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Combined effect of the growth temperature and salinity of the medium on the accumulation of compatible solutes by *Rhodothermus marinus* and *Rhodothermus obamensis*

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Abstract In this study we propose revised structures for the two major compatible solutes of *Rhodothermus marinus.* We have also examined the accumulation of compatible solutes by the type strains of the slightly halophilic and thermophilic species *Rhodothermus marinus* and *Rhodothermus obamensis* at several growth temperatures and salinities. The major solutes of *R. marinus* were identified as α-mannosylglycerate (α-MG) and α-mannosylglyceramide (α-MGA), whereas *R. obamensis* accumulated only α-mannosylglycerate. The total osmolyte content was higher during the early exponential phase and decreased abruptly as growth continued into the stationary phase. At low growth temperatures, *R. marinus* responded to water stress by accumulation of α-mannosylglycerate and its amide, in addition to low levels of trehalose, glutamate, and glucose. At the highest growth temperature, α-mannosylglycerate was the major compatible solute and α-mannosylglyceramide was not detected. When both compounds were present, an increase in the salinity of the growth medium favored the accumulation of α-mannosylglyceramide over α-mannosylglycerate. The absence of α-mannosylglyceramide in *R. obamensis* at all growth temperatures and salinities constituted the most pronounced difference in the profiles of compatible solute accumulation by the two strains. Trehalose was also a prominent solute in this organism. Both organisms accumulated higher levels of α-mannosylglycerate as the temperature was raised. The importance of the two compounds in the mechanisms of thermoadaptation and osmoadaptation is discussed.

Key words Compatible solutes · Osmoadaptation · Thermoadaptation · α-Mannosylglycerate · α-Mannosylglyceramide · *Rhodothermus marinus* · *Rhodothermus obamensis*

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Introduction

All known halotolerant and the majority of halophilic microorganisms accumulate compatible solutes in response to osmotic stress imposed by salt or sugars. Only the members of the halophilic bacterial family *Haloanaerobiales* and the extremely halophilic Archaea of the family *Halobacteriaceae* maintain a highly saline cytoplasm using K^+, Na^+, and Cl^- to balance changes in the extracellular salt concentration. Nevertheless, even some of these organisms, such as species of the genera *Natronococcus* and *Natronobacterium*, accumulate the compatible solute sulfotrehalose in addition to inorganic ions (Desmarais et al. 1997; da Costa et al. 1998).

The compatible solutes used by microorganisms include sugars, polyols, amino acids, and their respective derivatives, ectoines and betaines (da Costa et al. 1998). Many of these solutes, namely glycine betaine, are preferentially taken up from the medium rather than synthesized de novo. The number of known compatible solutes has increased substantially in recent years by means of the examination of osmoadaptation in thermophilic and hyperthermophilic bacteria and Archaea. These newly discovered compatible solutes include two isomers of di-*myo*-inositol phosphate, di-mannosyl-di-*myo*-inositol phosphate and di-glycerol phosphate (Scholz et al. 1992; Martins et al. 1996, 1997). Moreover, most compatible solutes identified in mesophilic bacteria and Archaea have not been found in thermophilic and hyperthermophilic organisms, leading to the hypothesis that some common osmolytes, such as betaine and ectoine,

cannot serve their usual function in organisms that grow at high temperatures. Some compatible solutes identified in hyperthermophilic organisms also increase in concentration as the temperature is raised above the optimum for growth. These solutes may, therefore, have a role in thermoprotection of enzymes or other macromolecules (Hensel and König 1988; Scholz et al. 1992; Ciulla et al. 1994; Martins and Santos 1995; Martins et al. 1996, 1997; Ramos et al. 1997).

The halothermophilic eubacterium *Rhodothermus marinus*, with an optimum growth temperature of about 65°–70°C, was found to accumulate two solutes, identified as α- and β-mannosylglycerate, during salt stress (Nunes et al. 1995). Other thermophilic or hyperthermophilic organisms, however, accumulate only one of these compounds (Martins and Santos 1995; Nunes et al. 1995; Martins et al. 1996, 1997). α-Mannosylglycerate had already been identified in several red algae, where it may function as a compatible solute (Karsten et al. 1994).

We have purified the solutes and reevaluated their structures, and shown that they are in fact α-mannosylglycerate and α-mannosylglyceramide. Recently, a new species of the genus *Rhodothermus*, designated *R. obamensis*, was reported to have an optimum growth temperature of about 80°C and grow at temperatures as high as 85°C (Sako et al. 1996). We have, therefore, investigated the accumulation of compatible solutes in these two related species in relation to growth temperatures and salinity.

Materials and methods

Bacterial strains and growth conditions

Rhodothermus marinus (DSM 4252^T) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, and strain *Rhodothermus obamensis* (JCM 9785^T) was obtained from the Japanese Collection of Microorganisms, Saitama, Japan. Both strains were grown on Degryse 162 medium, as modified by Nunes et al. (1992), containing 2.5 g tryptone and 2.5 g yeast extract per liter. *R. obamensis* was also grown on MJ medium previously utilized for the description of this strain (Sako et al. 1996). The effect of salinity on the synthesis of intracellular solutes by *R. marinus* and *R. obamensis* was examined in Degryse 162 medium containing 1.0%, 3.0%, and 5.0% NaCl (wt/vol), or 1.5%, 3.0%, and 5.0% NaCl (wt/vol), respectively. Cultures of *R. marinus* and *R. obamensis* were grown between 57.5° and 77.5°C and between 60.0° and 75.0°C, respectively, to examine the effect of temperature on growth and the accumulation of compatible solutes. Typically, cultures were grown in a water-bath shaker until the late exponential growth phase (turbidity at 660 nm, 0.6–0.7). Cells were harvested by centrifugation (6000 × *g*, 4°C for 10 min) and washed twice with a solution composed of the macronutrients of Degryse medium 162 and the appropriate concentration of NaCl. To examine the accumulation of solutes during the growth cycle, cultures were harvested at appropriate time intervals of growth, and washed as described.

Ethanol extraction

Extraction of compatible solutes was performed with boiling 80% ethanol (Reed et al. 1984). The extraction was repeated twice and the combined extracts evaporated to dryness under negative pressure; the residue was resuspended in water and chloroform, and centrifuged to remove lipid components. The aqueous extract was lyophilized and resuspended in ^2H_2O for nuclear magnetic resonance (NMR) analysis.

Purification of mannosylglycerate and mannosylglyceramide

A freeze-dried extract obtained from 90 g (wet mass) of *Rhodothermus marinus* grown at 65°C and in medium containing 4.0% NaCl was dissolved in 10 ml of water and loaded onto a QAE-Sephadex A25 (20 × 5 cm) column previously equilibrated with 5.0 mM sodium bicarbonate, pH 9.8. The elution was performed with one bed volume of the same buffer, followed by six bed volumes of a linear gradient of $NaHCO_3$ (5.0 mM to 1 M) at a flow rate of 10 ml min^{-1}. The eluted fractions were analyzed for carbohydrate by the method of Dubois et al. (1956). Two carbohydrate-containing fractions were obtained: the first fraction did not adsorb to the column and was, therefore, eluted at the ionic strength of the equilibrating buffer (5.0 mM $NaHCO_3$), and the second fraction was eluted at 200 mM $NaHCO_3$. The two fractions were lyophilized and analyzed by ^{1}H-NMR spectroscopy. Each sample was loaded onto a column of activated Dowex 50W-X8 resin (10 × 2.5 cm) and eluted with distilled water. Subsequently, the fractions were pooled, degassed under vacuum, and the pH raised to 3.5 with 1.0 M KOH. Samples were lyophilized and dissolved in water. The sample that adsorbed to the anionic resin was judged pure by ^{1}H-NMR spectroscopy, and the spectrum was identical to that of the compound earlier designated as β-mannosylglycerate (Nunes et al. 1995).

The major solute of the nonionic sample was an unknown compound, but NMR spectroscopy also revealed the presence of contaminating glucose and trehalose. This unidentified compound was further purified by adsorption chromatography. A silica gel S (0.032–0.063 mm in diameter; Riedel-de Häen) column (20 × 2 cm) was prepared in dichloromethane/methanol/acetic acid (25:15:10, v/v). This solvent system was also used to dissolve the sample and for the elution. Glucose was the first compound eluted, followed by the unknown solute, and finally by trehalose. The fractions containing the unknown compound were pooled and lyophilized. The lyophilized residue was dissolved in distilled water and loaded onto a QAE-Sephadex A25 (5 × 1 cm) column to remove residual acetate. The unknown compound was eluted with distilled water and its purity assessed by ^{1}H-NMR spectroscopy.

Enzymatic hydrolysis of mannosylglycerate

A pure sample of mannosylglycerate was subjected to enzymatic hydrolysis by jackbean α-mannosidase (Sigma, St Louis, MO, USA) and snail β-mannosidase (Sigma). The reaction mixture contained 50 μmoles of mannosylglycerate, and 25 U of either enzyme in 50 mM citrate buffer at pH 4.5 for α-mannosidase and pH 4.0 for β-mannosidase at 25°C. After 6 h the reaction was stopped by the addition of HCl and the samples were analyzed by ^{1}H-NMR.

Analytical methods

Cell protein was determined by the Bradford method (1976) after sonicating the cells. ^{1}H-NMR spectra were recorded at 37°C on a Bruker AMX 300 or DRX 500 spectrometer with a 5-mm-diameter broadband inverse probe head. Spectra were acquired with water presaturation and the following parameters: spectral width, 10 kHz; pulse width, 6 μs, corresponding to a 60° flip angle; data size, 64 K; repetition delay, 25 s. Formate was added as a concentration standard. ^{1}H chemical shifts were relative to 3-(trimethylsilyl) propanesulfonic acid (sodium salt). Two-dimensional NMR spectra were obtained as described by Nunes et al. (1995) and Lamosa et al. (1998). Assignments of resonances to glutamate and trehalose were confirmed by the addition of small amounts of pure compounds. Quantification of organic solutes was based on ^{1}H-NMR of ethanol extracts; the amounts of α-glutamate and glucose in the extracts were also determined enzymatically using test kits as recommended by the manufacturer (Boehringer, Mannheim, Germany). Pure α-mannosylglycerate was kindly provided by O. Nixdorf (University of Bremen, Germany) and U. Karsten (Alfred Wegener Institute, Bremerhaven, Germany).

Peracetyl derivatives were prepared by treatment of the dry samples with 100 μl of trifluoroacetic anhydride/acetic acid (2:1, vol/vol) for 10 min at room temperature (Dell 1990). Reagents were removed by vacuum centrifugation and the derivatives were dissolved in 1 ml of chloroform, washed with water, evaporated to dryness, and redissolved in chloroform/methanol (1:1, vol/vol) for analysis by fast atom bombardment mass spectrometry (FAB-MS).

Fast atom bombardment mass spectra were obtained on a Kratos MS80 RFA spectrometer (Kratos, Manchester, UK), fitted with an Ion Tech saddle-field atom gun supplied with high-purity xenon gas. Underivatized samples were analyzed from glycerol matrix and the peracetyl derivatives from 3-nitrobenzyl alcohol. Collision-induced dissociation was performed in the field-free region between the ion source and the electrostatic analyzer. Daughter ion spectra were recorded by scanning the magnetic field (B) and the electrostatic analyzer voltage (E), while maintaining a constant ratio of B/E. The helium collision gas pressure was adjusted to give 50% attenuation of the precursor ion beam. Elemental analysis was performed on a Fisons E A 1108 cnNS-o (Italy) apparatus using the method described by Colombo et al. (1979).

Results

Effect of the temperature and salt concentration of the medium on the growth of *Rhodothermus marinus* and *Rhodothermus obamensis*

Our results show that the lowest growth temperature of the type strain of *R. marinus* was about 55°C, the optimum about 65°–70°C, and the maximum in the neighborhood of 77° to 80°C (Fig. 1). The only known strain of *R. obamensis*, which was isolated from a marine hot spring in Japan, was reported to have an optimum growth temperature about 80°C and a maximum growth temperature about 85°C (Sako et al. 1996). Our results showed that *R. obamensis* had an optimum growth temperature about 70°C and a maximum growth temperature in the vicinity of 77°C in medium 162 (Fig. 2).

Degryse medium 162 was chosen over MJ medium because growth of the two species was slightly better in the former medium under all conditions, and the results could be compared to those of Nunes et al. (1995). At the optimum growth temperature or below, both organisms grew well in Degryse medium 162 containing 1.0%–1.5% NaCl. However, at temperatures greater than the optimum for growth, the two species of *Rhodothermus* showed a marked

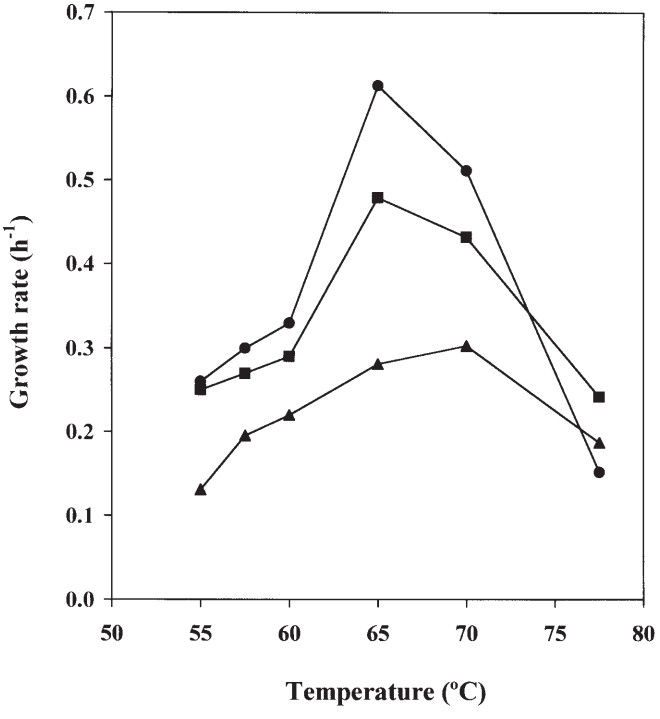

Fig. 1. Effect of temperature on the growth of *Rhodothermus marinus* in Degryse medium 162 containing 1.0% NaCl (*circles*), 3.0% NaCl (*squares*), and 5.0% NaCl (*triangles*)

decrease in the growth rate in medium with 1.0%–1.5% NaCl. In fact, *R. obamensis* did not grow at 75°C in this medium. Growth at supraoptimal temperatures was always better in Degryse medium 162 containing 3.0% NaCl than in media with lower salt concentrations.

Identification of mannosylglyceramide

The purified novel compound was subjected to a mild alkaline hydrolysis with NaOD in an ultrasonic bath. Samples were removed at intervals between 1 and 8 h and analyzed by ^{1}H-NMR spectroscopy. After 8 h, the alkaline hydrolysate of the unknown solute contained only a single ^{1}H-NMR-detectable compound whose spectrum coincided with that of mannosylglycerate previously identified in *R. marinus* and *Pyrococcus furiosus* (Martins and Santos 1995; Nunes et al. 1995). The identification of this compound was confirmed by spiking the hydrolyzed sample with pure mannosylglycerate purified from *P. furiosus*.

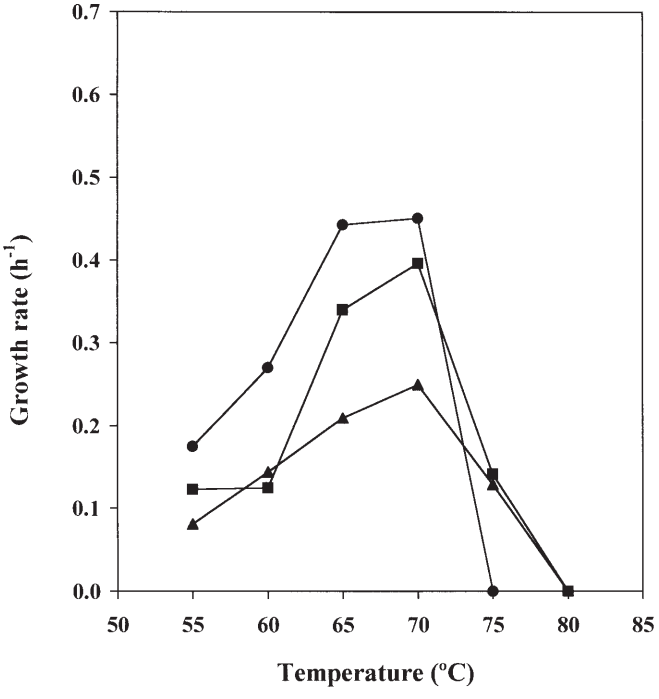

Fig. 2. Effect of temperature on the growth of *Rhodothermus obamensis* in Degryse medium 162 containing 1.5% NaCl (*circles*), 3.0% NaCl (*squares*), and 5.0% NaCl (*triangles*)

One-dimensional decoupled ^{13}C-NMR spectrum of the compound showed a set of nine resonances (175.5, 100.1, 77.9, 73.9, 70.7, 70.4, 67.1, 62.8, and 61.4 ppm), also detected by Nunes et al. (1995). The resonance at 100.1 ppm was assigned to an anomeric carbon. A heteronuclear multiple quantum coherence (HMQC) spectrum showed that the anomeric carbon was correlated to the proton at 4.92 ppm. A proton–homonuclear correlation (COSY) spectrum enabled assignment of most of the remaining protons. The three-carbon moiety was assigned from the analysis of heteronuclear multiple bond correlation (HMBC) and COSY spectra. From the HMQC spectrum, two of the signals present in the ^{13}C-NMR spectrum were assigned to methylene groups (62.8 and 61.4 ppm), and five were assigned to methine groups (77.9, 73.9, 70.7, 70.4, and 67.1 ppm). The assignment of all proton and carbon chemical shift values, as well as proton–proton and proton–carbon coupling constants, are listed in Table 1.

The position of the glycosidic bond between carbon 1 of the mannose and the hydroxyl group at position 2 of the three-carbon moiety was derived from HMBC spectrum (Fig. 3). Bock and Pedersen (1974) showed that the coupling constants between C_1 and H_1 could be used to assign the anomeric carbon configuration of pyranoside derivatives of carbohydrates. Characteristic coupling constants of 170 and 160 Hz were reported for α- and β-pyranosides, respectively (Bock and Pedersen 1983). The configuration of the glycosidic linkage in the newly discovered compound was identified as alpha, because of the value of 170.8 Hz determined for the respective anomeric coupling constant (Table 1).

Table 1. NMR data of mannosylglyceramide and mannosylglycerate

Moiety	Mannosylglyceramide				Mannosylglycerate			
	^{13}C-NMR		^{1}H-NMR		^{13}C-NMR		^{1}H-NMR	
	δ (ppm)	$^{1}J_{CH}$ (Hz)	δ (ppm)	$^{3}J_{HH}$ (Hz)	δ (ppm)	$^{1}J_{CH}$ (Hz)	δ (ppm)	$^{3}J_{HH}$ (Hz)[b]
Mannose								
C-1	100.1	170.8	4.92	$^{3}J_{1'2'} = 1.7$	99.1	171.8	4.89	$^{3}J_{1'2'} = 1.7$
C-2	70.4	149.7	4.10	$^{3}J_{2'3'} = 3.3$	70.5	149.2	4.07	$^{3}J_{2'3'} = 3.3$
C-3	70.7	144.5	3.90	$^{3}J_{3'4'} = 9.5$	70.8	143.5	3.90	$^{3}J_{3'4'} = 9.6$
C-4	67.1	146.4	3.67	nd[a]	67.2	146.0	3.65	$^{3}J_{4'5'} = 9.6$
C-5	73.9	148.8	3.74	nd	73.5	145.8	3.71	$^{3}J_{5'6'a} = 2.4$, $^{3}J_{5'6'b} = 6.2$
C-6	61.4	144.4	3.89, 3.75	nd	61.4	144.0	3.87, 3.74	$^{3}J_{6'a\,6'b} = -11.9$
Glycerate								
C-1	175.5				176.6			
C-2	77.9	146.8	4.32	$^{3}J_{23a} = 3.9$, $^{3}J_{23b} = 3.8$	77.8	146.4	4.27	$^{3}J_{23a} = 3.0$, $^{3}J_{23b} = 6.4$
C-3	62.8	145.9	3.87, 3.87	nd	63.3	145.4	3.86, 3.79	$^{3}J_{3a3b} = 12.3$

[a] nd, not determined
[b] Coupling constants determined from simulation of the proton spectrum with NMR-SIM (Bruker software)

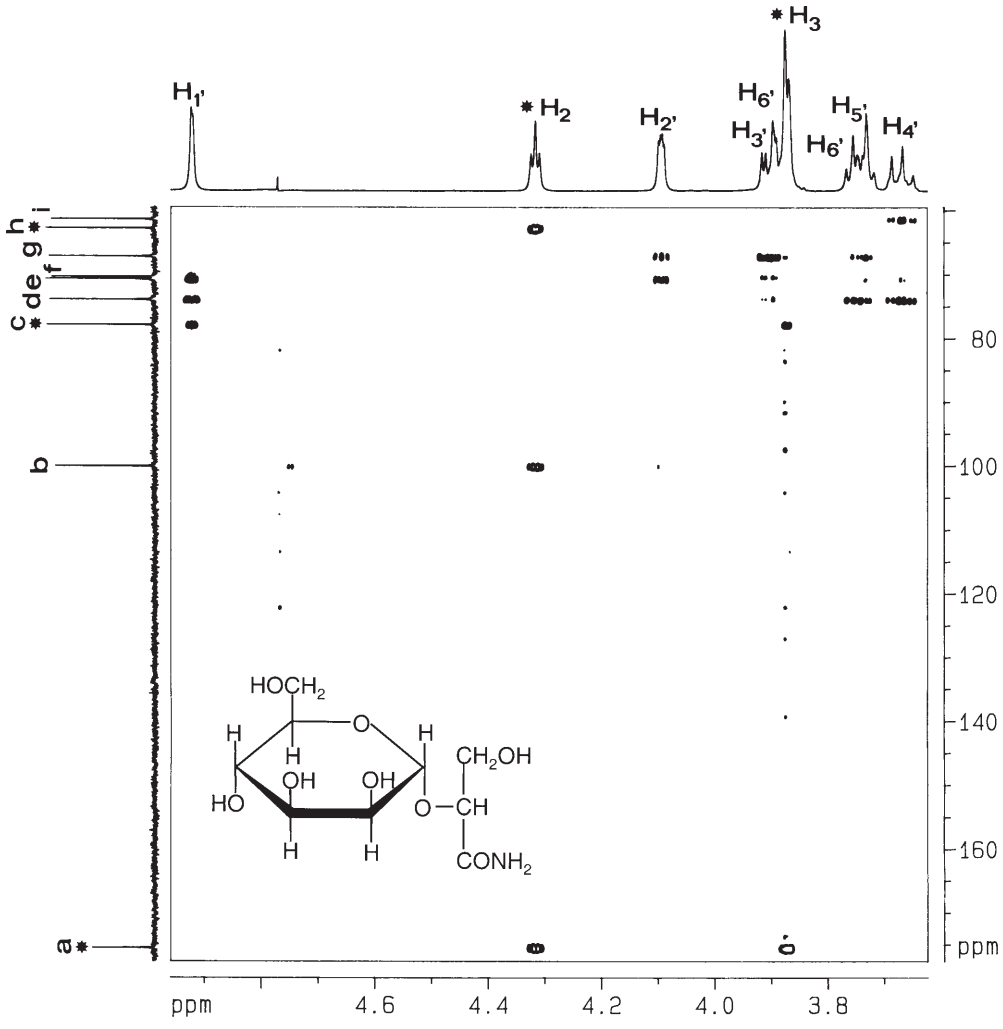

Fig. 3. ^{13}C–^{1}H correlation spectrum through long-range coupling (heteronuclear multiple bond correlation) of pure α-mannosylglyceramide. Peaks resulting from the glyceramide moiety are labeled with an *asterisk*. Specific assignments of carbon resonances are as follows: a, c, and h represent C_1, C_2, and C_3 of the glycerate moiety, respectively; b, d, e, f, g, and i represent C_1, C_5, C_3, C_4, and C_6 of the mannose moiety, respectively. The *inset* shows a schematic represention of α-mannosylglyceramide. Crosspeaks represent connectivities between proton and carbon atoms separated by two or three bonds

The positive FAB spectrum of the underivatized unknown compound was characterized by ions at *m/z* 268.1 ($[M + H]^+$), 290.2 ($[M + Na]^+$), and 306.1 ($[M + K]^+$), which were one dalton lower in mass than the corresponding ions from authentic mannosylglycerate. In the spectrum of its peracetyl derivative, abundant ions were observed at *m/z* 478.2 ($[M + H]^+$), 500.2 ($[M + Na]^+$), and 542.2, together with an abundant B_1 carbenium ion at *m/z* 331 (characteristic of a terminal hexose), which decomposed via loss of acetate and ketene giving secondary fragments at *m/z* 169 and 109 (Dell 1987).

Collisional activation of the protonated molecule at *m/z* 478 resulted in daughter ions at *m/z* 331, 169, and 109, further confirming the presence of a terminal nonreducing hexose and suggesting that the modification resulting in a 1-Da mass difference from mannosylglycerate must be confined to the glyceric acid moiety. One plausible structural alteration resulting in a loss of 1 Da is conversion of a COOH group to $CONH_2$, which was consistent with the presence of the signal at *m/z* 542 representing *N*-acetylation of the amide nitrogen in addition to the five hydroxyl groups.

Elemental analysis revealed a C:N ratio of 9:1. The unknown compound was, therefore, identified as α-mannosylglyceramide on the basis of NMR, mass spectrometry, and elemental analysis.

Reassessement of the configuration around the anomeric carbon in mannosylglycerate from *R. marinus*

The new identification of the configuration of the anomeric carbon of mannosylglyceramide as alpha, described in this study, led us to suspect that our previous identification of the configuration of mannosylglycerate in *R. marinus*, based on a weak NOE effect between H_1 and H_5, was incorrect. In fact, the J_{CH} of the anomeric carbon of the purified mannosylglycerate was 171.8 Hz (Table 1), a value indicative of an alpha configuration (Bock and Pedersen 1983). To confirm this identification a sample of *P. furiosus*, mannosylglycerate was spiked with pure red algal α-mannosylglycerate. Moreover, mannosylglycerate was hydrolyzed to mannose and glycerate by α-mannosidase, but not by β-mannosidase, corroborating the identification of

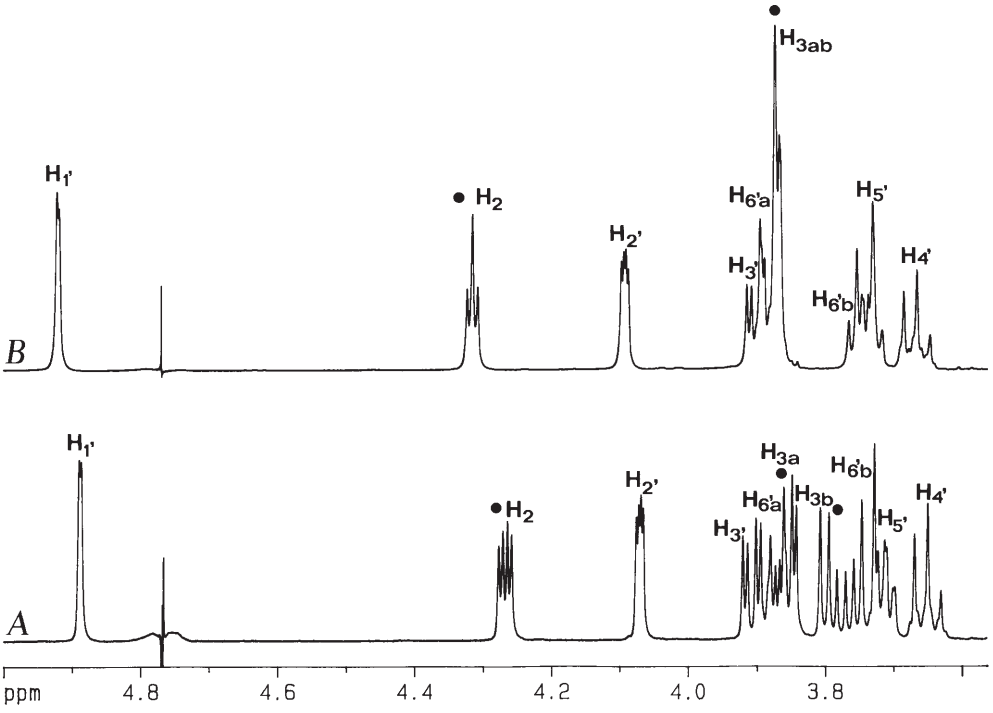

Fig. 4. ^{1}H-NMR spectra of purified α-mannosylglycerate (*A*) and α-mannosylglyceramide (*B*). The resonances from the glycerate moieties are labeled with *full circles*. The pH values of the solutions of mannosylglycerate and mannosylglyceramide were 4.0 and 8.0, respectively

this solute as α-mannosylglycerate (results not shown). This confirmed that the configuration of mannosylglycerate in thermophilic and hyperthermophilic organisms (Martins and Santos 1995; Nunes et al. 1995) is identical to that of the solute identified earlier in red algae of the order Ceramiales (Karsten et al. 1994). A comparison of the ^{1}H-NMR spectra of mannosylglycerate and mannosylglyceramide is presented in Fig. 4.

Accumulation of compatible solutes during the growth cycle of *Rhodothermus* spp.

Alterations in compatible solute accumulation were examined in both organisms during the growth cycle at the optimum growth temperature and in medium containing 5.0% NaCl. The inocula used to assess the alterations in the compatible solute composition in both organisms were derived from stationary phase cells cultivated for 16–18 h in medium containing 5.0% NaCl.

The major compatible solutes of the inoculum of *R. marinus* were α-mannosylglyceramide and α-mannosylglycerate, together with very minor amounts of glutamate and glucose (Fig. 5). After 2 h of incubation there was a steep rise in the levels of α-mannosylglycerate, as well as the accumulation of trehalose. During exponential growth, there was a small, but steady, decrease in most solutes, while at the onset of the stationary phase and during prolonged incubation of the cultures all solutes, except α-mannosylglyceramide, which remained fairly constant during the growth cycle, decreased considerably.

The accumulation of compatible solutes during the growth cycle of *R. obamensis* was similar to that of *R. marinus*, except for the absence of α-mannosylglyceramide (Fig. 6). The inoculum had large concentrations of glucose, in addition to α-mannosylglycerate, and low levels of

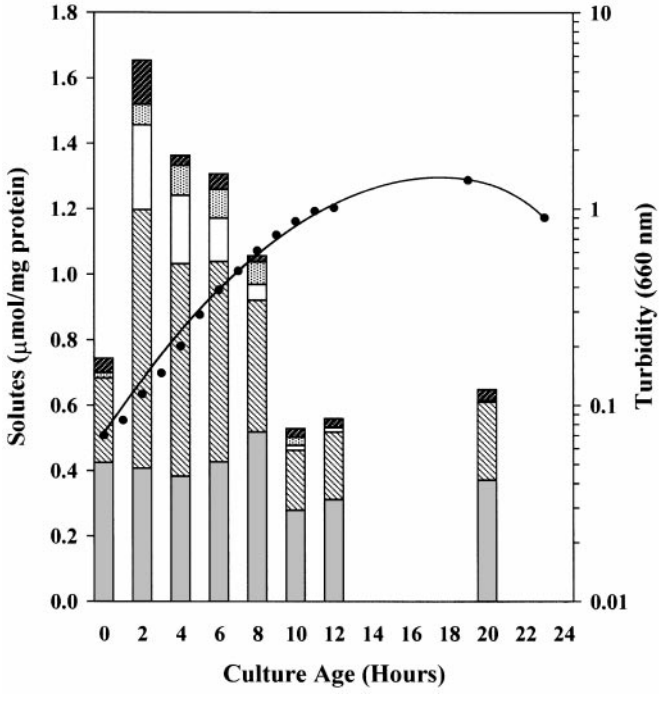

Fig. 5. Relationship between the growth phase and accumulation of compatible solutes by *Rhodothermus marinus*; growth in medium containing 5% NaCl at 65°C (*circles*). *Bars* represent the intracellular concentration of: α-mannosylglyceramide (▩), α-mannosylglycerate (▨), trehalose (□), glutamate (▧), and glucose (▩)

glutamate and trehalose. As growth resumed the levels of trehalose increased steeply, approaching those of α-mannosylglycerate. Large concentrations of trehalose and α-mannosylglycerate were maintained throughout the exponential phase, while the levels of glucose and glutamate remained low. An abrupt decrease in the levels of all solutes followed the onset of the stationary phase of growth, although prolonged incubation of the culture resulted in decrease of the trehalose levels to practically undetectable and a rise in the levels of glucose.

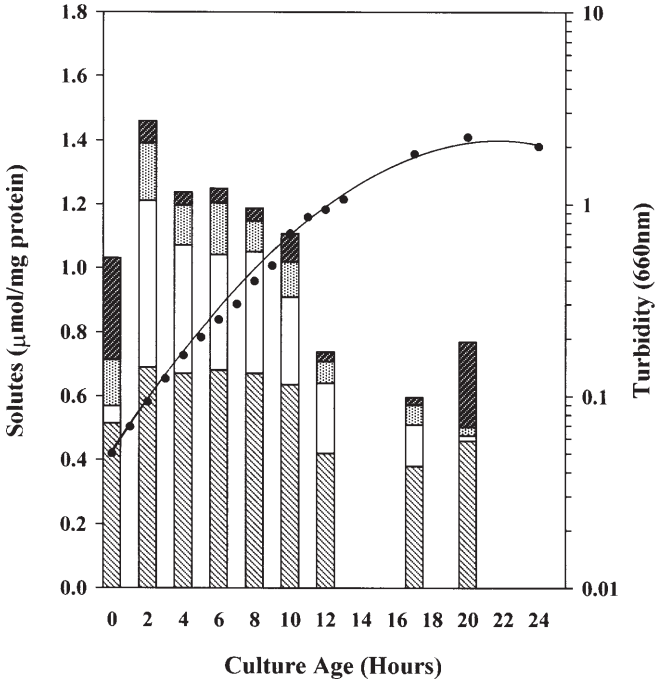

Fig. 6. Relationship between the growth phase and accumulation of compatible solutes by *Rhodothermus obamensis*; growth in medium containing 5% NaCl at 65°C (*circles*). *Bars* represent the intracellular concentration of: α-mannosylglycerate (▨), trehalose (□), glutamate (⊡), and glucose (▩)

Effect of the growth temperature and NaCl of the medium on the accumulation of compatible solutes

The absence of α-mannosylglyceramide, throughout the growth temperature range of *R. obamensis*, was the most significant difference in the compatible solute composition of the two organisms. At low temperatures and in medium containing 1.0% NaCl, glutamate was the sole compatible solute in *R. marinus* (Table 2). Increasing the salinity of the medium to 3.0% NaCl led primarily to the accumulation of α-mannosylglycerate, instead of α-mannosylglyceramide. However, this response was reversed in medium with 5.0% NaCl where α-mannosylglyceramide was as abundant as α-mannosylglycerate. At the highest growth temperature (77.5°C), α-mannosylglyceramide was never found in *R. marinus*, and α-mannosylglycerate was the major solute detected, together with small amounts of trehalose, glutamate, and glucose. By contrast, *R. obamensis* responded to salt stress by increasing the intracellular levels of α-mannosylglycerate and, to a lesser extent, trehalose (Table 3). The levels of α-mannosylglycerate accumulated by *R. obamensis* also increased as the growth temperature was raised from 60° to 75°C at all salinities.

Discussion

Several years ago we detected two major compounds in ethanolic extracts of *Rhodothermus marinus*, exhibiting very similar ^{1}H-NMR spectra, and identified as two forms of mannosylglycerate (Nunes et al. 1995). One of them was

Table 2. Accumulation of compatible solutes in *Rhodothermus marinus* DSM 4252^{T} grown at different temperatures and NaCl concentrations

Growth conditions	Solutes[a] (μmol/mg protein)					
	Mannosylglyceramide	Mannosylglycerate	Glutamate	Trehalose	Glucose	Total solutes
57.5°C						
1.0% NaCl	–[b]	–	0.045	–	–	0.045
3.0% NaCl	0.077	0.197	0.066	–	0.012	0.352
5.0% NaCl	0.520	0.244	0.085	–	0.103	0.952
65.0°C						
1.0% NaCl	–	–	0.031	–	–	0.031
3.0% NaCl	0.058	0.331	0.050	–	0.016	0.455
5.0% NaCl	0.517	0.404	0.068	–	0.077	1.066
70.0°C						
1.0% NaCl	–	0.017	0.013	–	0.003	0.033
3.0% NaCl	0.007	0.390	0.007	0.009	0.023	0.436
5.0% NaCl	0.430	0.432	0.039	0.061	0.035	0.997
77.5°C						
1.0% NaCl	–	0.120	0.010	–	0.005	0.135
3.0% NaCl	–	0.701	0.073	–	0.010	0.784
5.0% NaCl	–	1.170	0.104	0.163	0.050	1.487

[a] Mannosylglycerate, mannosylglyceramide, and trehalose were quantified by ^{1}H-NMR; glutamate and glucose were quantified enzymatically
[b] Not detected by ^{1}H-NMR

Table 3. Accumulation of compatible solutes in *Rhodothermus obamensis* JCM 9785^T grown at different temperatures and NaCl concentrations

Growth conditions	Solutes[a] (μmol/mg protein)				
	Mannosylglycerate	Glutamate	Trehalose	Glucose	Total solutes
60.0°C					
1.5% NaCl	–[b]	0.160	–	–	0.160
3.0% NaCl	0.165	0.290	0.004	0.013	0.472
5.0% NaCl	0.490	0.120	0.240	0.071	0.921
65.0°C					
1.5% NaCl	0.029	0.190	–	0.004	0.223
3.0% NaCl	0.217	0.290	0.009	0.022	0.538
5.0% NaCl	0.635	0.110	0.274	0.088	1.107
70.0°C					
1.5% NaCl	0.043	0.087	–	0.001	0.131
3.0% NaCl	0.360	0.110	0.074	0.033	0.577
5.0% NaCl	0.941	0.068	0.465	0.103	1.577
75.0°C					
3.0% NaCl	0.385	0.063	0.030	0.041	0.519
5.0% NaCl	0.982	0.017	0.337	0.041	1.377

[a] Mannosylglycerate and trehalose were quantified by ^{1}H-NMR; glutamate and glucose were quantified enzymatically
[b] Not detected by ^{1}H-NMR

identified as β-mannosylglycerate on the basis of a weak Overhauser effect observed between protons H_1 and H_5 of the mannose ring. Consequently, the other compound was proposed to be α-mannosylglycerate. In the present study the compatible solutes of *R. marinus* were purified, allowing a thorough characterization of their structures not only by NMR but also by mass spectrometry and elemental analysis. During the purification of these compounds it was immediately evident that the initial identification of α-mannosylglycerate could not be correct, because it did not bind to anionic exchange resins as expected for a negatively charged compound. As a consequence of the re-evaluation of the structure of these compounds, we show that the two forms of mannosylglycerate in *R. marinus* are, in fact, α-mannosylglycerate and α-mannosylglyceramide. Therefore, we conclude that thermophilic and hyperthermophilic organisms, like some red algae, accumulate only α-forms of mannosylglycerate (Martins and Santos 1995; Nunes et al. 1995; Martins et al. 1996, 1997).

Our results show that *R. marinus* accumulates primarily α-mannosylglyceramide at high salinities and low growth temperatures, while α-mannosylglycerate is the major intracellular solute at high growth temperatures and α-mannosylglyceramide is not detectable. We also show that *R. obamensis*, being no more thermophilic than *R. marinus*, accumulates only α-mannosylglycerate over the entire temperature and salinity range for growth. The inability of the type strain of *R. marinus* to accumulate α-mannosylglyceramide at high temperatures came as a complete surprise, because without doubt α-mannosylglyceramide functions as a compatible solute in *R. marinus* at low growth temperatures. The absence of this solute in *R. obamensis* may be a strain-specific phenomenon or may be a stable characteristic of the species. Nevertheless, the inability of *R. obamensis* to accumulate α-mannosylglyceramide provides important insights into the strategies developed by both organisms to cope with salt stress.

The alteration in the type and the levels of compatible solutes during the growth cycle of the two *Rhodothermus* spp. was not unexpected because the levels of compatible solutes in the stationary phase of bacteria and archaea, yeast, and fungi are often lower than in the exponential phase of growth. Moreover, the major compatible solutes, or their relative proportions, may differ between exponential and stationary phase in many yeasts (Mackenzie et al. 1988; da Costa and Nobre 1989; Brown 1990; Luxo et al. 1993). Similar phenomena have been observed in mesophilic bacteria and hyperthermophilic archaea, where the total levels of compatible solutes decrease during the stationary phase with sometimes drastic alterations in composition of the major solutes (Whatmore et al. 1990; Martins and Santos 1995; Smith 1996; Lamosa et al. 1998). The lower levels of compatible solutes during the stationary phase may be the result of loss to the medium, or metabolism of the primary compatible solutes to levels that are sufficient to maintain cell viability under osmotic stress until favorable conditions allow the organisms to resume growth.

The increase in the levels of glucose was not expected, because glucose was not deliberately added to the growth medium. The presence of glucose in fairly high levels during the stationary phase of *R. obamensis* is probably related to the breakdown of trehalose. By virtue of its concentration, however, glucose acts as a compatible solute during the stationary phase of *R. obamensis*, when other organic solutes are present in lower amounts than in the exponential phase. It should also be noted that trehalose contributes significantly to the compatible solute levels in *R. obamensis*.

α-Mannosylglyceramide has only been identified in strains of *R. marinus*; other slightly thermophilic, thermo-

philic, and hyperthermophilic organisms such as *Petrotoga myotherma*, *Thermus thermophilus*, *Pyrococcus furiosus*, and several species of the genus *Thermococcus* accumulate α-mannosylglycerate in response to salt stress (Martins and Santos 1995; Nunes et al. 1995; Martins et al. 1997; Lamosa et al. 1998). This compatible solute has not been detected, thus far, in mesophilic bacteria, but has been identified in red algae of the order Ceramiales. In these organisms α-mannosylglycerate may serve as a compatible solute during the initial stages of osmoadaptation, but is subsequently replaced by mannitol (Karsten et al. 1994). For unknown reasons α-mannosylglyceramide is the primary compatible solute at low growth temperatures in *R. marinus*, while α-mannosylglycerate is preferentially synthesized at supra-optimum growth temperatures, indicating this compound may represent a more efficient strategy to stabilize macromolecules from the effects of intracellular dehydration during osmoadaptation at high temperatures. One can also argue that α-mannosylglycerate serves a dual role in osmoadaptation and thermoadaptation in *Rhodothermus* spp. and that the need to accumulate other solutes at high temperatures is not warranted. In fact, this contrasts with observations that other slightly halophilic hyperthermophilic organisms accumulate different compatible solutes under water stress and temperature stress (Ciulla et al. 1994; Martins and Santos 1995; Martins et al. 1996). The large alteration in the relative proportions of α-mannosylglyceramide and α-mannosylglycerate in *R. marinus* when the salinity or the temperature is altered may indicate that the enzyme responsible for the synthesis of α-mannosylglyceramide is not active or is not expressed at high growth temperatures.

The accumulation of organic solutes above the optimum growth temperature coupled to the thermostabilizing properties of a few of them led to the hypothesis that some compatible solutes contribute to growth at very high temperatures (Hensel and König 1988; Scholz et al. 1992; Ramos et al. 1997). Nonhalophilic thermophiles and hyperthermophiles do not accumulate organic solutes at the optimum growth temperature, nor do they accumulate organic solutes as the temperature is raised above the optimum for growth (Martins et al. 1996, 1997). These results indicate that the accumulation of specific organic solutes, under temperature stress in halophilic thermophiles and hyperthermophiles, may serve other roles. However, different solutions to cope with stress are possible and there is no evidence, at present, that solutes that accumulate at supraoptimal growth temperatures serve roles unrelated to thermostabilization. Further studies are necessary to resolve the question of the thermostabilizing effect of α-mannosylglycerate and di-*myo*-inositol phosphate, and these studies will be essential to broaden our understanding of how organisms grow at high temperatures.

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