic mechanism of further desaturation (Δ^{12} and Δ^{15}) in yeasts. The work of Wisnieski, Keith & Resnick (1970) suggests there was little or no conversion of the added unsaturated fatty acid into other fatty acids.

Unfortunately, the fatty acids used in our study were not 100 % pure. Nevertheless, a number of valid observations can be made. Firstly, when grown on palmitoleic acid ($C_{16:1}$) no longer chain unsaturated fatty acids were found, indicating that conversion of palmitoleic to oleic ($C_{18:1}$) acid does not occur. Secondly, although the oleic acid used as a growth supplement was contaminated with linoleic acid ($C_{18:2}$) this was too low to account for the amount of linoleic acid found in these cells, particularly at the lower concentration. Thirdly, the samples of oleic and linoleic acids did not contain any linolenic acid ($C_{18:3}$), yet this fatty acid was found in extracts from cells grown with these supplements. These last two observations indicate that, in *C. albicans*, a different set of enzymes from those involved in the initial Δ^9 desaturation step control the conversion of oleic acid to linoleic and linolenic acids. The resolution of the $C_{18:2}$ peak into components by gas-liquid chromatography, probably representing $\Delta^{9,12}$ and $\Delta^{9,15}$ isomers of $C_{18:2}$, suggests that the conversion of oleic to linolenic acid may occur via two intermediates.

In terms of polyene sensitivity, exponential-phase cells of strain A'44 grown in the presence of oleic or linoleic acid were significantly more resistant than the parent strain grown in unsupplemented SY medium. Protoplasts derived from these cells were, within experimental error, equally resistant. With all four unsaturated fatty acids, stationary-phase cells were more resistant than the corresponding exponential-phase cells. This resistance could again be largely attributed to the plasma membrane rather than the cell wall, since protoplasts derived from these cells were almost equally resistant.

Some variation in sterol composition was observed when strain A'44 was grown in the presence of different unsaturated fatty acids. However, these variations were not sufficient to account for the large variations in polyene sensitivity of the corresponding cells or protoplasts. The variation in sensitivity thus appears to be due to the differing fatty acid compositions, although no direct correlation can be made. HsuChen & Feingold (1973) and Archer (1976) have suggested that sterol-phospholipid interaction, rather than the presence of sterol *per se*, is important in determining the binding of polyenes to membranes. It would appear that our results reflect these observations; however, confirmation of this would require a detailed chemical analysis of the plasma membrane from resistant cells.

This work was supported by a grant from the Medical Research Council.

REFERENCES

- ARCHER, D. B. (1976). Effect of the lipid composition of *Mycoplasma mycoides* subspecies *capri* and phosphatidylcholine vesicles upon the action of polyene antibiotics. *Biochimica et biophysica acta* **436**, 68-76.
- BLOOMFIELD, D. K. & BLOCK, K. (1960). The formation of Δ^9 unsaturated fatty acids. *Journal of Biological Chemistry* **235**, 337-345.
- BREIVIK, O. N. & OWADES, J. L. (1957). A semi-micromethod for determining the percentages of ergosterol and 24,28 dehydroergosterol. *Journal of Agricultural and Food Chemistry* **5**, 360-363.
- DAVIES, R. (1953). Enzyme formation in *Saccharomyces fragilis*. I. Invertase and raffinase. *Biochemical Journal* **55**, 484-497.
- DOUGLAS, L. J., ATKINSON, D. M., HOSSACK, J. A. & ROSE, A. H. (1975). A possible role for lipid unsaturation in the biosynthesis of wall polymers in *Saccharomyces cerevisiae*. In *Abstracts of the Fourth International Symposium on Yeast and Other Protoplasts*, p. 71.
- FOLCH, J., LEES, M. & SLOANE-STANLEY, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* **226**, 497-509.
- GALE, E. F. (1974). The release of potassium ions from *Candida albicans* in the presence of polyene antibiotics. *Journal of General Microbiology* **80**, 451-465.
- GALE, E. F., JOHNSON, A. M., KERRIDGE, D. & KOH, T. Y. (1975). Factors affecting the changes in amphotericin sensitivity of *Candida albicans* during growth. *Journal of General Microbiology* **87**, 20-36.
- HUSCHEN, C. C. & FEINGOLD, D. S. (1973). Polyene antibiotic action on lecithin liposomes: effect of cholesterol and fatty acyl chains. *Biochemical and Biophysical Research Communications* **51**, 972-978.
- KEITH, A. D., RESNICK, M. A. & HALEY, A. B. (1969). Fatty acid desaturase mutants of *Saccharomyces cerevisiae*. *Journal of Bacteriology* **98**, 415-420.

- KOH, T. Y. (1975). The isolation of obligate osmophilic mutants of the yeast *Saccharomyces rouxii*. *Journal of General Microbiology* **88**, 184-188.
- MARRIOTT, M. S. (1975). Isolation and chemical characterization of plasma membranes from the yeast and mycelial forms of *Candida albicans*. *Journal of General Microbiology* **85**, 115-132.
- MARRIOTT, M. S. (1977). Mannan-protein location and biosynthesis in plasma membranes from the yeast form of *Candida albicans*. *Journal of General Microbiology* (in the Press).
- NICHOLS, B. W. & MOORHOUSE, R. (1969). The separation, structure and metabolism of monogalactosyl diglyceride species in *Chlorella vulgaris*. *Lipids* **4**, 311-316.
- RESNICK, M. A. & MORTIMER, R. K. (1966). Unsaturated fatty acid mutants of *Saccharomyces cerevisiae*. *Journal of Bacteriology* **92**, 597-600.
- VANDENHEUVAL, F. A. & COURT, S. A. (1968). Reference high-efficiency nonpolar packed columns for the gas-liquid chromatography of nanogram amounts of steroids. I. Retention time data. *Journal of Chromatography* **38**, 439-459.
- WISNIESKI, B. J., KEITH, A. D. & RESNICK, M. A. (1970). Double bond requirement in a fatty acid desaturase mutant of *Saccharomyces cerevisiae*. *Journal of Bacteriology* **101**, 160-165.