Effect of Glucose and Long-chain Fatty Acids on Synthesis of Long-chain Alcohols by *Candida albicans*

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Candida albicans, grown aerobically in glucose-containing media, produced C₁₄, C₁₆ and C₁₈ saturated long-chain alcohols only after the end of exponential growth. Contents of C₁₄ alcohols were always lowest, and C₁₆ and C₁₈ alcohol contents about equal. Contents of all three classes of alcohol increased as the concentration of glucose in aerobic cultures harvested after 168 h incubation was raised from 1.0 to 30.0% (w/v). However, in 168 h anaerobic cultures, greatest long-chain alcohol contents in organisms were obtained using media containing 10% (w/v) glucose. Substituting glucose (10%, w/v) with the same concentration of galactose in aerobic cultures greatly decreased contents of long-chain alcohols, while inclusion of 10% (w/v) glycerol virtually abolished their synthesis. Supplementing anaerobic cultures with odd-chain fatty acids induced synthesis of odd-chain alcohols. Maximum conversion of fatty acid to the corresponding long-chain alcohol was observed with heptadecanoic acid. The effect of glucose on production of heptadecanol from exogenously provided heptadecanoic acid was similar to that observed on synthesis of the three major even-chain alcohols in media lacking a fatty-acid supplement. Cell-free extracts of organisms catalysed *in vitro* conversion of palmitoyl-CoA to 1-hexadecanol.

INTRODUCTION

A number of micro-organisms are known to synthesize long-chain alcohols. Among prokaryotes, they include mycobacteria (Ratledge, 1976) and other bacteria with the capacity to oxidize alkanes (Ratledge, 1978; Britton, 1984). When grown on carbon sources other than alkanes, some bacteria synthesize long-chain primary alcohols by reduction of CoA esters of long-chain carboxylic acids which arise during endogenous production of fatty-acyl residues (Day *et al.*, 1970; Naccarato *et al.*, 1972; Lloyd & Russell, 1983) or which are supplied exogenously (Naccarato *et al.*, 1974; Lloyd & Russell, 1983). Less has been reported on long-chain alcohol production by yeasts. Some species can grow on alkanes (Britton, 1984) and certain of these, including *Candida guilliermondii* (Muratov *et al.*, 1979) and *Rhodotorula glutinis* (Zalashko *et al.*, 1979; Zalashko & Salokhina, 1982), may accumulate wax esters containing long-chain alcohols. Davidova *et al.* (1978) reported briefly that glucose-grown *C. tropicalis* synthesized wax esters, although greater amounts were produced when the yeast was grown on n-octadecane. White *et al.* (1987) detected unesterified long-chain alcohols in several yeast strains grown on glucose. They screened 14 yeast strains from six genera for ability to synthesize these alcohols, and found that six strains from three genera had this ability, highest levels being

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found in *Candida albicans*. The alcohols were predominantly C₁₄, C₁₆ and C₁₈ saturated primary alcohols; unsaturated alcohols were not detected. Long-chain alcohol synthesis was favoured by anaerobic conditions during growth, and occurred only after the end of exponential growth. The present paper reports on the effect of glucose concentration and exogenous provision of long-chain fatty acids on long-chain alcohol synthesis by *C. albicans* grown aerobically or anaerobically.

METHODS

Organism. The organism used was *Candida albicans* NCYC 1467, kindly provided by L. Julia Douglas, University of Glasgow, UK, before it was deposited in the collection. It was maintained at 4 °C on slopes containing (l⁻¹): malt extract, 3.0 g; yeast extract (Lab M), 3.0 g; glucose, 10 g; peptone, 0.5 g; and agar, 20 g (MYGP; Wickerham, 1951).

Experimental cultures. Organisms were grown aerobically or anaerobically in a medium containing (l⁻¹): (NH₄)₂SO₄, 3.0 g; KH₂PO₄, 4.5 g; yeast extract (Lab M), 4.0 g; MgSO₄ · 7H₂O, 25 mg; and CaCl₂ · 2H₂O, 25 mg (adjusted to pH 4.5 with HCl). Unless otherwise indicated, the carbon source was glucose, at the concentration stated in the text. Portions (1 l) of medium were dispensed into 2-litre round flat-bottomed flasks and sterilized by autoclaving at 10 lbf in⁻² (6.89 × 10⁴ Pa) for 1 min. In certain experiments, the medium was supplemented with a long-chain fatty acid (10 mg l⁻¹) added either as a 1 ml solution in ethanol at the start of growth or 2 mg (24 h)⁻¹ in ethanol to a final concentration of 10 mg l⁻¹ after an initial 24 h incubation. Starter cultures (100 ml medium in a 250 ml flask) were inoculated with a pinhead of organisms from a slope culture, and incubated for 48 h at 30 °C on an orbital shaker (200 r.p.m.). Experimental cultures were inoculated with a portion of starter culture containing 10 mg dry wt organisms, and incubated aerobically or anaerobically as described by White *et al.* (1987). Growth was followed by measuring optical density at 600 nm, measurements being related to dry wt by a standard curve. At times indicated, organisms were harvested by centrifugation at 6000 g at 4 °C and washed twice with water.

Analysis of long-chain alcohols. Methods for extracting lipids from organisms, separating long-chain alcohols from extracts and analysing the alcohols were as described by White *et al.* (1987).

Preparation and fractionation of cell-free extracts. Anaerobically grown organisms (2 g dry wt) from 72 h cultures were washed twice with 100 mM-potassium phosphate buffer (pH 6.5) containing 5 mM-2-mercaptoethanol, suspended in 10 ml buffer and the suspension shaken in a Braun homogenizer for four periods of 30 s at speed 2 (4000 r.p.m.) with 40 g glass beads (Sigma type V; 0.45–0.50 mm diameter). During cell disruption, the sample bottle was cooled with expanding CO₂. The homogenate was suspended in 120 ml buffer and whole organisms and debris removed by centrifugation at 500 g for 5 min. The supernatant was centrifuged at 18000 g for 30 min to remove mitochondria or promitochondria. A microsomal fraction was obtained by centrifuging the post-mitochondrial supernatant at 100000 g for 90 min and suspended in 6 ml buffer. Protein in the supernatant obtained after removal of the microsomal fraction was precipitated as described by Naccarato *et al.* (1974). Ammonium sulphate (heavy-metal-free) was added to the supernatant to give 55% saturation, the suspension stirred for 15 min at 4 °C, and the precipitated protein collected by centrifugation at 12000 g for 20 min. The protein was redissolved in 6 ml buffer. All centrifugations were done at 4 °C.

Enzyme assay of 1-hexadecanol formation. 1-Hexadecanol formation by cell-free fractions was assayed using [¹⁴C]palmitoyl-CoA. The reaction mixture (final volume 2 ml), in a 5 ml Reacti-Vial (Pierce), consisted of 100 mM-potassium phosphate buffer (pH 6.5) containing 5 mM-2-mercaptoethanol, 2 mg bovine serum albumin (fatty-acid-free), 0.3 µmol NADH or NADPH, 20 nmol [¹⁴C]palmitoyl-CoA (2.15 GBq mmol⁻¹; Amersham) and 2 mg microsomal or soluble protein. The reaction mixture was incubated for 1 h at 30 °C on an orbital shaker (200 r.p.m.) after the head space had been flushed with nitrogen gas. The reaction was terminated by adding 3 ml methanol, and lipids extracted by adjusting the solvent concentration to 1:1:2 (by vol.) water/methanol/chloroform. 1-Hexadecanol was added as carrier to the extraction solvent such that 25 nmol was present in each reaction mixture. The lower organic phase was removed, concentrated under a stream of nitrogen gas and spotted onto a precoated Silica Gel 60 TLC plate (20 cm × 20 cm × 0.25 mm thick; Merck). Plates were developed with a hexane/diethyl ether/acetic acid (30:70:1, by vol.) solvent mixture, and long-chain alcohols removed and prepared for GLC analysis as described by White *et al.* (1987). A 1 µl portion was analysed by GLC to determine the extent of carrier recovery (estimated from the ratio of the peak area of the 1-hexadecanol in the sample to the peak area of the 1-hexadecanol present in the extraction solvent). Radioactivity in the remaining samples was assayed using a LKB 1217 Rackbeta liquid scintillation spectrometer, each vial containing 7.5 ml Optiphase Safe scintillant. The amount of 1-hexadecanol synthesized was calculated by dividing the corrected radioactivity by the specific activity of the sample substrate.

Protein assay. Protein contents were determined using a dye-binding assay (Bio-Rad). Protein was extracted from the microsomal fraction by boiling a portion (0.1 ml) with an equal volume of 0.1 M-NaOH for 5 min, then neutralizing with 0.1 M-HCl (0.1 ml) and 0.2 ml acetic acid/sodium acetate buffer (0.2 M; pH 5.0). Portions (0.1 ml) of protein solution, diluted as necessary, were mixed with Bio-Rad dye reagent (5 ml; 20%, v/v, in water), the

solution vortexed and the colour allowed to develop for 5 min at room temperature. Absorbance values (A_{595}) were related to protein content by a calibration curve prepared using bovine serum albumin as a standard.

Chemicals. All chemicals used were AnalaR or of the highest grade available.

RESULTS

Effect of glucose on long-chain alcohol production

White *et al.* (1987) largely confined their study of long-chain alcohol production by *C. albicans* to anaerobically grown organisms. Since the media used by these workers for anaerobic and aerobic growth differed in glucose concentration, a study was made of the effect of glucose concentration on production of long-chain alcohols by this yeast. Aerobic growth in media containing 200 g glucose l^{-1} showed that organisms produced the three major classes of alcohol only after cessation of exponential growth (Fig. 1). While the content of C_{14} alcohols was always lower than those of C_{16} and C_{18} alcohols, the pattern of alcohol production differed from that encountered during anaerobic growth (White *et al.*, 1987) in that the contents of C_{16} and C_{18} alcohols were very similar. As the concentration of glucose in media used for aerobic growth of organisms was raised from 1.0 to 30.0% (w/v), the contents of all three major classes of long-chain alcohol increased in organisms from 168 h cultures (Fig. 2a) to a maximum total content of approximately 900 μg (g dry wt organisms) $^{-1}$. In cultures grown aerobically and containing any of the glucose concentrations, the content of C_{18} alcohol was slightly greater than that of the C_{16} alcohol and, as the glucose concentration was increased, so was the relative proportion of C_{14} alcohol. However, in cultures grown anaerobically for 168 h, peak contents of each of the three alcohol classes were greatest in organisms grown in media containing 10% (w/v) glucose (Fig. 2b), the maximum being approximately 980 μg (g dry wt organisms) $^{-1}$. In cultures containing each of the glucose concentrations, except 1.0% (w/v), C_{16} alcohols predominated in organisms. All cultures harvested after 168 h incubation had been in the stationary phase of growth for approximately the same length of time.

Fig. 1. Time-course of aerobic growth (○) and of synthesis of $C_{14:0}$ (●), $C_{16:0}$ (□) and $C_{18:0}$ (■) long-chain alcohols by *C. albicans*. Values quoted are means of three independent determinations $\pm$ SD.

Fig. 2. Effect of glucose concentration on the long-chain alcohol contents of *C. albicans* grown aerobically (a) or anaerobically (b) for 168 h. Numbers at the base of the histogram indicate the chain length of the alcohol. Each value quoted is the mean of at least three independent determinations. Bars at the top of each histogram indicate SD.

Table 1. *Effect of the nature of the carbon source on the long-chain alcohol composition of Candida albicans*

Organisms were grown aerobically for 168 h. Values quoted are the means of three independent determinations $\pm$ SD. ND indicates that the alcohol was not detected. Tr. indicates that a trace was detected.

Carbon source (10%, w/v)	Long-chain alcohol composition [$\mu\text{g (mg dry wt organisms)}^{-1}$]		
	C _{14:0}	C _{16:0}	C _{18:0}
Glucose	37.1 $\pm$ 5.3	130.0 $\pm$ 3.9	184.0 $\pm$ 11.2
Galactose	3.7 $\pm$ 0.3	33.7 $\pm$ 2.7	95.3 $\pm$ 11.2
Glycerol	ND	Tr.	Tr.

Effect of the nature of the carbon source on long-chain alcohol production

Substituting glucose (10%, w/v) with the same concentration of galactose greatly decreased the content of long-chain alcohols in organisms grown aerobically for 168 h (Table 1). Proportionally, the effect was greatest for C₁₄ alcohols and smallest for C₁₈ alcohols. Replacing glucose by glycerol (10%, w/v) virtually abolished production of long-chain alcohols (Table 1). All cultures harvested after 168 h incubation had been in the stationary phase of growth for approximately the same length of time.

Long-chain alcohol production in anaerobic cultures supplemented with saturated fatty acids

Organisms grown for 168 h in media containing 200 g glucose l⁻¹ and supplemented with 10 mg C_{14:0}, C_{16:0}, C_{16:1}, C_{18:0} or C_{18:1} fatty acid l⁻¹ had the same long-chain alcohol contents as organisms grown in unsupplemented media. However, supplementing media with the odd-chain fatty acids C_{13:0}, C_{15:0}, C_{17:0} or C_{19:0} caused the appearance of corresponding long-chain alcohols in organisms from 168 h anaerobically grown cultures (Table 2). These odd-chain alcohols co-chromatographed with authentic alcohol standards, and their identities were confirmed by GLC-MS (White *et al.*, 1987). Maximum conversion of odd-chain fatty acid to the corresponding alcohol was achieved with heptadecanoic acid. There was some evidence for chain elongation and β -oxidation when shorter and longer odd-chain fatty-acid substrates were used (Table 2). The amounts of odd-chain alcohols in organisms were very small compared with the amounts of the three major even-chain alcohols. Supplementing cultures every 24 h with

Table 2. Long-chain alcohol composition of *Candida albicans* grown in media supplemented with long-chain fatty acids

Cultures were grown anaerobically for 168 h in media containing 20% (w/v) glucose and 10 mg of the long-chain fatty acid indicated l⁻¹. Values quoted are the means of three independent determinations $\pm$ SD. ND indicates that the alcohol was not detectable. Tr. indicates that a trace was detected.

Fatty-acid supplement	Long-chain alcohol composition of organisms [μ g (mg dry wt organisms) ⁻¹]							Total
	C _{13:0}	C _{14:0}	C _{15:0}	C _{16:0}	C _{17:0}	C _{18:0}	C _{19:0}	
None	ND	129.9 $\pm$ 4.1	ND	312.4 $\pm$ 19.2	ND	213.1 $\pm$ 22.0	ND	655.4 $\pm$ 32.2
C _{13:0}	26.3 $\pm$ 0.2	100.2 $\pm$ 5.0	22.4 $\pm$ 0.3	296.9 $\pm$ 6.1	Tr.	146.2 $\pm$ 5.1	ND	592.1 $\pm$ 4.8
C _{15:0}	Tr.	115.4 $\pm$ 1.6	28.5 $\pm$ 1.0	319.4 $\pm$ 14.5	Tr.	206.8 $\pm$ 5.8	ND	670.2 $\pm$ 22.8
C _{17:0}	ND	133.1 $\pm$ 5.7	Tr.	307.2 $\pm$ 6.0	85.5 $\pm$ 4.5	202.4 $\pm$ 7.6	Tr.	728.1 $\pm$ 11.6
C _{19:0}	ND	132.7 $\pm$ 9.3	Tr.	323.1 $\pm$ 3.2	9.6 $\pm$ 1.0	200.0 $\pm$ 7.6	5.25 $\pm$ 0.3	670.7 $\pm$ 18.8

Table 3. Effect of glucose concentration on the long-chain alcohol composition of *Candida albicans* grown anaerobically in cultures repeatedly supplemented with a long-chain fatty acid

Cultures were grown for 168 h, and were supplemented every 24 h, after the first 24 h incubation, with 2 mg C_{17:0} fatty acid to a final concentration of 10 mg l⁻¹. Values quoted are the means of three independent determinations $\pm$ SD.

Concn of glucose in medium (% w/v)	Long-chain alcohol composition of organisms [μ g (mg dry wt organisms) ⁻¹]				
	C _{14:0}	C _{16:0}	C _{17:0}	C _{18:0}	Total
1.0	6.8 $\pm$ 0.9	24.0 $\pm$ 1.8	31.2 $\pm$ 6.3	17.0 $\pm$ 0.6	79.0 $\pm$ 9.5
5.0	56.0 $\pm$ 7.2	204.7 $\pm$ 20.8	38.6 $\pm$ 3.1	134.7 $\pm$ 16.7	434.0 $\pm$ 36.5
10.0	151.9 $\pm$ 6.4	427.0 $\pm$ 4.6	183.8 $\pm$ 8.6	228.5 $\pm$ 1.6	994.9 $\pm$ 14.7
20.0	144.1 $\pm$ 7.3	221.3 $\pm$ 10.7	153.9 $\pm$ 7.5	133.2 $\pm$ 8.1	652.5 $\pm$ 29.9
30.0	124.9 $\pm$ 19.9	114.9 $\pm$ 10.4	62.7 $\pm$ 8.1	46.6 $\pm$ 9.7	349.1 $\pm$ 47.2

2 mg C_{17:0} fatty acid l⁻¹ to a final concentration of 10 mg l⁻¹ increased the amount of C₁₇ alcohol synthesized (Tables 2 and 3). Moreover, the effect of glucose concentration on synthesis of C_{17:0} alcohols (Table 3) by organisms grown anaerobically for 168 h was similar to the effect on production of the three major even-chain alcohols (Fig. 2b). Production of C_{17:0} alcohols was least in organisms grown in medium containing 1.0% (w/v), rose to a maximum with medium containing 10.0% (w/v) and declined with media containing more than 10.0% (w/v) glucose. Production of C_{17:0} alcohols under these conditions also led to a decrease in the contents of C_{16:0} and C_{18:0} alcohols, while the C_{14:0} alcohol content remained unaltered (Fig. 2b, Table 3).

Reduction of palmitoyl-CoA to 1-hexadecanol by cell-free systems from anaerobically grown organisms

Both microsomal and soluble protein fractions catalysed reduction of palmitoyl-CoA to 1-hexadecanol. The mean rate of production using microsomal preparations was 0.14 $\pm$ 0.04 nmol h⁻¹ (mg protein)⁻¹ irrespective of whether NADH or NADPH was used as the reductant. A faster rate [0.70 $\pm$ 0.06 nmol h⁻¹ (mg protein)⁻¹] was obtained with protein from the soluble fraction. Again, the same rate was obtained whether NADH or NADPH was used as the reductant.

DISCUSSION

The discovery that *C. albicans*, when grown anaerobically in media supplemented with odd-chain saturated fatty acids, synthesized long-chain alcohols of corresponding chain length is particularly significant. When grown in the absence of fatty-acid supplements, this yeast does not synthesize odd-chain fatty-acyl residues (White *et al.*, 1987). With most yeasts, odd-chain fatty-acyl residues are detected in their lipids only when they are grown in the presence of odd-

chain hydrocarbons (Rattray *et al.*, 1975; Ratledge & Evans, 1988). In this connexion, our evidence for β -oxidation and chain elongation by organisms grown in the presence of certain odd-chain fatty acids is of considerable interest, particularly with regard to the specificity of the enzymes involved in the conversion. Our conclusion is that long-chain alcohols are synthesized in *C. albicans* by reduction of fatty acids, which can be supplied exogenously or which are synthesized endogenously as CoA esters in stationary-phase organisms that have ceased production of phospholipids, triacylglycerols and sterol esters. Naccarato *et al.* (1974) reported that stationary-phase cultures of *Escherichia coli* can convert exogenous fatty acids into long-chain alcohols. White *et al.* (1987) suggested that long-chain alcohols synthesized by *C. albicans* in anaerobic cultures are secondary metabolites. Data reported here indicate that this conclusion holds also for this yeast grown under aerobic conditions.

Synthesis of long-chain alcohols by *C. albicans* was clearly favoured by anaerobic as compared with aerobic conditions as well as by high concentrations of glucose, both of which are conditions which repress formation of mitochondria (Käppeli, 1986). Production of these long-chain alcohols might therefore be explained by the repressive conditions diverting acyl-CoA derivatives of fatty acids to reductive routes. It has been shown that many *Candida* species (Einsele *et al.*, 1972; Johnson *et al.*, 1972; Skipton *et al.*, 1974) have a repressed oxidative metabolism when grown in the presence of high concentrations of glucose and under oxygen limitation. It is possible that the shift observed in long-chain alcohol content with different glucose concentrations, between aerobic and anaerobic conditions, could be explained by the fact that, even at very high concentrations of glucose (10%, w/v), aerobic conditions are still less repressive than the self-induced anaerobic conditions.

The discovery that substituting galactose or glycerol for glucose in the medium greatly decreased or eliminated long-chain alcohol production suggests that alcohol synthesis is subject to carbon catabolite control. Galactose, another fermentable carbon source, is not as efficient as glucose in causing respiratory repression in *C. albicans* (Meyer *et al.*, 1984). Brown & Johnson (1970) found that higher concentrations of galactose, as distinct from glucose, were required to induce fermentative activity in *Saccharomyces cerevisiae*. It would, therefore, seem not unreasonable to suggest that the inefficiency of galactose in causing mitochondrial repression could be responsible for the decreased content of alcohols in *C. albicans* grown on this substrate. *Candida albicans* is unable to use glycerol as a fermentable carbon source, but it is capable of respiring it (Meyer *et al.*, 1984). That this organism would have an active respiratory system when grown on glycerol may explain its inability to synthesize significant amounts of long-chain alcohol. The finding that glucose concentration, in aerobic or anaerobic cultures, markedly affects alcohol content indicates that the regulatory effect of glucose in long-chain alcohol synthesis is complex. Synthesis of another secondary metabolite, a glycolipid, by the yeasts *Torulopsis bombicola* and *Torulopsis magnoliae* has been reported to be affected by the concentration of glucose in the growth medium (Spencer *et al.*, 1979). Medium containing 10% (w/v) glucose generally gave better yields than media containing lower concentrations of glucose, although the mechanism of action is unknown. Moreover, Martín (1979) reported that macrolide antibiotic biosynthesis by *Streptomyces fradiae* is inhibited by high concentrations of glucose. This is believed to be due to its inhibitory effect on fatty-acid degradation, the products of which act as precursors for production of these secondary metabolites. Our ability to demonstrate cell-free conversion of palmitoyl-CoA to 1-hexadecanol means that it will be possible to study experimentally the effect of glucose on synthesis and activity of the enzymes that catalyse conversion of fatty acids into long-chain alcohols in *C. albicans*.

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