The inability of psychrophilic microorganisms to grow at moderate temperatures (>20 °C) presently represents an unresolved thermodynamic paradox. Here we report for the psychrophilic yeast *Rhodotorula aurantiaca* A19, isolated from Antarctic ice, that the inability to grow at temperatures close to 20 °C is associated with profound alterations in cell morphology and integrity. High performance liquid chromatography analysis of the intracellular acyl-CoA esters revealed an abnormal accumulation of myristoyl-CoA (C14-CoA) in cells cultivated close to the nonpermissive temperature. Its concentration (500 μM) was found to be 28-fold higher than in cells cultivated at 0 °C. If one considers its ability to disrupt membrane bilayers and to inhibit many cellular enzymes and functions, intracellular myristoyl-CoA accumulation in the psychrophile *R. aurantiaca* represents one of the principal causes of growth arrest at moderate temperatures. Intracellular acyl-CoA concentrations are believed to be regulated by thioesterase activity. Thus in an attempt to explore the mechanism by which temperature disrupts myristoyl-CoA metabolism, we isolated and characterized a long chain acyl-CoA thioesterase. The monomeric 80-kDa thioesterase from the psychrophilic yeast shows a very strong specificity for myristoyl-CoA. The affinity for substrate and the catalytic efficiency of the thioesterase are optimal below 5 °C (temperatures habitually experienced by the strain) and dramatically decrease with increasing temperature. The loss of affinity for substrate is related to the intracellular increase of myristoyl-CoA concentration. Our observations reveal one of the probable mechanisms by which temperature fixes the limit of growth for this psychrophilic yeast.

Earlier studies, reviewed by Inniss (10), report that the biochemical basis for the maximum growth temperature of psychrophiles is likely to be complex, involving a number of interacting phenomena. Temperature changes cause alterations in the structural and molecular components of cells, for example membrane integrity and permeability, functional stability of ribosomes, and enzyme activity and stability.

Recently, a large body of evidence has been accumulated indicating that long chain acyl-CoA esters have an important function in the regulation of a large number of cellular systems and functions, including ion channels, ion pumps, translocators, enzymes, membrane fusion, and gene regulation (for review see Ref. 11). Because of the amphipathic nature of acyl-CoA esters, excessive increases in their concentration can cause important cellular damage, including membrane disruption and a nonspecific inhibition of a variety of enzymes (11–14). A number of recognized metabolic diseases including Reye’s syndrome and sudden infant death syndrome can be attributed to specific enzyme deficiencies in acyl-CoA catabolic pathways, which result in the accumulation of toxic acyl-CoA thioesters (15).

The intracellular concentration of free acyl-CoA esters is tightly controlled by feedback inhibition of the acyl-CoA synthetase and is buffered and transported by the specific acyl-CoA-binding proteins (11, 16). Under normal physiological conditions the total acyl-CoA ester content in cells is in the range 5–160 μM, with the free cytosolic concentration in the low nanomolar range being unlikely to exceed 200 nM (11). Abnormal increases in the concentration are expected to be prevented by conversion into acylcarnitines or by hydrolysis by acyl-CoA hydrolases (17).

At present, for psychrophilic microorganisms, no data are available on the effects of growth temperature on the cellular metabolism of acyl-CoA esters.

In this paper we report that moderate temperatures, above 20 °C, cause an abnormal accumulation of myristoyl-CoA (tetradecanoyl-CoA) in *Rhodotorula aurantiaca*. The excessive concentration at moderate temperatures is probably one of the principal causes of cell death.

Acyl-CoA thioesterases are enzymes that cleave thioester bonds of fatty acyl-CoA and liberate free fatty acids and CoASH. Thioesterase activity is widely distributed in both prokaryotes and eukaryotes (18). In eukaryotes, acyl-CoA thioesterase activity is detected in various subcellular organelles (19, 20) including lysosomes (21), peroxisomes, and mitochondria (22) as well as in the cytosol (23). Although all the physiological functions of acyl-CoA thioesterases have not yet been clearly understood, previous data suggest (11, 17, 22) that they are involved in lipid metabolism and modulation of cellular con-

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**Influence of Moderate Temperatures on Myristoyl-CoA Metabolism and Acyl-CoA Thioesterase Activity in the Psychrophilic Antarctic Yeast *Rhodotorula aurantiaca***

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concentrations of acyl-CoA derivatives.

To assess further the mechanism by which temperature disrupts the regulation of myristoyl-CoA metabolism, we isolated and characterized a R. aurantiaca thioesterase with a particular focus on its thermodynamic properties.

MATERIALS AND METHODS

Microorganism and Culture Conditions—R. aurantiaca A19 was isolated at the Laboratory of Biochemistry, University of Liege, Belgium, from ice near the French Antarctic Station at Dumont d’Urville (66° 40’S, 140° 01’E) and identified by the Mycothèque of the University of Louvain-la-Neuve, Belgium; the registration number is 40267.

The growth medium, YPD, contains 2% dextrose, 2% casein peptone, and 1% yeast extract. For thioesterase purification R. aurantiaca A19 was produced in a 400-liter bioreactor (Biolaflite, France). Culturing was carried out aerobically (0.5 volume of air per volume of culture per minute) at 12 °C, in the YPD medium. After 100 h of growth 3.5 kg of cells were recovered by centrifugation (Sharpleis centripetal, Alphalav, Sweden).

Extraction and HPLC Analysis of Acyl-CoA Esters—Culturing was carried out at 0 or 18 °C until cell concentration in cultures reached 107 cells/ml. Cells were harvested by centrifugation, and acyl-CoA esters were extracted using a method adapted from a previously published extraction protocol (24). Cells were washed three times with 100 ml of 50 mM potassium phosphate, pH 7.2, containing 10 mM MgCl2 and 4% (v/v) glacial acetic acid. 3 ml of the same buffer and 40 ml of chloroform: methanol (1:2) were then added to 3 g of the cells, and the mixture was homogenized using a blender (Ultra-Turrax T25, Van der Heyden) for 3 min at 8000 rpm. Extraction was completed by adding 12.7 ml of distilled water and 12.7 ml of chloroform. After centrifugation the aqueous phase containing acyl-CoA esters and proteins was washed distilled water and 12.7 ml of chloroform. After centrifugation the supernatant was removed by centrifugation (30 min, 10,000 g).

Cell disruption and protein extraction—400 g of cells were suspended in 1 liter of 100 mM phosphate buffer, pH 7.0, containing 3 mM dithiothreitol and 10 mM EDTA and disrupted by pressure at 1500 bar with a Niro homogenizer (Panda). The cell extract was obtained by centrifugation at 40,000 × g for 60 min. Nucleic acids were removed by addition of potassium sulfate (0.1% w/v) and centrifugation for 10 min at 10,000 × g.

Thioesterase Precipitation—Ammonium sulfate salt was added to the cell extract under constant stirring until 30% saturation was obtained. The precipitate was removed by centrifugation (30 min, 10,000 × g). The resulting supernatant was saturated to 80% by addition of (NH4)2SO4. The resulting precipitate was solubilized in 200 ml of 50 mM phosphate buffer, pH 7.

Hydrophobic Interaction Chromatography with Phenyl-Sepharose Column—Samples were applied to a phenyl-Sepharose Fast Flow XK26/10 column (Amersham Pharmacia Biotech) equilibrated with 50 mM phosphate buffer, pH 7.0, containing 0.1 mM NaCl. A flow rate of 3 ml/min was used. The unadsorbed material was washed successively with 1 liter of the equilibration buffer and with 600 ml of distilled water.

Desorption of the bound proteins was performed with 200 ml of 20 mM Tris-HCl buffer at pH 8.5.

Ion-exchange Chromatography with Mono Q Column—Active fractions from the phenyl-Sepharose column were pooled, dialyzed overnight with distilled water, and lyophilized. The dried sample was solubilized in 20 ml Tris-HCl buffer, pH 7.7, and applied to a Mono Q HR5/5 column (Amersham Pharmacia Biotech) previously equilibrated with the same buffer. Proteins were eluted with a gradient of NaCl in 20 ml Tris-HCl buffer, pH 7.7, as follows: 0 mM NaCl for 20 min, 0–0.15 mM NaCl for 20 min, 0.15–1 mM NaCl for 5 min, and 1 mM NaCl for 10 min. The flow rate was 0.8 ml/min, and fractions of 2 ml were collected.

Hydrophobic Interaction Chromatography with Phenyl-Superose Column—Active fractions eluted from the Mono Q column were pooled, concentrated by ultrafiltration on an Amicon YM 10 membrane, and mixed with 4% NaH2SO4 to give a final (NaH2SO4) concentration of 1 M. The sample was then applied to a phenyl-Superose column HR5/5 previously equilibrated with 50 mM phosphate buffer, pH 7.0, containing 1 M (NH4)2SO4. Proteins were eluted with a gradient of (NH4)2SO4 in 50 mM phosphate buffer, pH 7.0, as follows: 1 M (NH4)2SO4 for 20 min, 1 to 0.6 M (NH4)2SO4 for 20 min, 0.6 to 0 M (NH4)2SO4 for 30 min, 0.6–0 M (NH4)2SO4 for 5 min, and 0 M (NH4)2SO4 for 10 min. The flow rate was 0.5 ml/min, and fractions of 2 ml were collected.

Protein Assay—Protein concentrations were determined by the Bradford (25) method using Coomassie Blue G.

Electrophoresis—Analysis of column fractions during purification was performed by gel electrophoresis using the LKB Multitof II electrophoresis system (Amersham Pharmacia Biotech). Polyacrylamide gels (Excigel SDS 8–18, Amersham Pharmacia Biotech) were used for SDS-PAGE following the standard procedure of Laemmli (27). Polyacrylamide gels (ExcelGel SDS 8–18, Amersham Pharmacia Biotech) were used for SDS-PAGE following the standard procedure of Laemmli (27).

RESULTS

Influence of Culture Temperature on Growth and Acyl-CoA Ester Metabolism—The Antarctic yeast R. aurantiaca A19 is unable to grow above 20 °C and is therefore referred to as a psychrophilic strain (28). Cultures are highly temperature-sensitive, and cell density drastically decreases when temper-
nature increases (29). Moreover, temperatures higher than those normally experienced by the strain (2 to 4 °C) have pronounced effects on physiological processes and cell morphology. Examination by phase contrast microscopy shows major cell morphological changes when the cultures are grown near 20 °C (Fig. 1). Budding is inhibited, and cells are larger than at low temperatures. Membrane integrity could also be affected since cellular content release was observed (arrows in Fig. 1B).

A similar behavior is reported for several temperature-sensitive mutants of *Saccharomyces cerevisiae*. The mutants are affected in long chain acyl-CoA metabolism and showed a temperature-dependent auxotrophy for long chain saturated fatty acids (30–35). A thermal shift from 24 to above 30 °C causes growth arrest associated with an increase in cell size and membrane lysis (33). With *R. aurantiaca* A19, exogenous fatty acids (C12, C14, C16, and C18) do not improve cell growth at nonpermissive temperatures (Fig. 2).

To determine whether acyl-CoA metabolism is affected by growth temperature, intracellular acyl-CoA esters were extracted and analyzed by HPLC (Fig. 3). The extract of cells grown at 18 °C showed an important peak at a retention time of 39.7 min. The peak was identified as myristoyl-CoA (C14-CoA) by comparison with an authentic standard. Fast atom bombardment-mass spectrometry confirmed the identification.

The mass spectrum and suggested fragmentation pattern are shown in Fig. 4. The peak at *m/z* 978 corresponds to the protonated molecular ion (MH+). Cleavages producing ions at *m/z* 471, 508, 428, and 330 are significant. The ion at *m/z* 471 is of particular significance because it preserves the identity of the acyl group (25). Cleavage at the adenine-ribose bond produces a positive ion at *m/z* 136.

The intracellular concentration of myristoyl-CoA in cells cultivated at 18 °C is 500 μM and is 28-fold higher than in cells grown at 0 °C. Retention times for octanoyl-CoA, decanoyl-CoA, palmitoyl-CoA, and stearoyl-CoA are 16.2, 25.1, 45.2, and 52.1 min, respectively, and no significant difference is observed in their intracellular concentrations at 0 and 18 °C.

Several mechanisms have been suggested for the regulation of cellular acyl-CoA ester concentration. Berge and Aarsland (17) proposed that acyl-CoA pools are regulated by acyl-CoA thioesterase, thus we purified and characterized the *R. aurantiaca* A19 thioesterase.
Thioesterase—Despite the negative effect of the increasing temperature on cell growth, thioesterase production is not directly influenced by temperature, and it decreases proportionally with cell density (data not shown). At all experimental temperatures production was 1 unit/10^9 cells.

Following cultivation of *R. aurantiaca* A19 at 12 °C, the intracellular long chain acyl-CoA thioesterase was released by mechanic cell disruption and purified by ammonium sulfate precipitation and three successive fast protein liquid chromatography steps. Recoveries calculated after each purification step are given in Table I. A final yield of 32% was obtained, and the purified enzyme had a specific activity of 108 units/mg of protein. Specific activity is 351 times higher after purification than in the crude extract.

SDS-PAGE analysis shows that the enzyme is pure at homogeneity, with an apparent molecular mass of about 80 kDa (Fig. 5). Gel filtration on Sephacryl S-200 shows an apparent molecular mass of 85 ± 6 kDa indicating that the purified thioesterase is monomeric. The pI of the enzyme is 4.4, and the enzyme is stable and active over a broad pH range (5–10) with an optimal activity at pH 8 (data not shown). Enzyme activity is completely inhibited by diisopropyl fluorophosphates, indicating that the thioesterase has a serine residue in its active site. The thioesterase is a highly glycosylated protein, and the glycosidic portion, removed by endoglycosidase H, represents about 19% of its molecular weight.

TABLE I
Purification of thioesterase from the psychrophilic yeast *R. aurantiaca* A19
Activity was determined with myristoyl-CoA as the substrate.

|                  | Total protein | Total activity | Specific activity | Yield | Purification |
|------------------|---------------|----------------|-------------------|-------|--------------|
|                  | mg            | units          | units/mg          | %     | -fold        |
| Crude extract    | 11,080        | 3,400          | 0.3               | 100   | 1            |
| Ammonium sulfate | 2,680         | 1,900          | 0.7               | 56    | 2.3          |
| Phenyl-Sepharose | 169           | 1,837          | 10.8              | 54    | 35.4         |
| Mono Q           | 21.6          | 1,811          | 83.8              | 53    | 273          |
| Phenyl-Superose  | 10            | 1,080          | 108               | 32    | 351          |

*Fig. 4. Full mass spectrum and suggested fragmentation pattern for myristoyl-CoA eluted at 39.7 min on reverse-phase chromatography (Fig. 3) and analyzed by positive ion fast atom bombardment.*

*Fig. 5. SDS-PAGE analysis.* Fractions pooled after each purification step of acyl-CoA thioesterase are analyzed by SDS-PAGE and silver-stained for protein revelation. Lane 1, pooled fractions after ammonium sulfate precipitation; lane 2, pooled fractions after phenyl-Sepharose chromatography; lane 3, pooled fractions after anion exchange (Mono Q) chromatography; lane 4, pooled fractions after phenyl-Superose chromatography; lane 5, molecular mass standards in kDa (LMW calibration kit, Amersham Pharmacia Biotech).

The intact enzyme has an N terminus blocked to protein sequencing. However, analysis of two internal peptide sequences obtained by endoproteinase Lys-C digestion (LRERALMRY and QAAYDTTAEFA) exhibited, respectively, 77 and 72% identity with two amino acid sequences located between
residues 179–187 and 252–262 of the *S. cerevisiae* Mvp1 protein (36). This protein interacts, by unknown mechanism, with Vps1p; a protein essential for vacuolar protein sorting and cell growth at high temperature (36, 37).

Substrate specificity was determined using several acyl-CoA ester derivatives. Kinetic parameters for hydrolysis are given in Table II. Thioesterase is active on thioesters with carbon chain lengths ranging from 8 to 18. No activity is detected with C2-CoA or C4-CoA. The preferred substrates are C14-CoA, C16-CoA, and C18-CoA, and their *Kₐₐₚ* values range from 18 to 24 μM. The best substrate is myristoyl-CoA which is hydrolyzed with the higher catalytic efficiency (*kₐₐₚ/Kₐₚ*) of about 10 s⁻¹μM⁻¹. The *Kₐₚ* and *kₐₐₚ/Kₐₚ* values calculated for the hydrolysis of the unsaturated palmiteoyl-CoA (C16:1) are 2-fold higher and 3.5-fold lower, respectively, than those calculated for the corresponding saturated thioester (C16:0).

**Thermodependence of Thioesterase Activity**—Thermal stability of the thioesterase activity was examined by heating the enzyme solution to 40, 50, and 60 °C for different times (Fig. 6A). The remaining activities after 60 min of incubation are 82, 52, and 3%, respectively. The temperature profile of thioesterase activity was determined at temperatures ranging from 5 to 60 °C (Fig. 6B) and shows an optimal activity at 45 °C.

Thermodependence of the kinetic parameters *Kₐₚ* and catalytic efficiency (*kₐₐₚ/Kₐₚ*) for myristoyl-CoA hydrolysis shows that the best physiological efficiency of the thioesterase is reached near 0 °C (Fig. 7). The *Kₐₚ* value is 0.4 μM at 5 °C and increases exponentially with temperature; at 20 °C it is 59-fold higher. Catalytic efficiency is also largely affected by a temperature increase; its value is 28-fold higher at 5 than at 20 °C (Fig. 7).

**DISCUSSION**

The inability to grow above 20 °C is common in psychrophilic microorganisms (10, 28, 38) and seems to be thermodynamically a paradox, especially if one considers the favorable effect of moderate temperatures on enzyme reactions and biological processes. Temperatures higher than 5 °C have a negative effect on the growth of the antarctic yeast *R. aurantiaca* A19, and cell lysis occurs when cultures are grown close to the upper temperature limit for growth.

Because of their amphipathic and toxic property, an excess of acyl-CoA esters is likely to disrupt membrane bilayers and to impair several cellular enzymes and functions (11, 16). HPLC analysis of the intracellular acyl-CoA esters from *R. aurantiaca* A19 reveals an abnormal accumulation of myristoyl-CoA (C14-CoA) in cells cultivated close to the nonpermissive temperature (20 °C). Its concentration is 500 μM (28-fold higher than at 0 °C), whereas it has been reported that the total cellular acyl-CoA ester content is unlikely to exceed 160 μM, even under the most extreme conditions (11).

Myristoyl-CoA is an essential compound for cell growth because it contributes to the activation, by N-myristoylation, of several proteins regulating cell growth and signal transduction (31, 33).

**Table II**

|          | *Kₐₚ* (μM) | *kₐₐₚ* (s⁻¹) | *kₐₐₚ/Kₐₚ* (μM⁻¹s⁻¹) |
|----------|-------------|---------------|-----------------------|
| Octanoyl-CoA (C8:0) | 118 ± 15 | 159 ± 25 | 1 |
| Decanoyl-CoA (C10:0) | 67 ± 12 | 136 ± 23 | 2 |
| Myristoyl-CoA (C14:0) | 18 ± 2 | 183 ± 16 | 10 |
| Palmitoyl-CoA (C16:0) | 23 ± 4 | 153 ± 12 | 7 |
| Palmitoleoyl-CoA (C16:1) | 58 ± 14 | 106 ± 12 | 2 |
| Stearoyl-CoA (C18:0) | 24 ± 4 | 121 ± 14 | 5 |

In *S. cerevisiae* there are at least two metabolic pathways that produce myristoyl-CoA, de novo synthesis or activation of free myristate by acyl-CoA synthetases (30–33). The de novo pathway uses malonyl-CoA produced by acetyl-CoA carboxylase (39) to generate long chain saturated acyl-CoAs through the cytosolic fatty-acid synthetase complex (40). Palmitoyl-CoA and stearoyl-CoA are the main products of fatty-acyc synthetase, whereas myristoyl-CoA represents only 3–5% of the total acyl-CoAs synthesized (41, 42).

In *R. aurantiaca* A19 myristoyl-CoA accumulation at non-

**FIG. 6.** Thermal stability and optimal temperature of long chain acyl-CoA thioesterase. *A*, thermal inactivation of the acyl-CoA thioesterase from *R. aurantiaca* A19 at 40 °C (●), 50 °C (○), and 60 °C (■). Residual activity was determined using myristoyl-CoA as the substrate. *B*, effect of temperature on the activity of acyl-CoA thioesterase from *R. aurantiaca* A19. Activity was determined at different temperatures as described under "Material and Methods." Myristoyl-CoA was used as the substrate.

**FIG. 7.** Thermodependence of *Kₐₚ* and catalytic efficiency. The kinetic parameters (*Kₐₚ* (○) and *kₐₐₚ/Kₐₚ* (●)) were determined at the temperature range from 5 to 35 °C. The substrate was myristoyl-CoA at the concentrations of 10, 25, 50, 75, 100, and 150 μM in 20 mM Tris-HCl buffer, pH 7.2. Initial velocity values were fitted to the Hanes transformation of the Michaelis-Menten equation.
permissive temperatures could not be due to an increase in the fatty-acid synthetase activity or acyl-CoA synthetase as it represents a minor product of these enzymes in comparison with palmitoyl-CoA and stearoyl-CoA (41–43).

Intracellular concentrations of acyl-CoA esters are regulated by their rate of synthesis, utilization, and degradation, and excessive increases in their concentration are prevented by hydrolysis by thioesterases (11, 16, 17, 22). At present, myristoyl-CoA metabolism is not completely understood; however, the increase in concentration at 18 °C indicates a deficiency in its utilization and/or hydrolysis at high temperatures.

Because of their putative contribution to the control of myristoyl-CoA levels, an 80-kDa long chain acyl-CoA thioesterase could not be related to a thermal inhibition of the genetic expression of the purified thioesterase because its production is about 1 unit/10⁹ cells and varies proportionally with cell density at various culture temperatures.

On the other hand, great structural flexibility is a rather common characteristic of the psychrophilic enzymes (1–5). Hence, it is tempting to speculate that the lower thermal stability of mon characteristic of the psychrophilic enzymes (1–5). Hence, it is tempting to speculate that the lower thermal stability of myristoyl-CoA at nonpermissive temperatures. However, the thermostability of myristoyl-CoA could not be related to a thermal inhibition of the genetic expression of the purified thioesterase because its production is about 1 unit/10⁹ cells and varies proportionally with cell density at various culture temperatures.

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Effect of Moderate Temperatures on R. aurantiaca Metabolism

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