JOURNAL OF BACTERIOLOGY, Oct. 1976, p. 56-68

Vol. 128, No. 1
Printed in U.S.A.

Thermal Adaptation in Yeast: Growth Temperatures, Membrane Lipid, and Cytochrome Composition of Psychrophilic, Mesophilic, and Thermophilic Yeasts

HELEN ARTHUR AND KENNETH WATSON*

Department of Chemistry and Biochemistry, James Cook University of North Queensland, Townsville, 4811, Queensland, Australia

Received for publication 23 June 1976

The temperature limits of growth of a number of yeast species were examined, and on this basis the organisms were classified into different thermal categories. The following species were examined: *Leucosporidium frigidum* and *Leucosporidium nivalis*, psychrophilic, temperature limits of growth, −2 to 20°C; *Candida lipolytica*, mesophilic, temperature limits of growth, 5 to 35°C; *Candida parapsilosis* and *Saccharomyces telluris*, thermotolerant, temperature limits of growth, 8 to 42°C; *Torulopsis bovina* and *Candida slooffii*, thermophilic, temperature limits of growth, 25 to 45°C and 28 to 45°C, respectively. The membrane lipid and cytochrome composition of mitochondrial fractions isolated from these yeasts were compared. There was a direct correlation between the growth temperature and the degree of membrane lipid unsaturation; the lower the temperature, the greater the degree of lipid unsaturation. The membrane lipid composition of the thermophilic yeasts were distinguished by the high percentage (30 to 40%) of saturated fatty acid, as compared with the mesophilic and psychrophilic yeasts. The latter contained approximately 90% unsaturated fatty acid, 55% of which was linolenic acid, $C_{\alpha-18:3}$. Changes in phospholipid composition in relation to temperature were also noted. The respiratory-deficient thermophile, *C. slooffii*, was characterized by the absence of cardiolipin (sensitivity 0.1 μg of phosphorus) and cytochrome aa_3. The absence of conventional mitochondrial structures in this thermophilic microorganism is tentatively suggested, although low concentrations of cytochromes *b*, *c*, and c_1 were detected by low-temperature spectroscopy. On the other hand, the respiratory-competent thermophile, *T. bovina*, was characterized by a high cardiolipin (25% of the total phospholipid) and cytochrome aa_3 content (1 nmol/mg of mitochondrial protein). Low-temperature spectra showed the presence of one *b*-type cytochrome in the thermophilic yeasts, two *b*-type cytochromes in the mesophilic yeasts, and three *b*-type cytochromes in the psychrophilic yeasts. It was concluded that a knowledge of the properties of the biological membrane is fundamental to an understanding of the ability of a microorganism to grow and reproduce in different temperature environments.

The ability of microorganisms to adapt to different temperature environments has attracted considerable attention, but the mechanism(s) underlying this phenomenon is not well understood. The majority of studies have been conducted on procaryotes, particularly the thermophilic (32) and psychrophilic (26) bacteria. A substantial amount of data pertaining to temperature effects on eucaryotic microorganisms (14) and thermophilic fungi (9, 34) has also accumulated.

There have been numerous references in the literature to psychrophilic (33) and thermophilic (35) yeasts, but many of these studies have been primarily concerned with growth requirements (35) of ecological and taxonomic significance (15). A few workers have reported on the heat lability of protein synthesis (27) and substrate transport (1) in psychrophilic yeasts, as well as increased levels of unsaturated fatty acids in yeasts grown at low temperatures (21). The yeast cell with its wealth of membrane organization, together with its rapid growth, reproduction, and relative genetic simplicity, is an attractive system in which to study the mechanism of thermal adaptation in eucaryotic microorganisms.

As a working hypothesis, it is proposed that the properties of the biological membrane are fundamental to the ability of microorganisms to grow and reproduce in different temperature environments. In this respect the mitochon-

drial membrane and associated enzymes serve as an ideal prototype for such studies. Thus, a major advantage of yeasts is that they permit studies to be made on the effect of environmental conditions on mitochondrial membrane structure and composition.

In the present communication we have examined in some detail the temperature limits of growth of various yeast species, on the basis of which they have been placed into different thermal categories. The membrane lipid and cytochrome composition of mitochondrial fractions isolated from these yeasts are also reported.

MATERIALS AND METHODS

Yeast cultures. Seven strains of yeast were used in these studies and their characteristics and sources are listed below. *Leucosporidium frigidum* (CBS 5270) was collected by di Menna (12) from soil at Scott Base, Antarctica, and was originally described as *Candida frigida* but was reclassified on the basis of its heterobasidiomycetous life cycle (15). *C. frigida* is considered to be the imperfect form of *L. frigidum*. *L. nivalis*, strain 3AH17, was received from di Menna. As with *L. frigidum*, this strain, originally described as *C. nivalis*, has been reclassified (15). *Candida lipolytica*, strain NCYC 153, was used. *C. parapsilosis* was a laboratory-isolated strain and was received from P. R. Stewart, Australian National University, Canberra. *C. slooffii* CBS 4068 was originally isolated from the cecum of swine (39). Bulder (4) has shown that *C. slooffii* is a respiratory-deficient organism. *Saccharomyces telluris* (CBS 2622) was originally isolated by van der Walt (36) from soil. *Torulopsis bovina* (CBS 2760), when originally isolated from bovine cecal contents (38), was described as *Candida bovina*, which is considered to be the imperfect form of *S. telluris* (23). *C. bovina* was transferred to the genus *Torulopsis* on the basis of its relation to *T. pintolopesii* and the findings of van Uden and do Carmo-Sousa (38) on pseudomycelium formation.

Maintenance of yeast cultures. Stock cultures were maintained on agar slopes containing 20 g of glucose, 10 g of yeast extract, 5 g of peptone, and 15 g of agar per liter of distilled water (pH 6.1).

With the psychrophilic yeasts, *L. frigidum* and *L. nivalis*, cultures were maintained at 4°C. The mesophilic yeast, *C. lipolytica*, was incubated for 2 to 3 days at 25°C before transferral to 4°C. The thermotolerant strains, *C. parapsilosis* and *S. telluris*, were incubated for 2 to 3 days at 34°C and then transferred to 4°C. In contrast, it was necessary to maintain the thermophilic strains, *C. slooffii* and *T. bovina*, at 34°C as storage for a few days at 4°C resulted in cell death.

Monthly subcultures were carried out with all strains. The thermophiles, especially *C. slooffii*, exhibited short-lived growth on vitamin-free media, and periodic subcultures from vitamin-supplemented broth cultures were necessary to maintain growth.

Growth conditions. The effect of temperature on yeast growth was studied using basal media containing 1% yeast extract, 0.5% peptone and mineral salts (42), with either 0.5% ethanol (wt/vol) or 2% glucose (wt/vol) as the carbon source. With the respiratory-deficient thermophile *C. slooffii*, it was necessary to supplement the basal glucose medium with vitamins (35).

For growth studies, cells were grown in 100 ml of medium in 250-ml conical flasks and shaken at 180 rpm in Paton orbital shakers (Paton Industries Pty. Ltd., St. Peeters, South Australia) under controlled temperature conditions. Cells for the preparation of mitochondria were grown in 600 ml of medium in 2-liter baffled conical flasks to which a few drops of tributyl citrate had been added as an antifoaming agent. In both cases a 4% inoculum (wt/vol) from a starter culture grown to late exponential phase was used.

For both respiration and cytochrome studies, the psychrophilic yeasts were grown at −0.5°C, the mesophilic yeast was grown at 28°C, and the thermotolerant and thermophilic yeasts were grown at 37°C. *C. slooffii* was grown on 2% glucose (wt/vol) and the other strains were grown on 0.5% ethanol (wt/vol) as the carbon source.

Preparation of mitochondria. Cells were harvested in the late exponential-early stationary phase of growth, washed, and suspended in a solution containing 0.5 M mannitol–2 mM ethylenediaminetetraacetic acid–20 mM tris(hydroxymethyl)-aminomethane-hydrochloride buffer (pH 7.4) (MET buffer) and 0.1% bovine serum albumin.

Mitochondria were isolated by mechanical disruption of the cells with glass beads (0.45 to 0.50 mm in diameter) for 20 s in a Braun shaker at 4,000 rpm, followed by differential centrifugation. After three successive centrifugations at 3,000 × *g* to remove unbroken cells, cell debris, and nuclei, a mitochondrial pellet was obtained by centrifuging at 15,000 × *g* for 20 min using a Sorvall RC2-B refrigerated centrifuge. The crude mitochondrial pellet was washed and suspended in 2 mM ethylenediaminetetraacetic acid, 20 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.4), and 0.1% bovine serum albumin to a concentration of 20 to 40 mg of protein per ml.

Dry weight and protein estimations. The dry weight of intact cells was estimated by transferring a suitable portion of yeast suspension to calibrated, graduated tubes (millimeter graduations) and centrifuging at 3,000 × *g* for 5 min. The graduated tubes were calibrated by comparison of dry weights determined by direct weighing of cells after drying at 110°C for 24 h.

Protein was estimated using the Hartree modification of the Lowry method (17). Absorbances were read at 650 nm using a model 101 Hitachi UV-VIS spectrophotometer.

Lipid analysis. (i) Total lipid extraction. Lipids were extracted from mitochondrial fractions by a modification of the Folch procedure (16). Chloroform-methanol, 2:1 (vol/vol), containing 0.01% butylated hydroxytoluene to prevent auto-oxidation, was added to give a 20-fold dilution of the suspension.

The upper lipid-containing phase was removed by

aspiration and the lower phase was reextracted with chloroform-methanol, 2:1 (vol/vol), containing 10 mM HCl and 0.01% butylated hydroxytoluene. The HCl was added to preclude possible loss of phosphatidyl inositides in the initial extraction procedure. The upper phase was again removed by aspiration, and the lipid extracts were combined.

A volume of 0.88% KCl (wt/vol) equivalent to one-quarter of the total volume of extract was added, and, after shaking gently, the mixture was allowed to separate overnight at 8°C. The lower phase was then removed, dried over anhydrous sodium sulfate, and evaporated to a small volume in vacuo. If not used immediately, the extract was stored at −20°C in a nitrogen atmosphere.

A sample of the extract was used for thin-layer chromatography of phospholipids and the remainder for the preparation of the fatty acid methyl esters.

(ii) Thin-layer chromatography. All chromatographic solvents used were analytical reagent grade and were redistilled in glass before use. The developing solvent system in each case contained 0.005% butylated hydroxytoluene.

Phospholipid composition of the lipid extracts was determined quantitatively using two-dimensional thin-layer chromatography (30). Samples of 50 to 100 μl of lipid extract were applied to thin-layer plates coated with a 0.25-mm layer of MN-Silica Gel N-HR (Macherey, Nagel & Co., Düren, West Germany). The plates were developed in the first dimension with chloroform-methanol-28% aqueous ammonia (65:25:5). Ammonia was removed by exposing the plates to 110°C for 5 min. After cooling in a desiccator, the plates were developed in the second direction with chloroform-acetone-methanol-acetic acid-water (6:8:2:2:1). Phospholipids were detected by exposure to iodine vapor. For the detection of phospholipids containing free amino groups, the plates were sprayed with 2% ninhydrin (wt/vol) in acetone.

Before determination of phosphorous, iodine was removed by heating the plates at 110°C overnight. The plates were then sprayed with concentrated sulfuric acid, and the spots were developed by heating at 180°C for 30 min. Phospholipid concentrations were determined by analysis of the inorganic phosphorous content of components recovered from thin-layer chromatograms using a modified method of Rouser et al. (30).

(iii) Fatty acid analysis. Methyl esters of the fatty acids of the lipid extracts were prepared by acid-catalyzed esterification using methanolic-hydrochloride (0.5 M), and the esters were extracted into 5 ml of ultraviolet-grade *n*-hexane. The samples were dried over anhydrous sodium sulfate for 30 min and then evaporated to a small volume (0.5 to 1.0 ml) in vacuo. If not analyzed by gas-liquid chromatography immediately, the samples were stored at −20°C in a nitrogen atmosphere.

Analysis was carried out using a Schimadzu GC-4APF gas chromatograph fitted with a dual-flame ionization detector. Assays were carried out using a Silar 10C column (2 m), operating at 175°C. The injection and detection ports were set at 225°C. The carrier gas (nitrogen) flow rate was 40 ml/min.

Individual fatty acid methyl esters were identified by comparing the retention times with those of standard methyl esters, and the area percentage was estimated by triangulation.

Cytochrome spectra. Cytochrome compositions of both intact cell and mitochondrial suspensions were determined by difference spectroscopy. Cells were suspended in MET buffer to a concentration of 20 to 25 mg (dry weight)/ml and mitochondria were suspended to a concentration of 1 to 10 mg of protein/ml. With both cells and mitochondria, the contents of the sample cell were reduced with a few grains of sodium dithionite. In the case of the mitochondria, the contents of the reference cell were oxidized by aeration, whereas with the intact cells the contents were oxidized with 10 μl of hydrogen peroxide (30%, wt/vol).

Difference spectra at both room and liquid nitrogen (77 K) temperatures were recorded in a 4-mm light-path cell (final volume 0.3 to 0.4 ml) using a Cary 14 spectrophotometer equipped with a Cary 1462 scattering transmission accessory. Liquid nitrogen difference spectra were recorded in the apparatus essentially as described by Chance (7). Room temperature spectra were also determined in 10-mm light-path cells using a Unicam SP800 spectrophotometer.

For quantitative determination, the following wavelength pairs and extinction coefficients were used: cytochrome *c*, 550 − 540 nm, ϵ_{mM} = 19; cytochrome *b*, 560 − 540 nm, ϵ_{mM} = 22 (44); and cytochrome aa_3 605 − 630 nm, ϵ_{mM} = 24 (37).

RESULTS

Effect of temperature on growth. The growth curves shown are the average of two to three experiments and are plotted as log (dry weight) (milligrams per milliliter) against time (hours). In each experiment, triplicate samples were prepared, and the mean was determined for each point.

(i) Psychrophilic yeasts. The growth of *L. frigidum* and *L. nivalis* at −0.5°C and 17°C was essentially the same when either ethanol (Fig. 1) or glucose (Fig. 2) was used as the carbon source, except for a shorter lag phase with glucose as substrate. For both yeasts, the maximum cell yield was obtained at subzero temperatures, although the lag phase was considerably greater than at higher temperatures. Rapid growth of the psychrophiles occurred at 17°C; however, a slight increase in temperature to 18.5°C resulted in decreased growth. This was less marked when glucose was used as the carbon source. The inability of *L. nivalis* to grow at 19°C indicates that it is more heat sensitive than *L. frigidum*.

The lower limit of growth has not been determined due to technical difficulties (minimum −2°C), but strains of these yeasts have been reported to grow at −7°C (24).

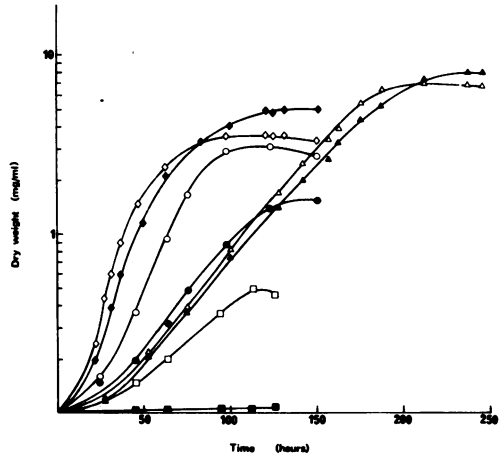

FIG. 1. *Growth curves of L. frigidum and L. nivalis on 0.5% ethanol. Symbols: L. frigidum* – (△) *–0.5°C;* (◇) *17°C;* (○) *18.5°C;* (□) *19°C; L. nivalis* – (▲) *–0.5°C;* (◆) *17°C;* (●) *18.5°C;* (■) *19°C.*

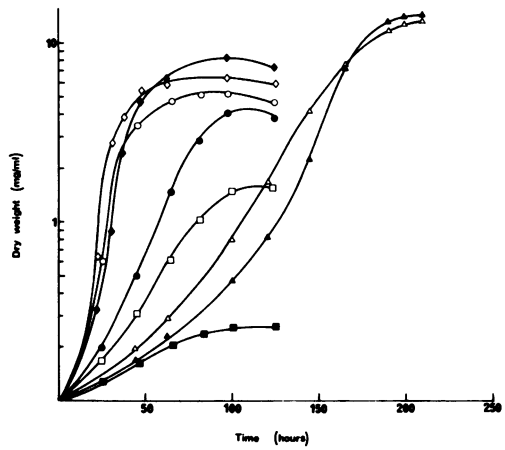

FIG. 2. *Growth curves of L. frigidum and L. nivalis on 2% glucose. Symbols: L. frigidum* – (△) *–0.5°C;* (◇) *17°C;* (○) *18.5°C;* (□) *19°C; L. nivalis* – (▲) *–0.5°C;* (◆) *17°C;* (●) *18.5°C;* (■) *19°C.*

(ii) Mesophilic yeast. With ethanol as the carbon source, the initial growth rate of *C. lipolytica* (Fig. 3) was found to be slightly slower at 17°C than at 28°C; however, the maximum cell yield was approximately the same at both temperatures. At 36°C growth was negligible, and only slight growth was observed at 5°C. Similar results were obtained when glucose was used as the carbon source.

(iii) Thermotolerant yeasts. The rates of growth of *C. parapsilosis* and *S. telluris* were found to be comparable at all temperatures tested (Fig. 4). The greater cell yield obtained at 28°C compared with that at 37°C for both strains serves to illustrate that they are thermotolerant and not true thermophiles. The upper temperature limit for growth of both yeasts was close to 42°C. Furthermore, both species are capable of growth at temperatures as low as 8°C (data not shown), well below the lower limit expected for true thermophilic yeasts.

(iv) Thermophilic yeasts. In contrast to the thermotolerant yeasts. *T. bovina* showed a

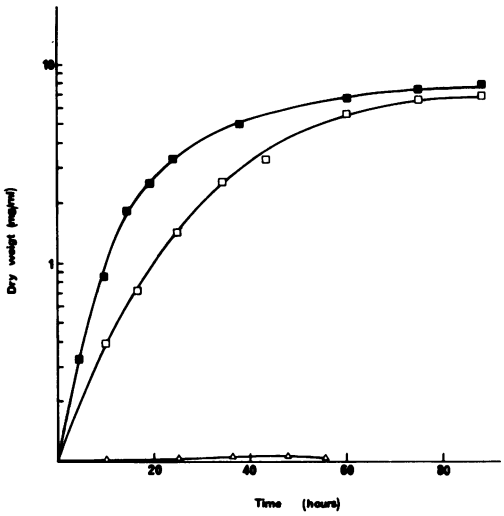

FIG. 3. *Growth curves of C. lipolytica using 0.5% ethanol as carbon source. Symbols:* (□) *17°C;* (■) *28°C;* (△) *36°C.*

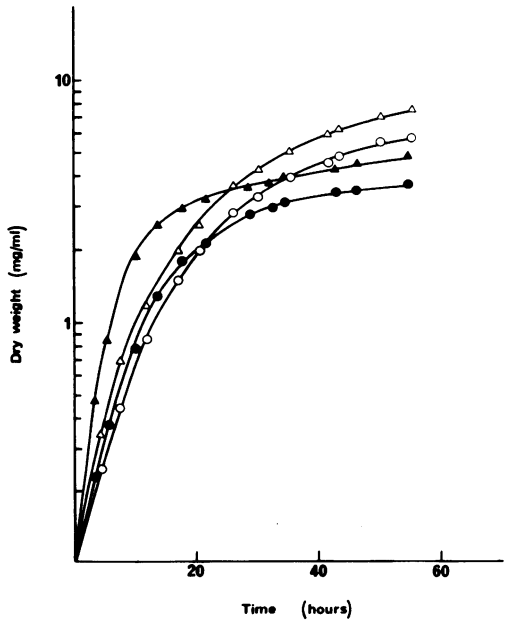

FIG. 4. *Growth curves of C. parapsilosis (28°C,* △; *37°C,* ▲) *and S. telluris (28°C,* ○; *37°C,* ●) *using 0.5% ethanol as carbon source.*

maximum cell yield at 37°C when grown on ethanol (Fig. 5). At 25°C growth was extremely slow, and at 20°C no growth was observed, thus indicating that this strain possesses a more thermophilic character than *C. parapsilosis* and *S. telluris*.

In vitamin-supplemented media, *C. slooffii* also gave a maximum cell yield at 37°C and exhibited only slow growth at 28°C (Fig. 5). No growth was observed at 25°C. In vitamin-free media, limited growth was possible at 37°C (data not shown), but no growth was observed at 28°C.

The upper temperature limit for growth of *T. bovina* and *C. slooffii* was close to 45°C.

These experiments were used to establish the temperature characteristics of growth and in determining the late-exponential phase of growth so that actively dividing cells could be harvested for lipid analysis and cytochrome determination of mitochondrial fractions.

Lipid analysis. (i) Phospholipid analysis. The phospholipid composition of the various strains was determined from the percentage of total phospholipid phosphorus of the individual components resolved by two-dimensional thin-layer chromatography. A summary of the results is presented in Table 1, and representative thin-layer chromatograms for the psychrophile, *L. frigidum* (Fig. 6a), the mesophile, *C. lipolytica* (Fig. 6c), and the thermophile, *C. slooffii* (Fig. 6d), are illustrated in Fig. 6. The individual components were identified either by comparison with authentic standards run under identical conditions or from the literature if standards were unavailable (30). One component, glycolipid (Fig. 6a and d), was tentatively identified as glycolipid on the basis of its initial reaction with concentrated sulfuric acid. Phospholipids containing free amino groups were identified by reaction with ninhydrin (Fig. 6b).

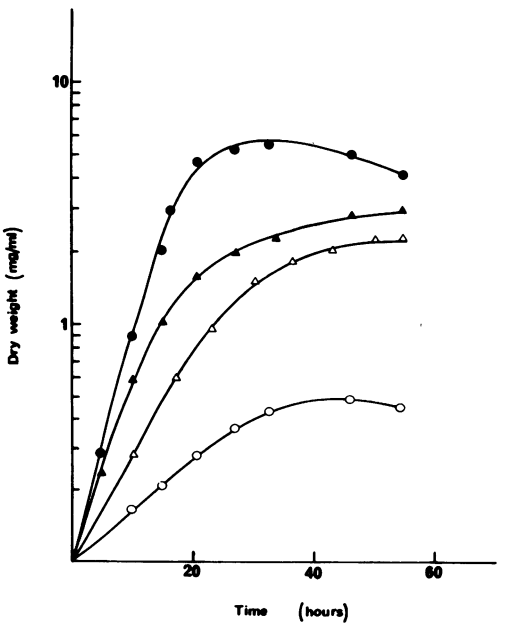

FIG. 5. *Growth curves of T. bovina (28°C, △; 37°C, ▲) on 0.5% ethanol and C. slooffii (28°C, ○; 37°C, ●) on 2% glucose as carbon source.*

Considerable interspecific variations in phospholipid distributions were observed in the strains examined. *L. frigidum* was chosen as representative of the psychrophilic strains and was found to contain a greater amount of phosphatidyl ethanolamine than did any of the other species (Fig. 6a). A concomitant decrease in phosphatidyl choline content was also observed. The thermotolerant strains, *C. parapsilosis* and *S. telluris*, and the mesophilic *C. lipolytica* were found to have comparable phospholipid compositions, apart from a decreased amount of phosphatidyl inositol in the mesophile (Fig. 6c).

Both of the thermophilic strains were found to have unusual phospholipid compositions. Although the phosphatidyl ethanolamine, phosphatidyl choline, and phosphatidic acid content of *T. bovina* and *C. slooffii* were similar, *T. bovina* was found to be rich in cardiolipin, whereas none could be detected in *C. slooffii* (Fig. 6d). On the other hand, *C. slooffii* was rich in phosphatidyl serine and phosphatidyl inositol, the latter a reflection of the inositol supplement in the growth medium. The unknown component, Y, was consistently observed in chromatograms of *C. slooffii* (Fig. 6d) and *T. bovina*. Analyses showed that this component contained no phosphorus but it was not further investigated.

In all strains, the low values obtained for the lyso-forms of the phospholipids indicate that negligible degradation had occurred during isolation and sample analysis.

(ii) Fatty acid composition. Table 2 shows the fatty acid composition of yeast mitochondrial fractions. With *L. frigidum*, the major components were linolenic ($C_{18:2}$) and α-linolenic ($C_{\alpha\text{-}18:3}$) acids, with less amounts of oleic ($C_{18:1}$) acid and tracts of γ-linolenic ($C_{\gamma\text{-}18:3}$) acid. Saturated fatty acids contributed less than 10% to the total fatty acid content.

In contrast, no trace of polyunsaturated fatty acids was observed with the thermophilic yeasts, *C. slooffii* and *T. bovina*, for which the major components were palmitoleic ($C_{16:1}$), oleic

TABLE 1. *Phospholipid composition of mitochondrial fractions*

Yeast	Percentage of total phospholipid phosphorous[a]							
	PS	PI	PA	PC	PE	CL	LPE	Others
L. frigidum	6 ± 1[b]	7 ± 1	2 ± 1	27 ± 2	43 ± 3	8 ± 110	2 ± 1	5 ± 3
C. lipolytica	2 ± 1	9 ± 1	2 ± 1	42 ± 3	32 ± 2	10 ± 1	1 ± 1	2 ± 1
C. parapsilosis	4 ± 1	14 ± 4	–	40 ± 2	26 ± 2	10 ± 1	2 ± 1	4 ± 3
S. telluris	2 ± 1	14 ± 3	2 ± 1	40 ± 3	25 ± 2	10 ± 2	2 ± 1	5 ± 3
T. bovina	2 ± 1	6 ± 1	7 ± 1	30 ± 2	20 ± 3	25 ± 4	–	10 ± 4
C. slooffii	12 ± 2	20 ± 3	8 ± 2	30 ± 1	20 ± 2	–	–	10 ± 5

[a] Abbreviations: PS, Phosphatidyl serine; PI, phosphatidyl inositol; PA, phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; CL, cardiolipin; LPE, lysophosphatidyl ethanolamine.
[b] Standard deviation for three to four values.

($C_{18:1}$), and tetradecanoic ($C_{14:0}$) acids. With these strains, saturated fatty acids contributed from 30 to 40% to the total fatty acid content.

The major component of the mesophilic yeast, *C. lipolytica*, and the thermotolerant strains, *S. telluris* and *C. parapsilosis*, was oleic acid (40 to 60%), with saturated fatty acids contributing from 10 to 30% to the total fatty acids.

It can clearly be seen that there is a direct correlation between the degree of fatty acid unsaturation and the temperature domain of the individual species; the lower the temperature domain, the greater the degree of membrane lipid unsaturation.

Cytochrome spectra. (i) Room temperature spectra. Difference spectra of intact cells (Fig. 7, 8) and mitochondrial fractions (Fig. 9) show essentially similar absorption maxima apart from a variable loss of cytochrome *c*, resulting from isolation procedures for mitochondria.

All strains, apart from *C. slooffii*, showed absorption bands at room temperature characteristic of cytochromes aa_3, *b*, and *c* (Table 3). The cytochrome aa_3 maxima (606 to 607 nm) of the psychrophilic and mesophilic yeasts were centered at wavelengths significantly longer than the corresponding maximum (603 nm) in *S. telluris* and *T. bovina*. Less marked differences in absorption maxima were observed with cytochromes *b* and *c* (Table 3). Intact cells of *C. slooffii* exhibited a maximum at 550 nm with a shoulder at 561 nm, indicative of cytochromes *c* and *b*, respectively. No absorption maximum corresponding to cytochrome aa_3 was noted even at low temperatures, and only a broad band around 585 nm was observed (Fig. 8c). A further notable feature was the broader cytochrome aa_3 peak observed for *C. lipolytica* compared with the other species (Fig. 7c). This may be attributable to a pigment absorbing at 585 to 590 nm, but this was not resolved even at low temperatures (Fig. 7c). For all species, the β-peaks of the cytochromes occurred at 520 to 530 nm.

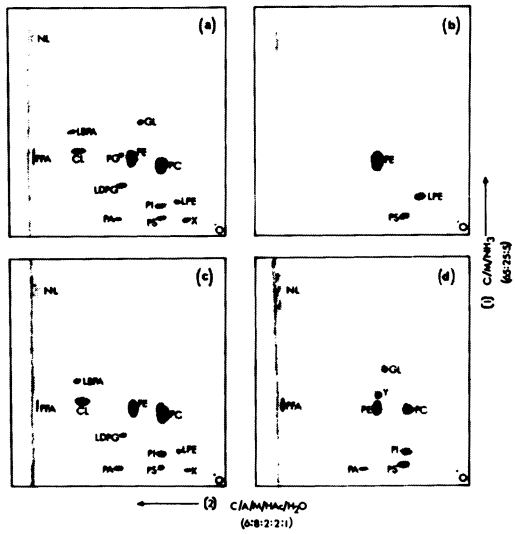

FIG. 6. *Phospholipid composition of psychrophilic, mesophilic, and thermophilic yeasts as resolved by two-dimensional thin-layer chromatography. Abbreviations: PA, Phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; PI, phosphatidyl inositol; PS, phosphatidyl serine; LDPG, lysodiphosphatidyl glycerol; LBPA, lysobisphosphatidic acid; CL, cardiolipin; FFA, free fatty acids; GL, glycolipid; NL, non-polar lipids; X, unidentified; Y, unidentified. The origin is denoted by O. (a) L. frigidum; (b) L. frigidum; detection of phospholipids containing free amino groups using the ninhydrin method (Stahl, 1969); (c) C. lipolytica; (d) C. slooffii.*

(ii) Low-temperature spectra. Typical difference spectra at 77°K for intact cells (Fig. 7, 8) and mitochondrial fractions (Fig. 9) were essentially the same, with the variable loss of cytochrome *c* again evident in the latter.

Low-temperature spectra characteristically resulted in improved resolution of peaks, increased peak height (two- to fivefold compared with room temperature spectra), and a shift of absorption maxima to shorter wavelengths (Table 3). The greatly increased resolution re-

TABLE 2. *Fatty acid composition of mitochondrial fractions*

Yeast	Percentage of total fatty acids										Degree of unsaturation[a]
	Saturated				Unsaturated						
	$<C_{14}$	C_{14}	C_{16}	C_{18}	$C_{14:1}$	$C_{16:1}$	$C_{18:1}$	$C_{18:2}$	$C_{\alpha 18:3}$	$C_{\gamma 18:3}$	
L. frigidum	1 ± 1[b]	2 ± 1	6 ± 1	Tr[c]	Tr	Tr	11 ± 1	27 ± 2	53 ± 2	Tr	2.3
C. lipolytica	2 ± 1	3 ± 1	6 ± 1	1 ± 1	3 ± 1	20 ± 2	44 ± 5	21 ± 4	Tr	–	1.1
C. parapsilosis	2 ± 1	2 ± 1	18 ± 2	6 ± 1	Tr	3 ± 1	53 ± 2	16 ± 1	Tr	–	0.9
S. telluris	3 ± 1	3 ± 1	12 ± 1	4 ± 1	Tr	3 ± 1	58 ± 2	17 ± 1	Tr	–	0.9
T. bovina	3 ± 1	16 ± 1	9 ± 1	3 ± 1	3 ± 1	42 ± 2	24 ± 3	–	–	–	0.7
C. slooffii	4 ± 1	26 ± 2	6 ± 1	4 ± 1	3 ± 1	34 ± 2	23 ± 3	–	–	–	0.6

[a] Degree of unsaturation, (percentage of monoene + 2[percentage of diene] + 3 [percentage of triene])/100.
[b] ± Standard deviation for four to six values.
[c] Tr, Trace, less than 1%.

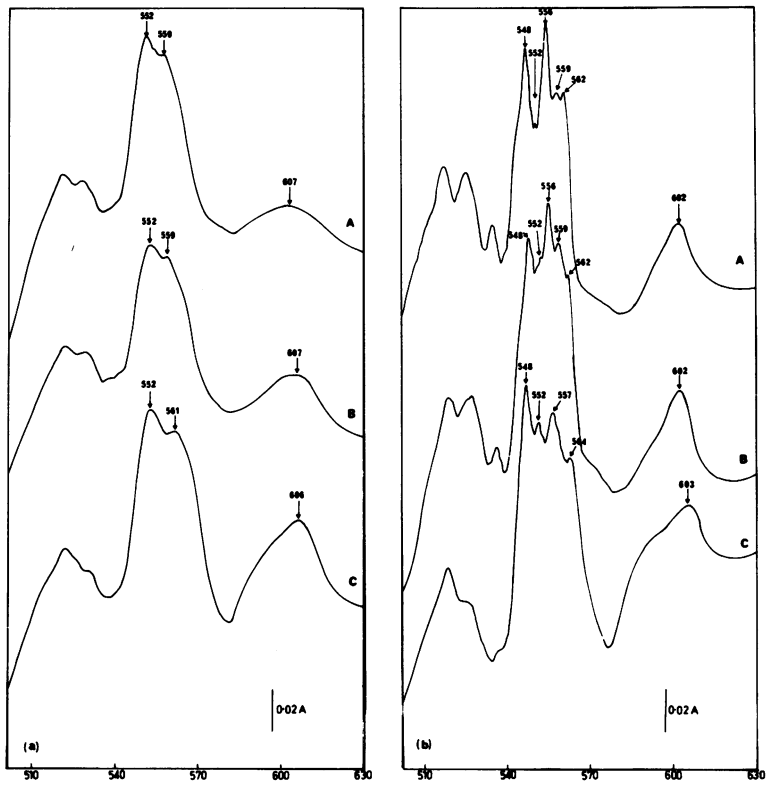

FIG. 7. *Difference spectra of intact yeast cells. Spectra were determined as outlined in text. A, L. frigidum; B, L. nivalis; C, C. lipolytica. (a) Room temperature (milligrams [dry weight] of cells per milliliter): A, 35.0; B, 40.0; C, 19.0. (b) Low temperature (77°K) (milligrams[dry weight] of cells per milliliter): A, 15.5; B, 20.0; C, 6.5.*

vealed that the psychrophilic yeasts possess three *b*-type cytochromes, together with cytochromes *c* and c_1. With the mesophilic strain, only two *b*-cytochromes together with cytochromes *c* and c_1 were detected, whereas the thermotolerant and thermophilic strains showed only one *b*-type cytochrome together with cytochromes *c* and c_1.

Increased resolution of the β-peaks of the cytochromes was also observed at low temperature (Fig. 7 and 8).

With *C. slooffii*, a small, broad peak occurred at 583 nm which may indicate the presence of an absorbing pigment. Due to the low cytochrome content of this organism, it was necessary to concentrate both intact cell and mito-

chondrial suspensions to obtain measurable peaks.

Quantitative estimations of the cytochrome content of the various strains are shown in Table 4. Marked interspecific variations were immediately apparent. A comparison of the values obtained for intact cells (based on dry weight) with those obtained for mitochondrial fractions (based on protein) give an indication of the high lipid content of the psychrophilic cells which resulted in apparent low values being obtained for intact cells. Although variable loss of cytochrome *c* may have occurred during isolation of the mitochondrial fractions, the values based on protein content give a more reliable indication of the extent of interspecific variations.

From the values shown in Table 4, it can be seen that with the respiratory-competent species there is a general trend toward increased cytochrome content with increased growth temperature, although the values for the thermotolerant *S. telluris* deviate slightly. The most marked differences were seen with the thermophilic yeasts. The cytochrome content of the respiratory-competent thermophile, *T. bovina*, was significantly greater than all other strains examined. In contrast, the paucity of cytochromes in the respiratory-deficient thermophile, *C. slooffii*, was the notable feature.

DISCUSSION

Temperature limits of growth. Procaryotic microorganisms have been reported to exist at temperatures close to that of boiling water (2). On the other hand, the upper temperature limit for eucaryotic microorganisms has been reported by Tansey and Brock (34) to be close to 60°C. The lower temperature limit for growth of microorganisms is difficult to determine accurately. The problem is primarily a technical one since the amount of antifreeze substance required to be added to the media to prevent

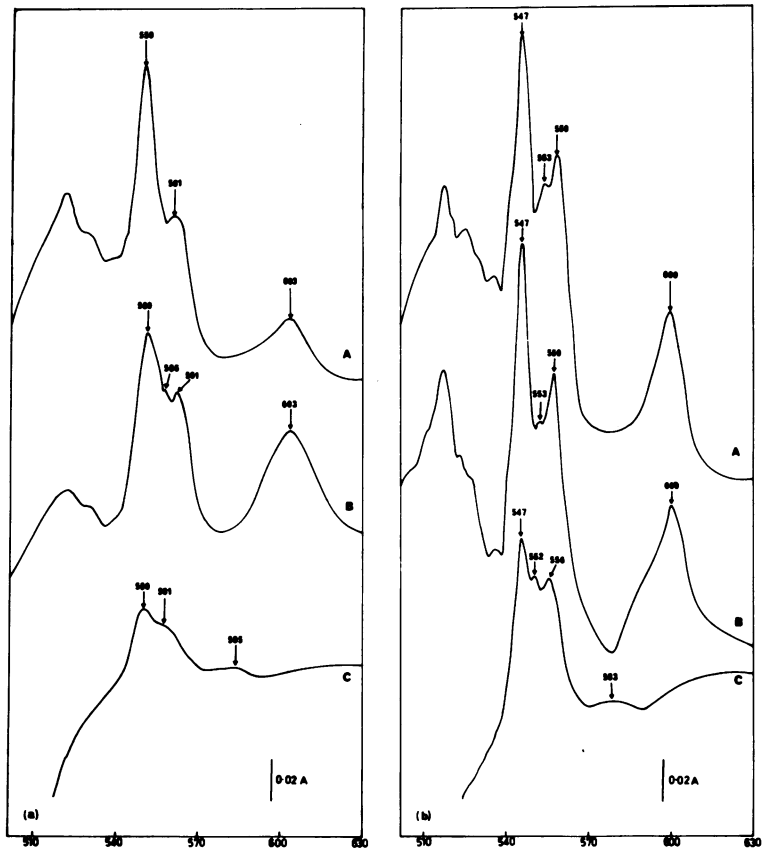

FIG. 8. *Difference spectra of intact yeast cells. Spectra were determined as outlined in text. A, S. telluris; B, T. bovina; C, C. slooffii. (a) Room temperature (milligrams [dry weight] of cells per milliliter): A, 17.8; B, 7.8; C, 85.8. (b) Low temperature (77°K) (milligrams [dry weight] of cells per milliliter): (A, 6.0; B, 2.0; C, 85.8.*

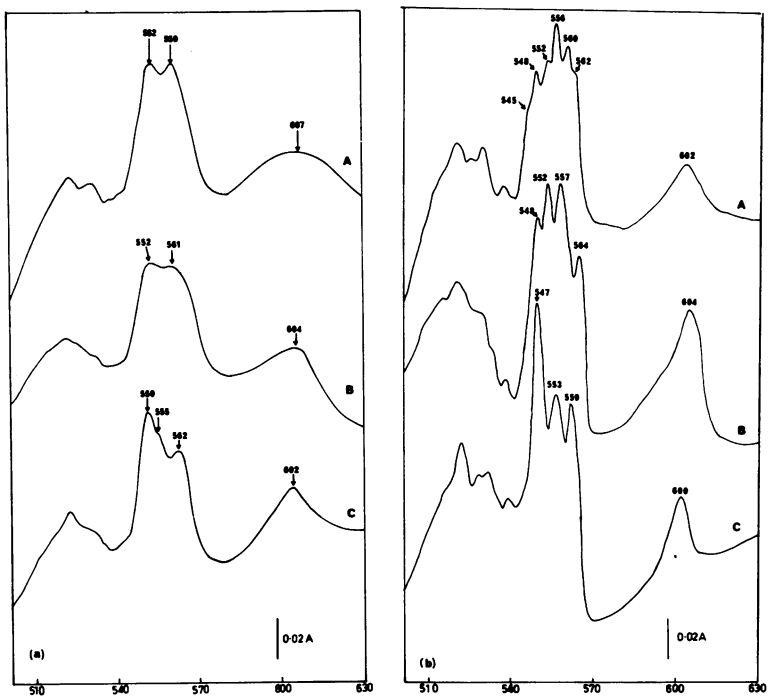

FIG. 9. *Difference spectra of isolated mitochondria. Spectra and isolation of mitochrondia were as outlined in text. A, L. frigidum; B, C. lipolytica; C, T. bovina. (a) Room temperature (milligrams of protein per milliliter): A, 6.0; B, 3.7; C, 2.8. (b) Low temperature (77°K) (milligrams of protein per milliliter): A, 1.9; B, 1.8; C, 0.9.*

TABLE 3. *Absorption maxima of intact cell cytochromes at room temperature and low temperature (77°K)*

Yeast	Wavelength (nm) of α-peaks of cytochrome: at room temp			Wavelength (nm) of α-peaks of cytochrome: at low temp			
	aa_3	*b*	*c*	aa_3	*b*	c_1	*c*
L. frigidum	607	559	552	602	562, 559, 556	552	548
L. nivalis	607	559	552	602	562, 559, 556	552	548
C. lipolytica	606	561	552	603	564, 557	552	548
S. telluris	603	561	550	600	559	553	547
T. bovina	603	561	550	600	559	553	547
C. slooffii	–	561(s)[a]	550	–	556	552	547

[a] s, Shoulder.

freezing is often inhibitory to growth (24). Within this limitation, a low temperature limit of between −5 and −7°C for growth of bacteria and yeasts has been reported (24).

It is clear that the definition of the terms psychrophilic and thermophilic as applied to microorganisms is dependent not only on whether the organism is a procaryote or a eucaryote but also on the species under consideration. With the yeasts, the upper temperature limit is close to 46°C (33), whereas the lower temperature limit is about −7°C (24). Applying these temperature limits for growth to the yeast strains examined in this report, it is possible to assign them to different thermal groups.

L. frigidum and *L. nivalis* are classified as obligate psychrophiles since they are unable to grow at temperatures above 20°C (12; this report). *T. bovina* and *C. slooffii* are classified as obligate thermophiles since they exhibit a narrow growth temperature range of between 28 to 45°C. The yeasts *C. parapsilosis* and *S. telluris* are designated as thermotolerant since, although capable of growth above 40°C (but not at 45°C), both are also capable of good growth

below 10°C. *C. lipolytica* was classified as a typical mesophilic yeast and showed no growth above 35°C and little growth below 5°C.

The placing of these various yeasts into different thermal groups was a prerequisite for studies on thermal adaptation in yeasts.

Membrane lipid composition. (i) Fatty acids. Marked variations in both phospholipid composition and the degree of fatty acid unsaturation of cold- and warm-adapted yeasts indicate that the ability to alter these cellular components may constitute an adaptation mechanism to suit a particular environment. The extent of such ability may be an important controlling factor which determines the growth temperature limits of the microorganisms, the lower limit being that at which the membrane lipids solidify and the upper limit being that at which the membrane lipids melt.

The membrane lipid composition of the different yeasts (Table 3) conforms well with this concept. There is a direct correlation between temperature adaptation and the degree of membrane lipid unsaturation; the lower the temperature, the greater the degree of fatty acid unsaturation.

The high $C_{18:2}$ (melting point, −5°C) and $C_{18:3}$ (melting point, −11°C) content of the psychrophilic yeasts would permit the cell membranes to remain in a sufficiently fluid state to allow unimpaired functioning of metabolic processes even at subzero temperatures. On the basis of melting points of the major fatty acids present, it could be proposed that the psychrophilic yeasts would be able to grow at temperatures as low at −10°C; however, both the saturated and the short-chain unsaturated fatty acids would tend to raise this temperature limit. In a similar fashion, temperature limits for the other strains could be estimated on the basis of the fatty acid content, but here again the heterogeneity of the membrane lipids precludes accurate estimations. For example, the melting points of mixtures of each of the C_{18} unsaturated fatty acids ($C_{18:1}$, $C_{18:2}$, $C_{18:3}$) with $C_{16:0}$ (melting point, 63.1°C) remain approximately constant only for concentrations of $C_{16:0}$ less than about 16%, with marked increases in melting point occurring as the concentration is increased above this critical limit (22).

An increase in the degree of fatty acid unsaturation with decreased temperature has frequently been noted in microbial (20, 41) and other systems (10, 5). The present studies are, therefore, not new in this respect. However, they do represent the first study of yeast strains adapted to defined temperature limits. Furthermore, the membrane lipid composition of the psychrophilic, thermotolerant (*S. telluris*) and thermophilic yeasts have not previously been reported, as far as is known to the authors.

Other parameters, apart from temperature, which are known to influence lipid composition in eucaryotic microorganisms, include the pH and composition of the growth medium, growth phase, and concentration of dissolved oxygen (3, 41). This last factor may be particularly important in regulating the degree of lipid unsaturation at various temperatures since (i) the solubility of oxygen decreases with temperature, and (ii) oxygen is obligatory in reactions involving the fatty acid desaturase enzymes of yeasts (13). It is noteworthy that Pugh and Kates (29) have recently reported that the rate of desaturation of oleoyl-coenzyme A by the acyl-coenzyme A desaturase of *C. lipolytica* was greater in cold-grown cells (10°C) than in warm-grown cells (25°C).

(ii) Phospholipids. Phospholipids are almost exclusively concentrated in the cellular membranes and phospholipid composition is, therefore, an excellent parameter for membrane composition and function. For example, it has been suggested that cardiolipin may be used as a marker component for, and is a good indicator of, the state of development of mitochondrial membranes (19). If the hypothesis that membrane function and temperature adaptation are closely related is correct, then changes in phos-

TABLE 4. *Cytochrome content of intact cells and mitochondrial fractions*

Yeast	Cytochrome content of intact cells (nmol/mg [dry wt])			Cytochrome content of mitochondrial fractions (nmol/mg of protein)		
	aa$_3$	*b*	*c*	*aa*$_3$	*b*	*c*
L. frigidum	0.08 ± 0.01[a]	0.18 ± 0.02	0.25 ± 0.02	0.70 ± 0.05	1.06 ± 0.01	1.30 ± 0.01
L. nivalis	0.05 ± 0.01	0.18 ± 0.03	0.26 ± 0.01	0.42 ± 0.02	0.84 ± 0.05	1.02 ± 0.07
C. lipolytica	0.29 ± 0.03	0.64 ± 0.08	0.89 ± 0.08	0.74 ± 0.05	1.49 ± 0.08	1.69 ± 0.04
S. telluris	0.23 ± 0.01	0.36 ± 0.01	0.58 ± 0.02	0.39 ± 0.05	1.12 ± 0.05	1.54 ± 0.08
T. bovina	0.35 ± 0.02	0.55 ± 0.02	1.19 ± 0.05	1.07 ± 0.05	1.85 ± 0.04	3.27 ± 0.06
C. slooffii	–	0.03 ± 0.01	0.06 ± 0.01	–	0.05 ± 0.01	0.08 ± 0.01

[a] ± Standard deviation for four to six determinations.

pholipid composition with temperature may give some insight in this direction.

The phospholipid composition of mitochondrial membranes of *L. frigidum* is unique in the exceptionally high concentration of phosphatidyl ethanolamine relative to phosphatidyl choline and other lipid components (Table 1). All other species of yeast so far examined have higher concentrations of phosphatidyl choline relative to phosphatidyl ethanolamine (18).

It is difficult to conceive a role for phosphatidyl ethanolamine which would constitute an advantage to yeasts growing at low temperatures. We have suggested that psychrophilic yeasts may synthesize cell wall glycoproteins which may act as freezing-point depressants at subzero temperatures (Watson and Arthur, manuscript in preparation) in an analogous manner to the glycoproteins found in the sera of Antarctic fish (11). The formation of hydrogen bonds between the amino groups of phosphatidyl ethanolamine and the polar water molecules may similarly serve to retard ice formation at subzero temperatures.

The phospholipid composition of the mesophilic yeast, *C. lipolytica*, was similar (apart from a lower phosphatidyl inositol content) to that of the thermotolerant species (Table 1). This was not entirely unexpected since there is considerable overlap in the growth temperature range of these organisms. On the other hand, the thermophile, *T. bovina*, was characterized by a high content of cardiolipin, thus reflecting a well-developed mitochondrial membrane system. Electron microscopy of intact cells (Watson and Arthur, unpublished data) and determination of cytochrome content (Table 4) confirm the presence of mitochondria with abundant cristae. The thermophilic properties of *T. bovina*, may, therefore, be related to its ability to carry out rapid synthesis and turnover of metabolites at high temperatures, a mechanism which has been proposed to account for the ability of microorganisms to adapt to high temperatures (32).

The absence of cardiolipin (sensitivity 0.1 μg of P) in mitochondrial fractions of *C. slooffii*, coupled with electron microscopy evidence (Watson and Arthur, unpublished data), suggest the absence of conventional mitochondrial structures in this respiratory-deficient organism.

Cytochrome composition. On a protein basis, there was a reasonable correlation between cytochrome concentration and the respiratory activity of the isolated mitochondria. *T. bovina* mitochondria, with the highest cytochrome concentration (Table 4), have the highest respiratory rate (Arthur, unpublished data). Conversely, *C. slooffii* shows the lowest cytochrome content with a complete absence of cytochromes aa_3, in keeping with its respiratory deficiency.

Low-temperature difference spectra revealed the presence of one *b*-type cytochrome in the thermophilic and thermotolerant yeasts, two *b*-type cytochromes in *C. lipolytica*, and three *b*-type cytochromes in the psychrophilic yeasts. The presence of one *b*-type cytochrome corresponds to the situation in *Saccharomyces* yeasts (6, 25), whereas the two *b*-type cytochromes in *C. lipolytica* correspond well to that reported for this yeast by Vollard and Chaix (40). The presence of three *b*-type cytochromes in the psychrophilic yeasts is unusual. It is interesting to note that Sato et al. (31) have recently reported, in room temperature spectral studies, the presence of three *b*-type cytochromes in *C. utilis* mitochondria. The significance of *b*-type cytochromes in yeasts adapted to different temperature remains to be resolved. It is not possible at this stage to develop any relationship between *b*-type cytochromes and temperature adaptation since the function of these cytochromes in mitochondria is uncertain (43).

The presence of adsorption bands corresponding to cytochrome c_1 (552 nm) and cytochrome *b* (556 nm) at liquid nitrogen temperatures is reported for the respiratory-deficient thermophile, *C. slooffii*. The unequivocal presence of cytochrome c_1 in respiratory-deficient cytoplasmic petite mutants of *S. cerevisiae* has only been recently demonstrated (8). These authors have also observed the presence of a small shoulder at 556 to 558 nm (presumably a *b*-type cytochrome) in aerobically grown petite mutants of *S. cerevisiae*. It appears, therefore, that the concept that cytoplasmic petite mutants of yeast have only cytochrome *c* may no longer be tenable. It remains to be demonstrated, however, that *C. slooffii* is a cytoplasmic mutant. The nature of the wild type corresponding to this respiratory-deficient yeast, if it exists at all, has not been established.

The natural habitat of *C. slooffii* is the extremely acid environment of the stomach of domestic animals, in particular the pig and the horse, and it is not difficult to conceive that it had adapted to this extreme environment in such a way as to preclude the necessity for conventional mitochondrial metabolism. Nevertheless, the presence of cytochrome *c* and absorption bands characteristic of cytochromes *b* (556 nm) and c_1 (552 nm) at low temperatures (Fig. 9), albeit in small amounts, does suggest the presence of some type of mitochondrial structure. Further work is required to establish

the subcellular localization of these cytochromes.

ACKNOWLEDGMENTS

This work was supported by a grant from the Australian Research Grants Committee. H.A. acknowledges the award of an Australian Government Postgraduate Scholarship.

LITERATURE CITED

1. **Baxter, R. M., and N. E. Gibbons.** 1962. Observations on physiology of psychrophilism in a yeast. Can. J. Microbiol. 8:511-517.
2. **Bott, T. L., and T. D. Brock.** 1969. Bacterial growth rates above 90°C in Yellowstone hot springs. Science 164:1411-1412.
3. **Brown, C. M., and A. H. Rose.** 1969. Influence of temperature and dissolved oxygen tension on fatty acid composition of *Candida utilis*. J. Bacteriol. 99:371-379.
4. **Bulder, C. J. E. A.** 1964. Induction of petite mutation and inhibition of synthesis of respiratory enzymes in various yeasts. Antonie van Leeuwenhoek J. Microbiol. Serol. 30:1-9.
5. **Caldwell, R. S., and F. J. Vernberg.** 1970. The influence of acclimation temperature on the lipid composition of fish gill mitochondria. Comp. Biochem. Physiol. 34:179-191.
6. **Cartledge, T. G., and D. Lloyd.** 1972. Subcellular fractionation by zonal centrifugation of glucose-repressed and anaerobically grown *S. carlsbergenesis*. Biochem. J. 127:693-703.
7. **Chance, B.** 1957. Techniques for the assay of the respiratory enzymes, p. 273-329. *In* S.P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 4. Academic Press Inc., New York.
8. **Claisse, M. L., and F. Pajot.** 1974. Presence of cytochrome c_1 in cytoplasmic "petite" mutants of *Saccharomyces cerevisiae*. Eur. J. Biochem. 49:49-59.
9. **Cooney, D. G., and R. Emerson (ed.).** 1964. Thermophilic fungi. W. H. Freeman & Co., San Francisco.
10. **Deuel, H. J. (ed.).** 1955. The lipids, vol. 2. Interscience Publishing Inc., New York.
11. **DeVries, A. L., S. K. Komatsu, and R. E. Feeney.** 1970. Chemical and physical properties of freezing-point-depressing glycoproteins from Antarctic fish. J. Biol. Chem. 245:2901-2908.
12. **di Menna, M. E.** 1966. Three new yeasts from Antarctic soils: *Candida nivalis, C. gelida, C. frigida*. Antonie van Leeuwenhoek J. Microbiol. Serol. 32:25-28.
13. **Erwin, J. A.** 1973. Comparative biochemistry of fatty acids in eucaryotic microorganisms, p. 41-143. *In* J. A. Erwin (ed.), Lipids and biomembranes of eucaryotic microorganisms. Academic Press Inc., New York.
14. **Farrell, J., and A. H. Rose.** 1967. Temperature effects on microorganisms, p. 147-218. *In* A. H. Rose (ed.), Thermobiology. Academic Press Inc., New York.
15. **Fell, J. W., A. C. Statzell, L. Hunter, and H. J. Phaff.** 1969. *Leucosporidium* gen. n.; the heterobasidiomycetous stage of several yeasts of the genus *Candida*. Antonie van Leeuweenhoek J. Microbiol. Serol. 35:433-462.
16. **Folch, J., M. Lees, and G. H. Sloane Stanley.** 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.
17. **Hartree, E. F.** 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. 48:422-427.
18. **Hunter, K., and A. H. Rose.** 1971. Yeast lipids and membranes, p. 211-270. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, vol. 2. Academic Press Inc., New York.
19. **Jakovcic, S., G. S. Getz, M. Rabinowitz, H. Jakob, and H. Swift.** 1971. Cardiolipin content of wild type and mutant yeasts in relation to mitochondrial function and development. J. Cell. Biol. 48:490-502.
20. **Kates, M.** 1964. Bacterial lipids. Adv. Lipid Res. 2:17-90.
21. **Kates, M., and R. M. Baxter.** 1962. Lipid composition of mesophilic and psychrophilic yeasts (*Candida* sp.) as influenced by environmental temperature. Can. J. Biochem. Physiol. 40:1213-1227.
22. **Keith, A. D., B. J. Wisnieski, S. Henry, and J. C. Williams.** 1973. Membranes of yeast and *Neurospora*: lipid mutants and physical studies, p. 259-321. *In* J. A. Erwin (ed.), Lipids and biomembranes of eucaryotic microorganisms. Academic Press Inc., New York.
23. **Kreger-van Rij, N. J. W.** 1958. The relationship between *Saccharomyces telluris* and *Candida bovina*. Antonie van Leeuwenhoek J. Microbiol. Serol. 24:137-144.
24. **Larkin, J. M., and J. L. Stokes.** 1968. Growth of psychrophilic microorganisms at subzero temperatures. Can. J. Microbiol. 14:97-101.
25. **Lindenmeyer, A.** 1965. Carbohydrate metabolism. 3. Terminal oxidation and electron transport, p. 301-347. *In* G. C. Ainsworth and A. S. Sussman (ed.), The fungi, vol. 1. Academic Press Inc., New York.
26. **Morita, R. Y.** 1975. Psychrophilic bacteria. Bacteriol. Rev. 39:144-167.
27. **Nash, C. H., D. W. Grant, and N. A. Sinclair.** 1969. Thermolability of protein synthesis in a cell-free system from the obligately psychrophilic yeast *Candida gelida*. Can. J. Microbiol. 15:339-343.
28. **Nash, C. H., and N. A. Sinclair.** 1968. Thermal injury and death in an obligately psychrophilic yeast, *Candida nivalis*. Can. J. Microbiol. 14:691-697.
29. **Pugh, E. L., and M. Kates.** 1975. Characterization of a membrane-bound phospholipid desaturase system of *Candida lipolytica*. Biochim. Biophys. Acta 380:442-453.
30. **Rouser, G., S. Fleischer, and A. Yamamoto.** 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5:494-496.
31. **Sato, N., T. Ohnishi, and B. Chance.** 1972. Spectral study of b-cytochromes in yeast mitochondria and intact cells. Biochim. Biophys. Acta 275:288-297.
32. **Singleton, R., and R. E. Amelunxen.** 1973. Proteins from thermophilic microorganisms. Bacteriol. Rev. 37:320-42.
33. **Stokes, J. L.** 1970. Influence of temperature on growth and metabolism of yeasts, p. 119-134. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, vol. 2. Academic Press Inc., New York.
34. **Tansey, M. R., and T. D. Brock.** 1972. The upper temperature limit for eucaryotic organisms. Proc. Natl. Acad. Sci. U.S.A. 69:2426-2428.
35. **Travassos, L. R. R. G., and A. Cury.** 1971. Thermophilic enteric yeasts. Annu. Rev. Microbiol. 25:49-74.
36. **van der Walt, J. P.** 1957. Three new sporogenous yeasts from soil. Antonie van Leeuwenhoek J. Microbiol. Serol. 23:23-29.
37. **van Gelder, B. F.** 1966. The extinction coefficient of cytochromes a and a_3. Biochim. Biophys. Acta 118:36-46.
38. **van Uden, N., and L. do Carmo-Sousa.** 1957. Yeasts from the bovine caecum. J. Gen. Microbiol. 16:385-395.
39. **van Uden, N., L. do Carmo-Sousa, and M. Farinha.** 1958. On the intestinal yeast flora of horses, sheep, goats and swine. J. Gen. Microbiol. 19:435-445.
40. **Vollard, C., and P. Chaix.** 1970. Étude de la chaine respiratoire de mitochondries de levures *Candida lipolytica* cultivées sur hexadécane. Bull. Soc. Chim. Biol. 52:581-584.

41. **Weete, J. D. (ed.).** 1974. Fungal lipid biochemistry, monographs in lipid research, vol. 1. Plenum Press, New York.
42. **Wickerham, L. J.** 1946. A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeasts. J. Bacteriol. **52:293-301.**
43. **Wikstrom, M. K. F.** 1973. The different cytochrome b components in the respiratory chain of animal mitochondria and their role in electron transport and energy conservation. Biochim. Biophys. Acta **301:155-193.**
44. **Wilson, D., and D. Epel.** 1968. The cytochrome system of sea urchin sperm. Arch. Biochem. Biophys. **126:83-90.**