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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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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.

The key of psychrophily in cold-adapted microorganisms, or psychrophiles, is their ability to produce cold-adapted enzymes (1–5) and to maintain high membrane fluidity at low temperatures (6–9). However, the incapacity to grow at moderate temperatures (>20 °C) remains a paradox and represents a barrier to their use in ecological and biotechnological processes. 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 the psychrophilic yeast *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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centrations 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 Mycotheque 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 (Biolaffite, 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 (Sharples centrifuge, Alpha-Laval, Sweden).

Extraction and HPLC[1] Analysis of Acyl-CoA Esters—Culturing was carried out at 0 or 18 °C until cell concentration in cultures reached 10^7 cells/ml. Cells were harvested by centrifugation, and acyl-CoA esters were extracted using a method adapted from a previously published extraction process (24). Cells were washed three times with 100 ml of 50 mM potassium phosphate, pH 7.2, containing 10 mM $MgCl_2$ 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 chloroform phase containing lipids was carefully removed, and the aqueous phase containing acyl-CoA esters and proteins was washed three times with 20 ml of chloroform. Proteins were precipitated by addition of 25 ml of acetonitrile. The mixture was allowed to stand for 20 min at room temperature before centrifugation. The supernatant was lyophilized before chromatography analysis.

The extracted acyl-CoA esters were analyzed by HPLC on a LiChrospher 100 RP-18 column (Merck). The mobile phases used were solvent A (20% acetonitrile in 25 mM KH_2PO_4, pH 5.3) and solvent B (80% acetonitrile in 25 mM KH_2PO_4, pH 5.3). The acyl-CoA esters were eluted with the following gradient of solvent B in solvent A: 0% B for 10 min, 0–60% B for 55 min, 60% B for 10 min, 60–100% B for 10 min, 100% B for 10 min, and 100 to 0% B for 5 min. The flow rate was 1 ml/min. Acyl-CoA esters were detected by UV absorption at 254 nm. Peaks were identified using standards (Sigma). For quantification of acyl-CoA esters the recorded chromatograms were integrated, and the amounts of individual acyl-CoA esters were determined by comparing peak areas to those obtained by injection of known quantities of commercial myristoyl-CoA. Data acquisition and integration were performed using the Softron PC Integration Software.

The peak eluted at the expected myristoyl-CoA elution time (39.7 min) was analyzed and identified by fast atom bombardment-mass spectrometry using a method previously described by Norwood *et al.* (25).

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 protamine 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 $(NH_4)_2SO_4$. 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 XK26/40 column (Amersham Pharmacia Biotech) equilibrated with 50 mM phosphate buffer, pH 7.0, containing 0.1 M 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 mM 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 mM Tris-HCl buffer, pH 7.7, as follows: 0 M NaCl for 20 min, 0–0.15 M NaCl for 20 min, 0.15 M NaCl for 30 min, 0.15–1 M NaCl for 5 min, and 1 M 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 a Amicon YM 10 membrane, and mixed with a 4 M $(NH_4)_2SO_4$ solution to give a final $(NH_4)_2SO_4$ 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, containing 1 M $(NH_4)_2SO_4$. Proteins were eluted with a gradient of $(NH_4)_2SO_4$ in 50 mM phosphate buffer, pH 7, as follows: 1 M $(NH_4)_2SO_4$ for 20 min, 1 to 0.6 M $(NH_4)_2SO_4$ for 20 min, 0.6 M $(NH_4)_2SO_4$ for 30 min, 0.6–0 M $(NH_4)_2SO_4$ for 5 min, and 0 M $(NH_4)_2SO_4$ 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 method (26) using Coomassie Blue G.

Electrophoresis—Analysis of column fractions during purification was performed by gel electrophoresis using the LKB Multifor II electrophoresis system (Amersham Pharmacia Biotech). Polyacrylamide gels (ExcelGel SDS 8–18, Amersham Pharmacia Biotech) were used for SDS-PAGE following the standard procedure of Laemmli (27). 15–40 μg of protein per sample were deposited on the gel. Proteins were revealed by silver nitrate staining.

Thioesterase Assays—Thioesterase activity was assayed spectrophotometrically at 25 °C by monitoring the increase in absorbance at 212 nm resulting from the generation of free CoASH in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma). Activities were determined with an $\epsilon_{412\,nm}$ of 13.6 mM^{-1} cm^{-1} for the 2-nitrobenzoate anion. A typical reaction mixture contained 100 μM myristoyl-CoA substrate in 20 mM Tris-HCl buffer, pH 7.4. A unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 μmol of myristoyl-CoA per min.

Molecular Mass Determination—The molecular mass of the native protein was determined by gel filtration on a Sephacryl S-200 column (Amersham Pharmacia Biotech). An aliquot of the purified enzyme from the phenyl-Superose chromatography step was applied to the column. Running buffer was 20 mM Tris-HCl, pH 7.7, and 150 mM NaCl. The flow rate was fixed at 0.3 ml/min, and fractions of 3 ml were collected. The molecular mass of the thioesterase was also estimated by SDS-PAGE under the conditions described above.

Substrate Specificity—Kinetic parameters for hydrolysis of acyl-CoAs were determined at concentrations of 10, 25, 50, 75, 100, and 150 μM in 20 mM Tris-HCl buffer, pH 7.2, as described for the thioesterase assay.

Thioesterase Thermodependence—The thermodependence of the kinetic parameters was determined in the temperature range from 5 to 35 °C. The substrate used was myristoyl-CoA at concentrations of 10, 25, 50, 75, 100, and 150 μM in 20 mM Tris-HCl buffer, pH 7.2. The study was carried out using a computer controlled LKB Ultrospec III spectrophotometer equipped with a thermostated cell changer base plate (Amersham Pharmacia Biotech). To calculate K_m and V_{max} values experimental data points were computer-fitted (Enzyme Kinetic Application Software 2.01, Amersham Pharmacia Biotech) to the Hanes transformation of the Michaelis-Menten equation.

The optimum temperature for activity was determined over the range 5–60 °C with the thermostated LKB Ultrospec III spectrophotometer (Amersham Pharmacia Biotech).

For thermostability, aliquots of purified thioesterase (28 μg per ml of 20 mM Tris buffer, pH 7.0) were incubated at 40, 50, and 60 °C, and samples were periodically withdrawn and chilled on ice before being assayed at 25 °C by the standard method.

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 as a psychrophilic strain (28). Cultures are highly temperature-sensitive, and cell density drastically decreases when temper-

[1] The abbreviations used are: HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

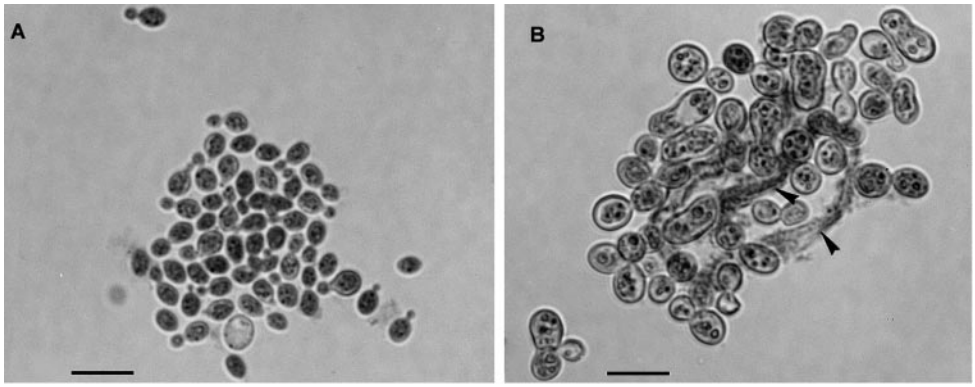

FIG. 1. **Morphological effect of growth temperature in *R. aurantiaca* A19.** Cells were grown at 0 (*A*) and 18 °C (*B*), stained by ethylene blue for 5 min, and visualized by contrast phase microscopy. The *arrows* in *B* indicate released cellular contents stained by ethylene blue. *Bar* represents 10 μm.

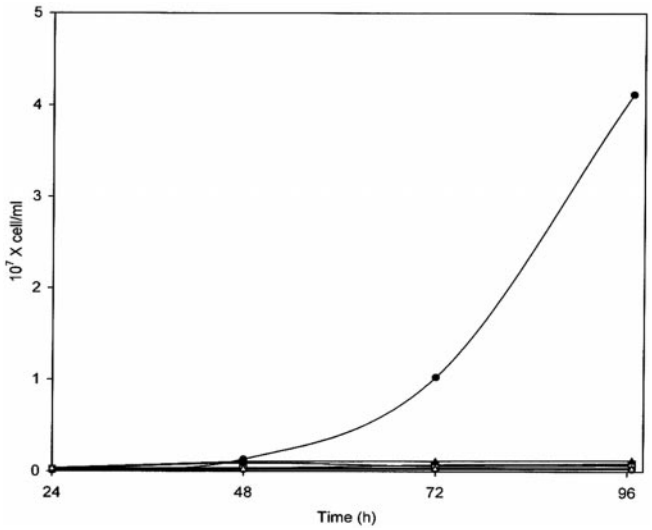

FIG. 2. **Effect of fatty acid addition on cell growth at nonpermissive temperatures.** Cultures are incubated at 18 °C on YPD medium with or without 500 μM of each fatty acid. ●, control cultivated at 4 °C on YPD medium without fatty acid; ○, YPD without fatty acid; ■, YPD+C12; □, YPD+C14; ▲, YPD+C16; △, YPD+C18.

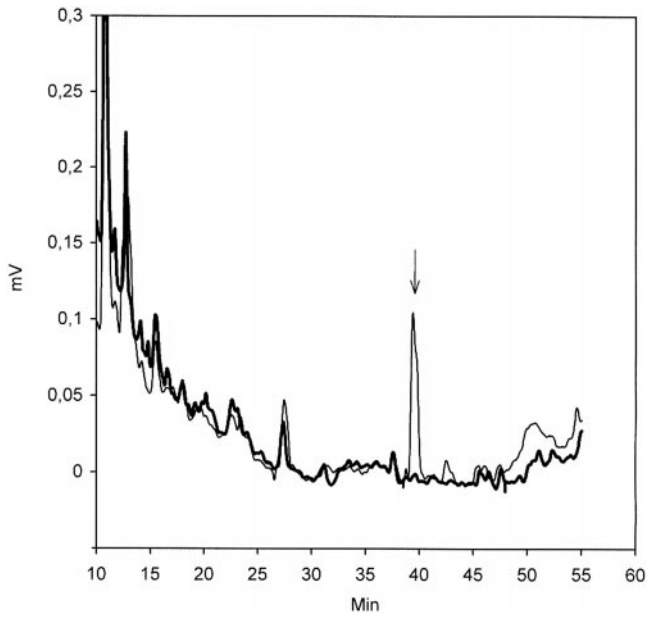

FIG. 3. **Reverse-phase HPLC of *R. aurantiaca* A19 extract.** Cell extracts of cells cultivated at 0 (*heavy line*) and 18 °C (*fine line*) were chromatographed on a LiChrospher 100 RP-18 column. The mobile phases are as follows: buffer A (20% acetonitrile in 25 mM KH_2PO_4, pH 5.3) and buffer B (80% acetonitrile in 25 mM KH_2PO_4, pH 5.3). The acyl-CoA esters were detected by UV absorption at 254 nm. The *arrow* indicates the putative myristoyl-CoA.

ature 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. 1*B*).

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 significance. The ion at m/z 471 is of particular significant 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.

Purification and Characterization of Long Chain Acyl-CoA

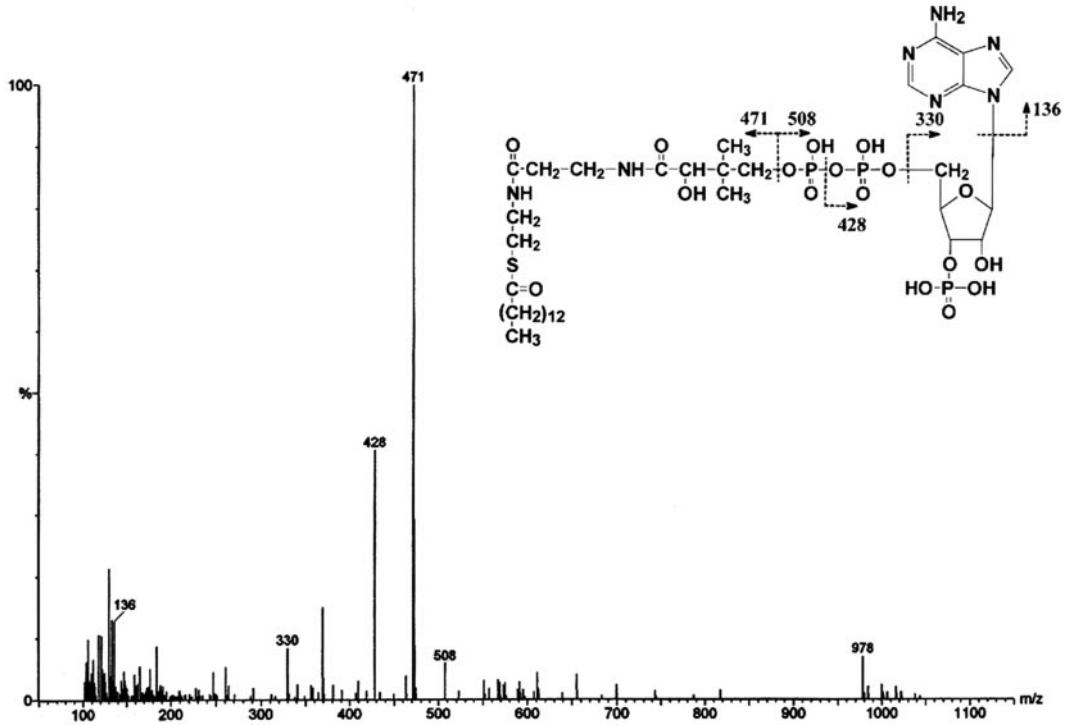

FIG. 4. **Full mass spectrum and suggested fragmentation pattern for myristoyl-CoA eluted at 39.7 min on reverse-phase chromatography (Fig. 3) and analyzed by positive ion fast atom bombardment.**

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

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.

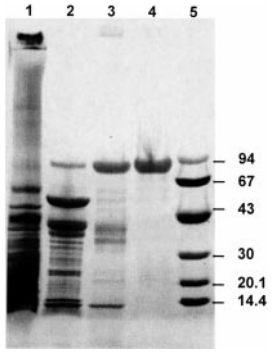

FIG. 5. **SDS-PAGE analysis.** Fractions pooled after each purification step of acyl-CoA thioesterase are analyzed by SDS-PAGE and silver-stained for protein revelation. *Lane 1,* pooled fractions after ammonium sulfate precipitation; *lane 2,* pooled fractions after phenyl-Sepharose chromatography; *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 (LREIALMRY 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 K_m values range from 18 to 24 μM. The best substrate is myristoyl-CoA which is hydrolyzed with the higher catalytic efficiency (k_{cat}/K_m) of about 10 $s^{-1}\cdot\mu M^{-1}$. The K_m and k_{cat}/K_m values calculated for the hydrolysis of the unsaturated palmitoleoyl-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. 6*A*). 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. 6*B*) and shows an optimal activity at 45 °C.

Thermodependence of the kinetic parameters K_m and catalytic efficiency (k_{cat}/K_m) for myristoyl-CoA hydrolysis shows that the best physiological efficiency of the thioesterase is reached near 0 °C (Fig. 7). The K_m 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
Kinetic parameters for thioesterase hydrolysis of different acyl-CoA esters at 25 °C

	K_m	k_{cat}	k_{cat}/K_m
	μM	s^{-1}	s^{-1} μM^{-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-acid 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-

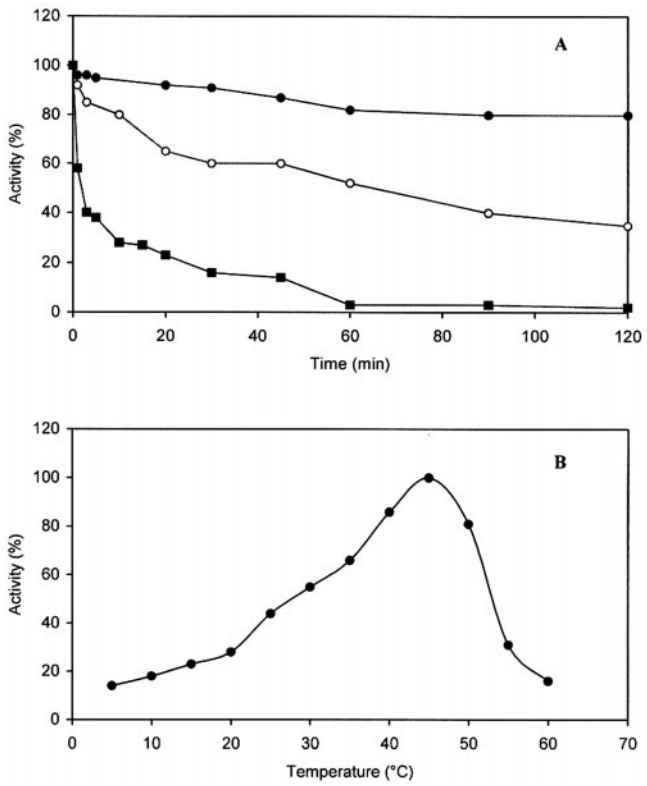

FIG. 6. **Thermal stability and optimal temperature of long chain acyl-CoA thioesterase.** *A,* thermal inactivation of the acyl-CoA thioesterase from *R. aurantiaca* A19 at 40 °C (●), 50 °C (○), and 60 °C (■). Residual activity was determined using myristoyl-CoA as the substrate. *B,* effect of temperature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

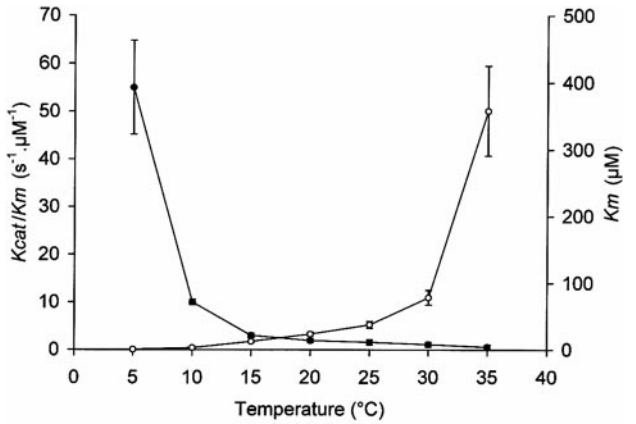

FIG. 7. **Thermodependence of K_m and catalytic efficiency.** The kinetic parameters (K_m (○) and k_{cat}/K_m (●)) were determined at the temperature range from 5 to 35 °C. The substrate was myristoyl-CoA at the concentrations of 10, 25, 50, 75, 100, and 150 μM in 20 mM Tris-HCl buffer, pH 7.2. Initial 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 was isolated and characterized. The intracellular accumulation 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/10^9 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 *R. aurantiaca* thioesterase could be a cause of the intracellular accumulation of myristoyl-CoA at nonpermissive temperatures. However, the thermostability of *R. aurantiaca* thioesterase contrasts with this suggestion. Enzyme-substrate interactions may also exhibit marked temperature dependence. The affinity and the catalytic efficiency generally give much information on this, especially in the case of regulatory intracellular enzymes that catalyze reactions at substrate concentration close to the K_m value (2, 44, 45). The temperature at which the affinity and the catalytic efficiency toward the myristoyl-CoA are highest closely coincides with the habitat temperature of the strain. At nonpermissive temperatures both parameters dramatically decrease, and the loss of catalytic efficiency correlates with the intracellular increase in myristoyl-CoA concentration. This behavior is consistent with the physiological temperature of the source organism; however, among the cold-adapted enzymes studied so far, this is the first intracellular enzyme found to display such a large variation in the K_m and catalytic efficiency with temperature. If one admits that the k_{cat}/K_m ratio is the operational parameter for regulatory intracellular enzymes, this finding is very important and reveals one of the probable mechanisms for the intracellular accumulation of myristoyl-CoA and cell death at moderate temperatures.

On the other hand, thioesterase is described as a "scavenger" in acyl-CoA metabolism, and its regulation role should only be utilized to avoid excessive concentrations (11, 16). Consequently, the pathological accumulation of myristoyl-CoA at high temperatures would not only be caused by the decrease in its hydrolysis by thioesterase but also by other, as yet unknown, factors. One such factor could be a deficiency in *N*-myristoyltransferase activity, which is an essential enzyme for cell growth and represents an important myristoyl-CoA utilizer. Recent work indicates that in mesophilic yeasts several *N*-myristoyltransferase mutations can cause a temperature-dependent affinity decrease for myristoyl-CoA and growth arrest above 30 °C (30, 31, 33). Moreover, a high myristoyl-CoA concentration without increase of the concentration of other long chain acyl-CoA esters, particularly palmitoyl-CoA, well known as the major product of the fatty acid synthase (41–43), suggests that the principal pathway of C14-CoA use in the psychrophilic *R. aurantiaca* is presumably temperature-sensitive and could be different from the metabolic pathways using other long chain acyl-CoA esters.

In conclusion, our results show for the first time that moderate temperatures cause a deficiency in the myristoyl-CoA metabolism in the psychrophile *R. aurantiaca*. Growth arrest at high temperatures is associated with a toxic intracellular accumulation of myristoyl-CoA. The purified long chain acyl-CoA thioesterase shows a temperature-dependent decrease in its catalytic efficiency and in its affinity for myristoyl-CoA. This result reveals one of the probable mechanisms by which high temperatures induce growth arrest.

Finally, our work should stimulate further studies of other enzymes that are implied in myristoyl-CoA metabolism and contribute to the understanding of the biochemical basis of the inability of psychrophilic microorganisms to grow at moderate temperatures.

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