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Accumulation of Mannosylglycerate and Di-*myo*-Inositol-Phosphate by *Pyrococcus furiosus* in Response to Salinity and Temperature

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^{13}C and ^{1}H nuclear magnetic resonance spectroscopy was used to identify and quantify organic solutes accumulated by the hyperthermophilic archaeon *Pyrococcus furiosus* in response to temperature and salinity. Di-*myo*-inositol-phosphate and 2-*O*-β-mannosylglycerate were the major organic solutes accumulated in these cells. The total intracellular organic solutes increased significantly in response either to an increase in temperature or to an increase in salinity, but β-mannosylglycerate accumulated mainly at high salinities, whereas the concentration of di-*myo*-inositol-phosphate increased dramatically at supraoptimal growth temperatures. Glutamate was present at concentrations detectable by nuclear magnetic resonance only in cells grown in low-salinity media. The intracellular levels of K^+ are clearly dependent on the salinity of the medium, and the concentrations of this cation are high enough to counterbalance the negative charges of β-mannosylglycerate and di-*myo*-inositol-phosphate in the cell. The results presented here together with those previously reported for *Pyrococcus woesei* (S. Scholz, J. Sonnenbichler, W. Schäfer, and R. Hensel, FEBS Lett. 306:239–242, 1992) strongly support a role for di-*myo*-inositol-phosphate in thermoprotection.

The archaeon *Pyrococcus furiosus* is an obligately anaerobic chemo-organotrophic microorganism capable of growth at temperatures above 90°C (1, 19) that was isolated from a geothermal marine environment and is strictly dependent on sodium for growth. Like other marine organisms, *P. furiosus* must cope with changes in the water activity by accumulating solutes, by either de novo synthesis or transport from the external medium (6, 11).

Life at high temperatures raises interesting questions about the strategies adopted for thermostabilization of cellular components such as proteins, nucleic acids, and membranes. Recently, we became interested in exploring the biochemical features of thermoadaptation and examined several strains of thermophilic bacteria for the presence of organic solutes that could play a role in thermophily (14). In this search, 2-*O*-β-mannosylglycerate was detected in all the halophilic or halotolerant thermophilic bacteria screened, and it may be relevant that this charged sugar derivative has not been observed in mesophilic organisms. This investigation has been extended to hyperthermophiles, and here we report on the use of nuclear magnetic resonance (NMR) to identify organic solutes in *P. furiosus* and to monitor the intracellular levels of these solutes as a function of salinity and temperature.

MATERIALS AND METHODS

Microorganism and growth conditions. *P. furiosus* DSM 3638 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Cells were cultivated in the medium described by Raven et al. (15), unless otherwise stated, at 95°C in a culture vessel with a total volume of 2 liters. The medium was continuously gassed with N_2 and stirred at 100 rpm. Cultures were grown at 88, 90, 95, 98, 100, and 101°C in medium containing 2.8% NaCl. To test the effect of osmotic stress on the synthesis of intracellular solutes, the salinity of the growth medium was varied between 1.0 and 5.0% NaCl. Cell growth was monitored by measuring the turbidity of the cultures at 600 nm. Cells were harvested by centrifugation (8,000 × *g*, 30°C, 15 min) at different stages of growth and washed once with an NaCl solution identical in NaCl concentration to the respective growth medium.

Cell protein determination. The protein contents of the cells were determined by the Bradford assay (3) after cell lysis with 1 M NaOH (100°C, 10 min) and neutralization with 1 M HCl.

Extraction and determination of intracellular solutes. Cell pellets from cultures of *P. furiosus* were extracted twice with boiling 80% ethanol (16). The solvent from the combined supernatants was removed by rotary evaporation, and the residue was freeze-dried. The dry residue was extracted twice with a mixture of water-chloroform (2:1). After centrifugation, the aqueous phase was lyophilized and the residue was dissolved in $^{2}H_2O$ for ^{1}H- and ^{13}C-NMR analyses. The final pH of the sample was adjusted to approximately 8 by the addition of $NaO^{2}H$. Mannosylglycerate and di-*myo*-inositol-phosphate were quantified by ^{1}H-NMR. Glutamate was determined in a Beckman model 6300 E amino acid analyzer.

Sample preparation for intracellular potassium determination. *P. furiosus* cells grown at 95°C in medium containing 1.0 or 4.0% NaCl were harvested by centrifugation as described above and were washed three times with an NaCl solution identical in NaCl concentration to the corresponding growth medium. The cell pellet was suspended in 2.5 ml of the same solution containing 5% $^{2}H_2O$ and was directly analyzed by ^{39}K-NMR.

Determination of intracellular volume. The intracellular volume of the cells was determined by the centrifugation method (20), with $^{3}H_2O$ and [^{14}C]polyethylene glycol for labelling total and extracellular volumes, respectively.

NMR spectroscopy. ^{13}C-NMR spectra were recorded at 121.77 MHz on a Bruker AMX500 spectrometer with a carbon-selective probe head. Spectra were acquired with a repetition delay of 3 s and a pulse width of 7 μs, corresponding to a 90° flip angle. Proton decoupling was applied during the acquisition time only, with the wideband alternating-phase low-power technique for zero-residue splitting sequence. Chemical shifts were referenced to the resonance of external methanol, designated at 49.3 ppm.

^{1}H-NMR spectra were recorded on a Bruker AMX300 or AMX500 spectrometer. For quantification, spectra were acquired with water presaturation, 6-μs pulse width (corresponding to a 60° flip angle), and a repetition delay of 16 s. Formate was added as a concentration standard. ^{1}H chemical shifts were relative to 3-(trimethylsilyl)propanesulfonic acid (sodium salt). Inverse detected ^{1}H-^{13}C heteronuclear multiple quantum coherence spectra (2) were acquired by collecting 4,096 (t_2) × 256 (t_1) datum points; 3.5 ms were used for evolution of $^{1}J_{CH}$.

^{39}K-NMR spectra were obtained at 23.33 MHz, with a 5-kHz sweep width, a 90° pulse width of 60 μs, and a repetition delay of 0.6 s. In order to quantify the NMR-detectable K^+, a known amount of KCl was added to the cell suspension and the intensities of the spectra that were run before and after the addition of the concentration standard were compared. All NMR spectra were run at 27°C.

RESULTS

Effects of NaCl and temperature on growth. Under the given conditions, *P. furiosus* had an optimum growth temperature in the range of 90 to 95°C when cultivated in the medium of

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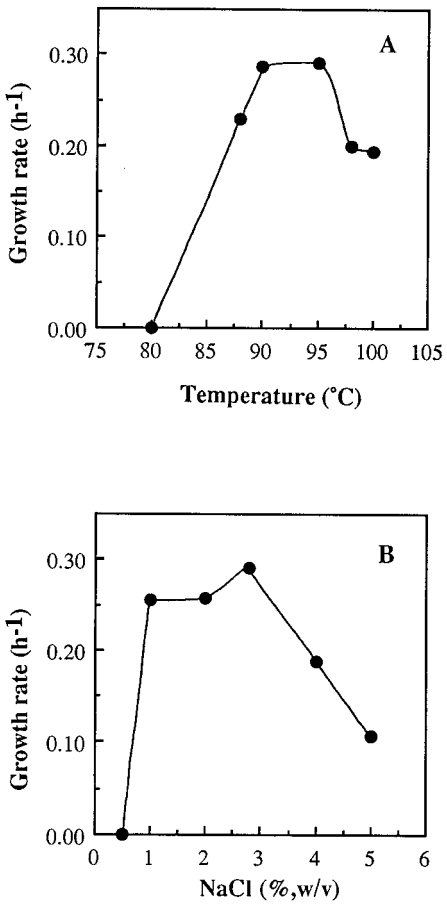

FIG. 1. Effects of temperature (A) and NaCl concentration (B) of growth medium on growth of *P. furiosus*.

Raven et al. (15) (Fig. 1A). Although lower specific growth rates were observed for cultures growing at 88 and 98°C, neither the lag phase nor the cell yield of the cultures was significantly affected. In contrast, at 100°C the cell yield was significantly lower, as evaluated either by turbidimetry or by total protein determination.

The effect of NaCl concentration on cell growth was also studied; *P. furiosus* showed a growth behavior typical of halophilic organisms, with no growth at low NaCl concentration (0.5%) and maximum growth rates at NaCl concentrations between 1.0 and 3.0% (Fig. 1B). At higher salinities, the specific growth rates decreased sharply; in 5.0% NaCl medium, cultures also showed a longer lag phase and the cell yield was 1.5-fold lower.

Identification of major intracellular solutes. Thick suspensions of *P. furiosus* grown at 95°C in 2.8% NaCl medium were analyzed by ^{31}P- and ^{13}C-NMR for the presence of major organic solutes in vivo. Spectra were dominated by resonances assigned to di-*myo*-inositol-phosphate by comparison with chemical shifts reported in the literature for the unusual inositol phosphodiester compound purified for the first time from *Pyrococcus woesei* and more recently also found in the hyperthermophile *Methanococcus igneus* (4, 18). ^{1}H- and ^{13}C-NMR analyses of ethanol extracts confirmed the assignment of these resonances. It was verified that the same isomer of di-*myo*-inositol-phosphate is synthesized by *P. furiosus* and *P. woesei*; this was done by adding small amounts of an ethanol extract of *P. woesei* and observing the effect on the ^{1}H-NMR spectrum of the *P. furiosus* extract.

^{1}H-NMR spectra of ethanol extracts (^{1}H projection in Fig. 2) also gave evidence for the presence of a second solute, which became predominant at high salinities (see below). The chemical shifts and the spin-spin splitting characteristics of the resonances due to this compound matched those recently observed by our research group for NMR spectra of ethanol extracts of *Rhodothermus marinus* and "*Thermus thermophilus*," in which the resonances were firmly assigned to 2-*O*-β-mannosylglycerate (14). The presence of this compound in *P. furiosus* cell extracts was confirmed by the addition of small amounts of an *R. marinus* ethanol extract and observation of the increased intensities of the corresponding resonances. Further confirmation for the assignments of both solutes was obtained from the ^{1}H-^{13}C connectivities observed in the heteronuclear multiple quantum correlation spectrum of an ethanol extract of cells grown in 4.0% NaCl, in which both major solutes occur in large amounts (Fig. 2).

Effects of temperature and NaCl on accumulation of intracellular solutes. The levels of intracellular solutes as functions of the growth temperature, NaCl concentration, and growth stage were quantified by ^{1}H-NMR with ethanol extracts. In order to check the reproducibility of the results, determinations of intracellular solutes were performed with three independent cell batches grown at 95°C and in 2.8% NaCl; the values for the replicates agreed within ±10%. The total amount of internal solutes increased remarkably at the temperatures and salinities above the optimum values for growth (Fig. 3 and 4). However, the nature of the solute accumulated was clearly dependent on the type of stress applied. For all the temperatures examined, di-*myo*-inositol-phosphate was the predominant solute in cells grown in 2.8% NaCl. With 95°C and 2.8% NaCl considered to be the optimum growth conditions for this medium, a 20-fold increase in the concentration of di-*myo*-inositol-phosphate (relative to that at 95°C) occurred at 101°C, whereas β-mannosylglycerate was not detected at this temperature or at 100°C. Conversely, β-mannosylglycerate was the major solute that accumulated intracellularly under salt stress (Fig. 4 and 5). In the medium containing 2.8% NaCl, the predominant solute found was di-*myo*-inositol-phosphate, but at higher salinities β-mannosylglycerate became the most abundant solute, despite the fact that the level of inositol phosphodiester also increased. At the highest salinity tested, 5.0% NaCl, β-mannosylglycerate represented 70% of the total solutes present and its concentration increased twofold compared with that under optimum growth conditions. Glutamate was found in reasonably high amounts (0.25 μmol mg of protein^{-1}) only in cells grown in 1.0% NaCl; for all the other media and temperature conditions tested, the level of glutamate was approximately 20 nmol mg of protein^{-1}.

The data presented above were obtained with cells harvested at the end of the exponential phase of growth. The quantification of internal solutes was also carried out with cells in the stationary phase, and the results shown in Fig. 4B illustrate the effects of salinity. The pattern is, in general, in agreement with that obtained for the exponential phase of growth. However, a clear decline in the amount of β-mannosylglycerate in the stationary phase was consistently observed.

Intracellular potassium determination. The potassium concentration in whole cells grown in medium containing 1.0 or 4.0% NaCl was measured by ^{39}K-NMR. Cells grown in 4.0% NaCl accumulated approximately 1.5 μmol of K^+ mg of protein^{-1}, and the amounts of di-*myo*-inositol-phosphate and β-mannosylglycerate determined in the same sample were 0.29 and 0.14 μmol mg of protein^{-1}, respectively. The intracellular

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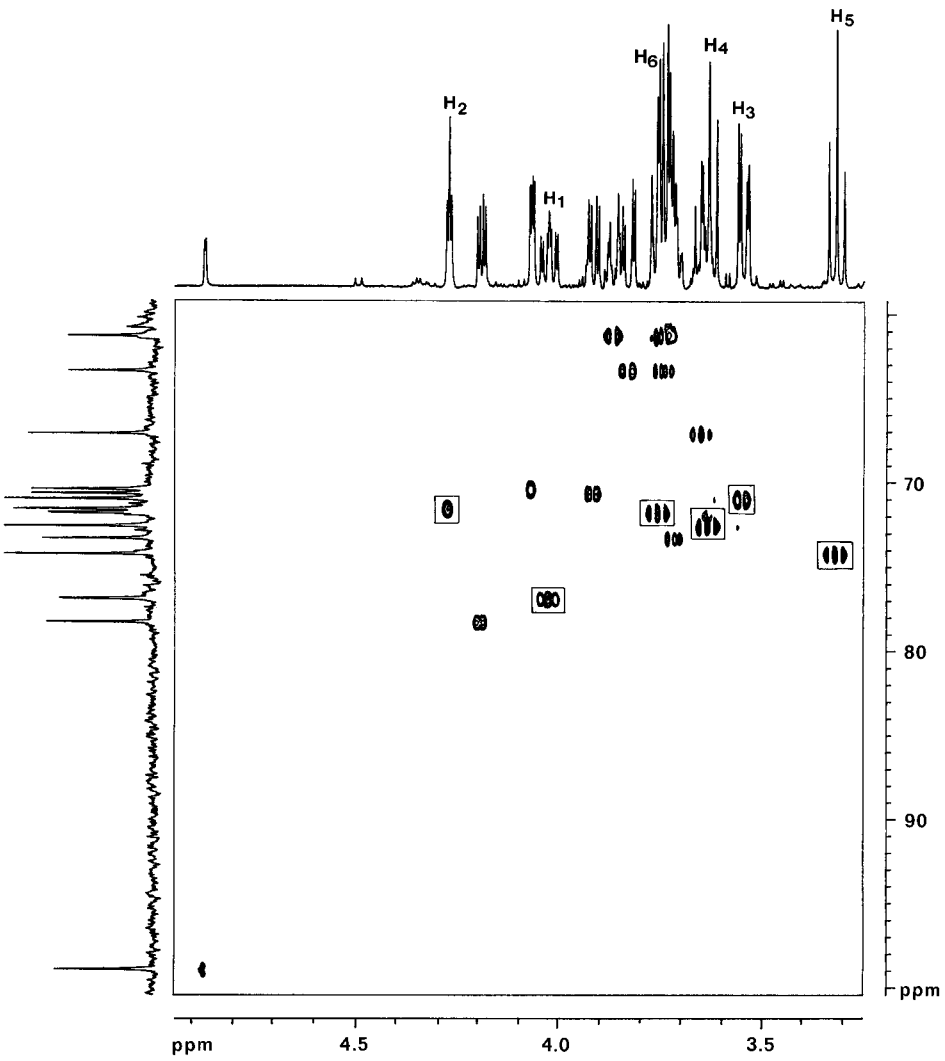

FIG. 2. ^{1}H-^{13}C correlation spectrum through one-bond coupling (heteronuclear multiple quantum correlation) of an ethanol extract of *P. furiosus* cells grown at 4% NaCl. Cross peaks representing connectivities between protons and carbon nuclei of di-*myo*-inositol-phosphate are enclosed in boxes.

volume of cells harvested at the end of the exponential phase of growth and cultivated at 95°C in the medium described by Raven et al. (15) was 4.5 ± 0.5 μl mg of protein^{-1}. This value represents the mean ± standard error of three independent determinations with different cell batches. Under the assumption that the intracellular volume of cells grown in 4% NaCl is not significantly different from that of cells grown in 2.8% NaCl, values of 330, 64, and 30 mM were calculated for the intracellular concentrations of K^+, di-*myo*-inositol-phosphate, and mannosylglycerate, respectively. The concentration of K^+ was significantly lower (0.3 μmol of K^+ mg of protein^{-1}) in cells grown in 1.0% NaCl, which accumulated 0.10 μmol of di-*myo*-inositol-phosphate mg of protein^{-1}. The intracellular concentration of K^+ in cells grown at 98°C in medium containing 2.8% NaCl was 3.0 μmol of K^+ mg of protein^{-1}.

DISCUSSION

Di-*myo*-inositol-phosphate and 2-*O*-β-mannosylglycerate were the major organic solutes accumulated in *P. furiosus*. The total intracellular organic solutes increased significantly in response either to an increase in temperature or to an increase in

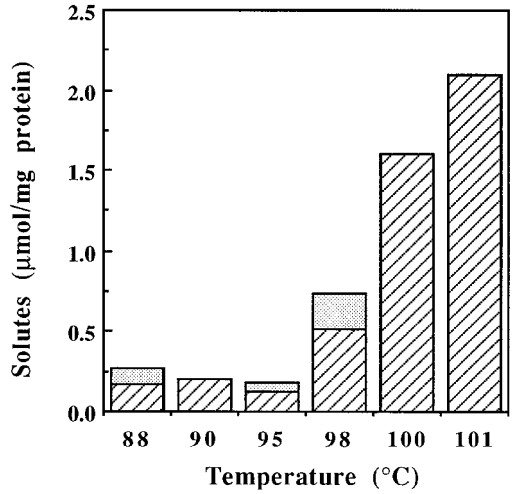

FIG. 3. Effects of growth temperature on accumulation of di-*myo*-inositol-phosphate (▨) and 2-*O*-β-mannosylglycerate (▩) by *P. furiosus* cells harvested at the end of the exponential phase of growth in medium containing 2.8% NaCl.

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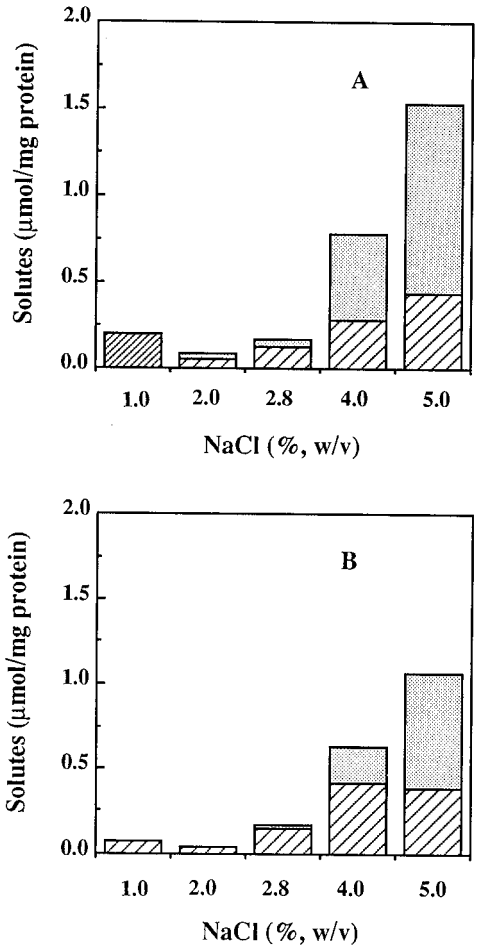

FIG. 4. Effects of NaCl concentration of growth medium on accumulation of di-*myo*-inositol-phosphate (▨), 2-*O*-β-mannosylglycerate (▩), and glutamate (▨) by *P. furiosus* cells harvested at the end of the exponential phase (A) or at the end of the stationary phase (B) of growth at 95°C. The levels of glutamate were not determined for the samples in panel B.

salinity, but β-mannosylglycerate accumulated mainly at high salinities, whereas the concentration of di-*myo*-inositol-phosphate increased dramatically at supraoptimal growth temperatures (Fig. 3 and 4). The level of di-*myo*-inositol-phosphate in *M. igneus* was also shown to respond primarily to an increase in the growth temperature, while β-glutamate was the major compatible solute that accumulated in response to salt stress (4).

Di-*myo*-inositol-phosphate was reported for the first time to occur in the hyperthermophilic archaeon *P. woesei* (18). The potassium salt of this organic anion was shown to have a thermostabilizing effect on glyceraldehyde-3-phosphate dehydrogenase from the same organism, and on the basis of these results, a role of this compound as a thermoprotector was suggested (18). Our data provide further support for the existence of a correlation between di-*myo*-inositol-phosphate and thermoprotection, and this hypothesis is reinforced by the recent detection of large amounts of this compound also in the hyperthermophilic bacterium *Thermotoga maritima* (13).

The accumulation of di-*myo*-inositol-phosphate in *P. furiosus* could be related to the synthesis of polar lipids containing *myo*-inositol-phosphate. In fact, these complex polar lipids are present in very large relative proportions in many archaea and are especially abundant in the species of the genus *Pyrococcus*, in which they reach 80 to 90% of the polar lipid fraction (7, 12).

β-Mannosylglycerate is a compatible solute found in thermophilic halophiles and halotolerant bacteria, such as *R. marinus*, "*Thermus thermophilus*," and *Rubrobacter* strains (14). A tentative finding of mannosylglycerate has been reported for *P. woesei* (9). In the present work, an unequivocal identification of this solute in *P. furiosus* was performed, and clear evidence supporting its role as the main osmolyte in this organism was obtained. The intracellular level of β-mannosylglycerate decreased significantly in the stationary phase of growth (Fig. 3); this effect cannot be attributed to cell lysis, since the level of di-*myo*-inositol-phosphate remained constant, and may indicate the utilization of β-mannosylglycerate by the cells in the late phase of growth.

The two major solutes found in the archaeon *P. furiosus* possess negative charges. It is interesting that charged organic solutes, such as β-glutamate, β-glutamine, cyclic 2,3-bisphosphoglycerate, and 1,3,4,6-hexanetetracarboxylic acid, are often found in methanogenic archaea, which are also capable of accumulating high concentrations of K^+ (8, 17); in general, bacteria preferentially accumulate neutral solutes (polyols, trehalose, sugar derivatives, amino acids, and amino acid derivatives) (5, 6). Our results show that the intracellular levels of K^+ in *P. furiosus* are dependent on the salinity of the medium and the temperature of growth and are high enough to counterbalance the negative charges of β-mannosylglycerate and di-*myo*-inositol-phosphate accumulated in the cell. As with *R. marinus* strains, the amount of positive charges due to K^+ exceeds the amount of negative charges in the organic solutes accumulated; chloride could be the anion required to fulfil the charge balance, but this hypothesis was not investigated.

The information available on the nature of compatible solutes in thermophilic halophiles is still limited, but it is worth noting that β-mannosylglycerate and di-*myo*-inositol-phosphate have been found only in thermophilic bacteria or archaea. The strong correlation between the accumulation of β-mannosylglycerate in response to salt stress in thermophiles and that in hyperthermophiles with no phylogenetic relationship suggests that this compatible solute may be better suited to play a role in osmoprotection in organisms that grow at high

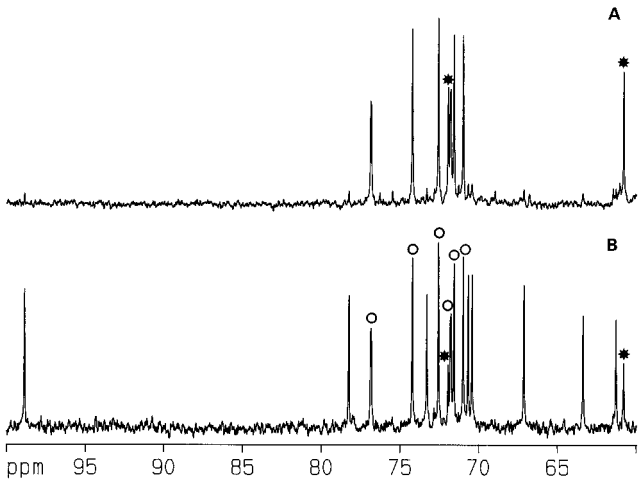

FIG. 5. Proton-decoupled ^{13}C-NMR spectra of ethanol extracts of *P. furiosus* grown in medium containing 2.8% NaCl (A) or 5.0% NaCl (B). Peaks due to di-*myo*-inositol-phosphate are labelled with open circles, and resonances due to 2-*O*-β-mannosylglycerate are not labelled. The asterisks indicate two resonances assigned to the contaminant diethylene glycol.

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temperatures than are other compatible solutes found in mesophilic organisms. There is also a clear correlation between temperature stress and the accumulation of di-*myo*-inositol-phosphate in *M. igneus* (4) and in *P. furiosus* (this work), which indicates that this solute could be necessary for growth at supraoptimal growth temperatures and may play a role in thermoprotection. A role in thermoadaptation has also been assigned to cyclic 2,3-bisphosphoglycerate, a solute that occurs in various amounts in several methanogenic genera of the archaea and acts as a thermostabilizer of enzymes in vitro (10).

Further research is in progress to determine how widespread the occurrence of β-mannosylglycerate and di-*myo*-inositol-phosphate among thermophilic organisms is and to test the efficiencies of these two solutes as stabilizing agents of macromolecules.

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