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New Compatible Solutes Related to Di-*myo*-Inositol-Phosphate in Members of the Order *Thermotogales*

LÍGIA O. MARTINS,[1] LAURA S. CARRETO,[2] MILTON S. DA COSTA,[2] AND HELENA SANTOS[1]

Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, 2780 Oeiras,[1] and Departamento de Bioquímica, Universidade de Coimbra, 3000 Coimbra,[2] Portugal

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The accumulation of intracellular organic solutes was examined in six species of the order *Thermotogales* by nuclear magnetic resonance spectroscopy. The newly discovered compounds di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate and di-*myo*-inositol-1,3′-phosphate were identified in *Thermotoga maritima* and *Thermotoga neapolitana*. In the latter species, at the optimum temperature and salinity the organic solute pool was composed of di-*myo*-inositol-1,1′(3,3′)-phosphate, β-glutamate, and α-glutamate in addition to di-*myo*-inositol-1,3′-phosphate and di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate. The concentrations of the last two solutes increased dramatically at supraoptimal growth temperatures, whereas β-glutamate increased mainly in response to a salinity stress. Nevertheless, di-*myo*-inositol-1,1′(3,3′)-phosphate was the major compatible solute at salinities above the optimum for growth. The amino acids α-glutamate and proline were identified under optimum growth conditions in *Thermosipho africanus*, and β-mannosylglycerate, trehalose, and glycine betaine were detected in *Petrotoga miotherma*. Organic solutes were not detected, under optimum growth conditions, in *Thermotoga thermarum* and *Fervidobacterium islandicum*, which have a low salt requirement or none.

Low-molecular-weight organic solutes accumulate to high levels in some microorganisms, where they generally serve as compatible solutes (osmolytes) in halotolerant or halophilic organisms or as carbon and energy reserves in many other organisms. Compatible solutes have a role in maintaining the appropriate turgor pressure and cell volume and permit normal enzyme activity under conditions of osmotic stress (4). With the exception of a few closely related anaerobic halophilic species of the domain *Bacteria* and the extremely halophilic species of the domain *Archaea* which accumulate K^+, Na^+, and Cl^- (30, 34), all other halotolerant and halophilic organisms examined accumulate organic compatible solutes under osmotic stress. These osmolytes include sugars and sugar derivatives, amino acids and amino acid derivatives, ectoine and hydroxyectoine, polyols, and small peptides (9).

Most studies on osmoregulation have been performed with mesophilic microorganisms, but several compatible solutes, some of which are unusual, have been identified in thermophilic and extremely thermophilic members of the domains *Archaea* and *Bacteria*. The bacterium *Rhodothermus marinus* accumulates the α- and β-isomers of mannosylglycerate, while trehalose and β-mannosylglycerate are the predominant compatible solutes in the bacterium "*Thermus thermophilus*" (25). The hyperthermophilic archaeon *Pyrococcus furiosus* also accumulates β-mannosylglycerate in response to salt stress (24), and other unusual compatible solutes, namely, β-glutamate, cyclic-2,3-bisphosphoglycerate, and 1,3,4,6-tetracarboxyhexane, have been detected in thermophilic and hyperthermophilic methanogens (7, 8, 19, 21).

It is also possible that some organic solutes play a role in the thermoprotection of macromolecules in thermophilic and hyperthermophilic organisms. Evidence for this is provided by the increase in the concentration of cyclic-2,3-bisphosphoglycerate in some thermophilic methanogens (13), and di-*myo*-inositol-1,1′-phosphate in the archaea *Methanococcus igneus* (8) and *P. furiosus* (24), in response to an increase in the growth temperature. Furthermore, in vitro experiments indicated that both compounds act as enzyme thermostabilizers (13, 32).

In order to explore biochemical features of thermoprotection and osmoprotection, we have recently examined the accumulation of organic solutes in response to growth temperature and salinity in the bacteria *R. marinus* and "*T. thermophilus*" (25) and the archaeon *P. furiosus* (24). Here we extend this investigation to the species of the order *Thermotogales*, which includes all the hyperthermophilic species described thus far within the domain *Bacteria*, with the exception of *Aquifex pyrophilus*, and constitutes a very ancient line of descent (38). The hyperthermophiles *Thermotoga maritima* and *Thermotoga neapolitana* have optimum growth temperatures of about 80°C and can grow at up to 90°C (14, 18), while *Thermotoga thermarum*, *Thermosipho africanus*, *Fervidobacterium nodosum*, and *Fervidobacterium islandicum* have optimum growth temperatures ranging between 65 and 75°C (15, 16, 27, 37). On the other hand, *Geotoga petraea*, *Geotoga subterranea*, and *Petrotoga miotherma* have significantly lower optimum growth temperatures of 45 to 55°C (10). The wide range of optimal growth temperatures found among the species of this order, as well as the very diverse range of halotolerance, makes the order *Thermotogales* an attractive group of organisms to pursue our current line of interest.

In this study we use nuclear magnetic resonance spectroscopy (NMR) to identify organic solutes in six species of the order *Thermotogales* and examine the effect of salinity and the growth temperature on the accumulation of intracellular organic solutes in *Thermotoga neapolitana*. An isomer of di-*myo*-inositol-phosphate and the solute di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate are described herein for the first time.

* Corresponding author. Mailing address: Instituto de Tecnologia Química e Biológica, Apartado 127, 2780 Oeiras, Portugal. Phone: 351 1 4426146. Fax: 351 1 4428766. Electronic mail address: santos @itqb.unl.pt.

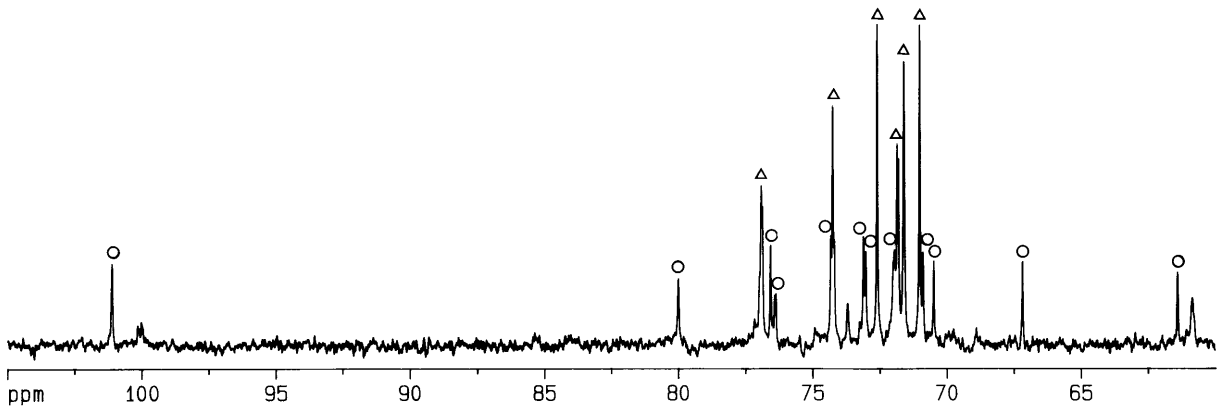


FIG. 1. Proton-decoupled ^{13}C NMR spectrum of an ethanol extract of *Thermotoga maritima* grown at 80°C in medium containing 2.7% NaCl. Resonances due to di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate are labelled with open circles, and resonances due to both isomers of di-*myo*-inositol-phosphate are labelled with open triangles.

MATERIALS AND METHODS

Strains and culture conditions. The type strains of *Thermotoga maritima* (DSM 3109^T), *Thermotoga neapolitana* (DSM 4359^T), *Thermotoga thermarum* (DSM 5069^T), *Thermosipho africanus* (DSM 5309^T), and *F. islandicum* (DSM 5733^T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. *P. miotherma* (ATCC 51224^T) was obtained from the American Type Culture Collection, Rockville, Md. Cultures of *Thermotoga maritima*, *Thermotoga neapolitana*, and *P. miotherma* were grown in the medium described by Huber et al. (14), supplemented with thiosulfate (20 mM) and biotin (20 μg/liter) to enhance cell yields and growth rates (6, 28). *Thermotoga thermarum* and *F. islandicum* were grown in the medium described by Windberger et al. (37) and Huber et al. (16), respectively. Cultures of *Thermosipho africanus* were grown in medium 141 (11), containing 1.0 g of $NaHCO_3$ per liter instead of 5.0 g/liter.

Cultures were routinely grown in a 2-liter fermentation vessel with continuous gassing with pure N_2 and stirred at 100 rpm. Cell growth was monitored by measuring the turbidity at 600 nm. Cells were harvested during the late exponential phase of growth by centrifugation (8,000 × *g*, 30°C for 15 min) and washed once with a NaCl solution identical in concentration to that of the medium in which the cells were grown. The effect of the salinity on the synthesis of intracellular solutes in *Thermotoga neapolitana* was examined in medium containing 1.5 to 4.7% (0.26 to 0.80 M) NaCl. To examine the effect of the growth temperature on the accumulation of intracellular solutes by *Thermotoga neapolitana*, cultures were grown between 65 and 88°C. The accumulation of organic solutes was examined in the other species at the optimum growth temperature and salinity.

Extraction of intracellular solutes, cell protein determination, and hydrolysis of the extracts. Cells were extracted twice with boiling 80% ethanol by the method of Reed et al. (29) slightly modified as previously described (24). NMR analysis of some extracts revealed high concentrations of polysaccharide, most probably starch from the growth medium, which in some cases would interfere with the identification of minor organic solutes. These extracts were eluted through a quaternary aminoethyl-Sephadex column equilibrated with NH_4HCO_3 buffer (5 mM, pH 8.0). The polysaccharide present in the extracts was eluted with water, and the low-molecular-weight solutes were then eluted with NH_4HCO_3 buffer (100 mM, pH 8.0). This fraction was freeze-dried and resuspended in a small amount of water, and the salt was removed by eluting the sample through a Sephadex G-25 column (PD-10; Pharmacia). The freeze-dried residue was dissolved in ^2H_2O for ^1H,^{13}C, and ^{31}P NMR analyses, and the final pH of the sample was adjusted to approximately 8 by the addition of NaO^2H.

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

Ethanol-soluble extracts were hydrolyzed with 6.0 N HCl at 100°C for 3 h under a N_2 atmosphere in sealed ampoules. The hydrolysates were dried under vacuum and dissolved in ^2H_2O for ^1H NMR.

NMR spectroscopy. All NMR spectra were acquired on a Bruker AMX500 spectrometer. ^{13}C NMR spectra were recorded at 125.77 MHz with a 5-mm carbon-selective probe head. Typically, spectra were acquired with a repetition delay of 5 s and a pulse width of 7 μs corresponding to 90° flip angle. Proton decoupling was applied during the acquisition time only, by 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.

^{31}P NMR spectra were recorded at 202.45 MHz, with or without proton broadband decoupling, with a repetition delay of 5 s and a pulse width of 16 μs corresponding to 60° flip angle. Chemical shifts were referenced with respect to external 85% H_3PO_4.

^1H NMR spectra were acquired with water presaturation, a 6-μs pulse width (corresponding to a 60° flip angle), and a repetition delay of 10 s. Formate was added as a concentration standard. Proton chemical shifts are relative to 3-(trimethylsilyl) propanesulfonic acid (sodium salt). Quantification of the organic solutes was based on ^1H and/or ^{31}P NMR spectra of ethanol extracts. Phase-sensitive nuclear Overhauser effect spectroscopy and proton-homonuclear shift correlation spectroscopy were performed by using standard Bruker pulse programs. Spectra were acquired over a 5-kHz bandwidth, collecting 4,096 (t_2) × 512 (t_1) datum points. ^1H-^{13}C heteronuclear multiple quantum coherence (HMQC) spectra (1) were acquired by collecting 4,096 (t_2) × 256 (t_1) datum points; 3.5 ms was used for evolution of $^1J_{CH}$. ^1H-^{31}P HMQC spectra (1) were acquired by collecting 4,096 (t_2) × 256 (t_1) datum points; 60 ms was used for evolution of $^3J_{PH}$. Spectra were run at 27 or 37°C.

^{39}K NMR spectra were obtained at 23.33 MHz as previously reported (24).

RESULTS

Identification of organic solutes in *Thermotoga maritima*. The ^{13}C NMR spectrum of an ethanol extract of *Thermotoga maritima* cells grown under optimal growth conditions was dominated by six resonances at 71.0, 71.6, 71.9, 72.6, 74.3, and 76.9 ppm (Fig. 1). These resonances were assigned to di-*myo*-inositol-1,1′-phosphate by comparison with the chemical shifts reported for this phosphodiester compound in *Pyrococcus woesei* (32), *M. igneus* (8), and *P. furiosus* (24).

However, the available NMR data are not sufficient to distinguish between the pair of enantiomers di-*myo*-inositol-1,1′-phosphate and di-*myo*-inositol-3,3′-phosphate; the signals could therefore arise from either enantiomer or a mixture of the two, and we shall refer to the compound as di-*myo*-inositol-1,1′(3,3′)-phosphate.

The proton spectrum of an ethanol extract of *Thermotoga maritima* confirmed the assignment made by ^{13}C NMR and also revealed the presence of a compound that appeared to be closely related to di-*myo*-inositol-1,1′(3,3′)-phosphate (Fig. 2). The ^{31}P NMR spectrum of the same ethanol extract showed only two intense resonances, at −0.69 and −0.81 ppm. However, the ^1H-^{31}P HMQC spectrum clearly showed that the resonance at −0.81 ppm was the result of two overlapping resonances (Fig. 3). Furthermore, one of the overlapping resonances correlated with the proton multiplet centered at 4.10 ppm, whereas the other correlated with the multiplet at 4.02 ppm. The remaining phosphorus resonance at −0.69 ppm correlated with a proton multiplet at 4.03 ppm which was firmly assigned to H_1 of di-*myo*-inositol-1,1′(3,3′)-phosphate by the addition of a small amount of a *P. furiosus* extract. Proton

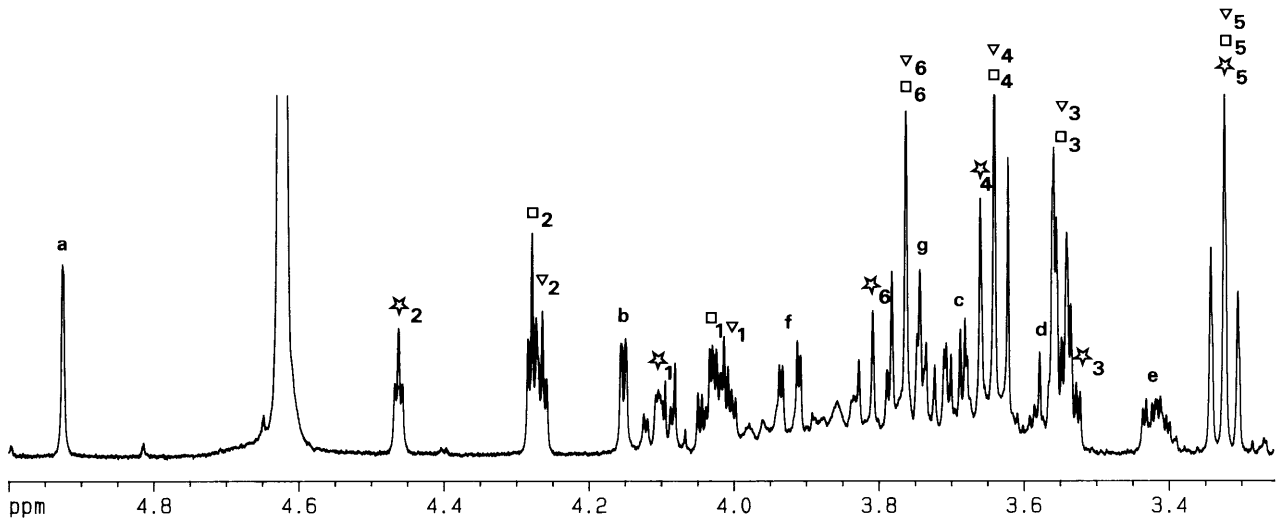


FIG. 2. ^{1}H NMR spectrum of an ethanol extract of *Thermotoga maritima* cells grown at 80°C and in medium containing 2.7% NaCl. Resonances due to protons of di-*myo*-inositol-1,1′(3,3′)-phosphate (□), di-*myo*-inositol-1,3′-phosphate (▽), and the inositol moiety of di-2-*O*-β-mannosyl-*myo*-inositol-1,1′(3,3′)-phosphate (☆) are labelled. Resonances due to the mannose moiety of di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate are designated a, b, c, d, e, f, and g representing H_1, H_2, H_3, H_4, H_5, H_6, and $H_{6'}$, respectively.

homonuclear correlation spectra (not shown) revealed the presence of a compound where resonances due to H_3, H_4, H_5, and H_6 of inositol overlapped the resonances of di-*myo*-inositol-1,1′(3,3′)-phosphate, while the resonances due to H_1 (at 4.02 ppm) and H_2 (at 4.26 ppm) were partially resolved from those of di-*myo*-inositol-1,1′(3,3′)-phosphate (H_1, 4.03 ppm; H_2, 4.28 ppm) (Fig. 2). ^{1}H-^{13}C-correlated HMQC spectra showed distinct resonances for C_1 and C_5 of these two compounds, whereas a single resonance was detected for each of the remaining carbon atoms in the inositol rings. In both compounds, resonances assigned to C_1 and C_6 were split because of coupling with phosphorus nuclei. Since the proton spin-spin coupling features were identical to those found for di-*myo*-inositol-1,1′(3,3′)-phosphate, the new resonances were as-

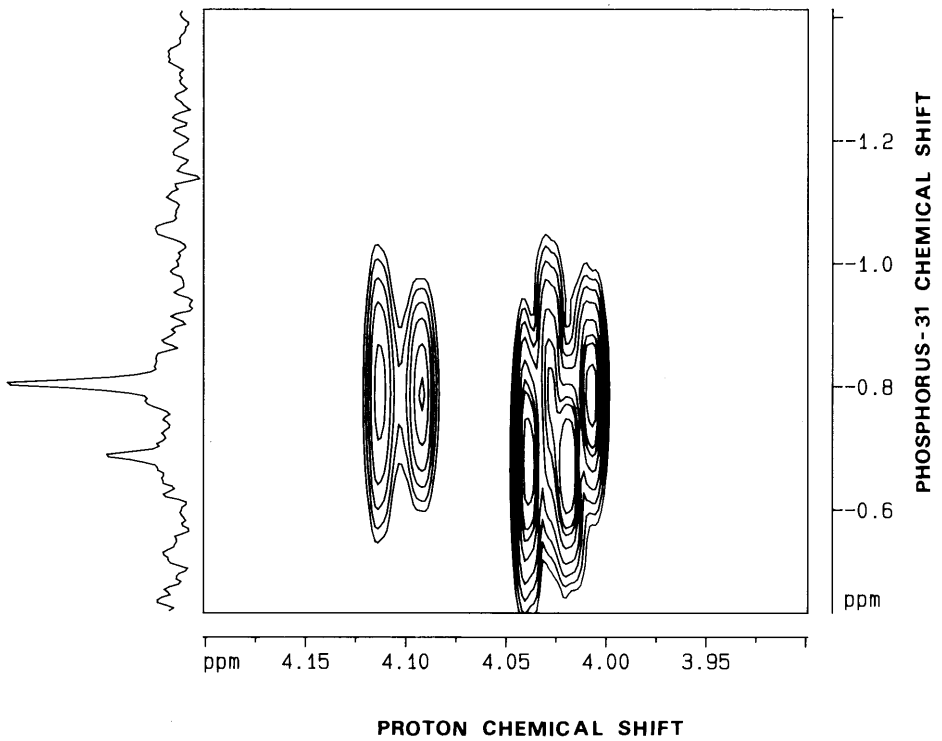


FIG. 3. ^{1}H-^{31}P correlation spectrum (HMQC) of an ethanol extract of *Thermotoga maritima* cells grown at 80°C. Cross peaks represent connectivities between proton and phosphorus nuclei of di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate (^{1}H, 4.10 ppm; ^{31}P, −0.81 ppm), di-*myo*-inositol-1,1′(3,3′)-phosphate (^{1}H, 4.03 ppm; ^{31}P, −0.69 ppm), and di-*myo*-inositol-1,3′-phosphate (^{1}H, 4.02 ppm; ^{31}P, −0.81 ppm).

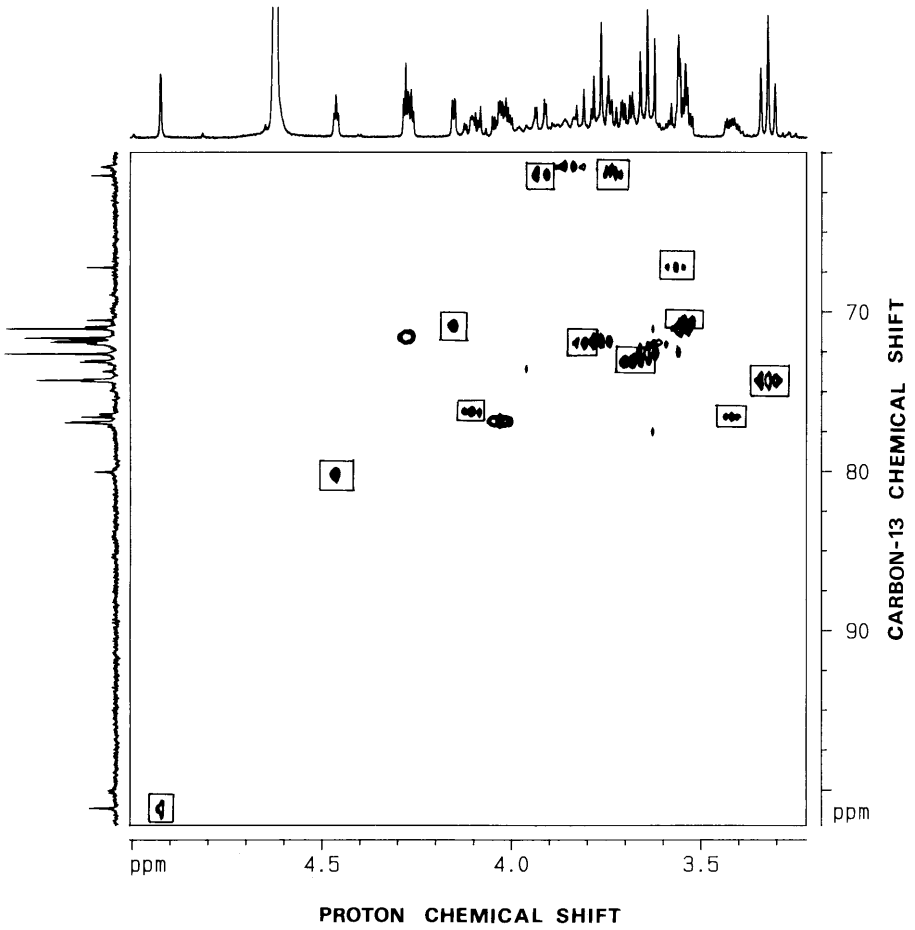


FIG. 4. ^{13}C-^{1}H correlation spectrum through one-bond coupling (HMQC) of an ethanol extract of *Thermotoga maritima* cells grown at 80°C. Cross peaks representing connectivities between proton and carbon nuclei of di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate are enclosed in boxes.

signed to the isomer of di-*myo*-inositol-phosphate with the phosphorus bridge positioned between carbon 1 of one inositol molecule and carbon 3 of the other. This compound will be designated di-*myo*-inositol-1,3′-phosphate.

The ^{13}C spectrum of the same ethanol extract also showed additional resonances at 101.1, 80.0, 76.6, 76.4, 74.3, 73.1, 73.0, 72.0, 70.9, 70.5, 67.2, and 61.4 ppm which could not be immediately assigned to a known compound (Fig. 1). The resonance at 101.1 ppm was assigned to an anomeric carbon on the basis of its chemical shift; proton coupling patterns, observed in ^{13}C NMR spectra acquired without proton broadband decoupling during the acquisition time, showed that the resonance at 61.4 ppm was due to a methylene group and the remaining 10 resonances were due to methine groups. Two of the resonances (at 76.4 and 72.0 ppm) were split because of phosphorus coupling. The sugar moiety was identified as mannose from the ^{1}H NMR spectra of acid hydrolysates spiked with the pure hexose.

The proton resonances due to mannose were easily identified from the connectivities in ^{1}H-^{1}H correlation spectra. Furthermore, the connectivity observed in the ^{1}H-^{31}P NMR correlation spectrum between the phosphorus resonance at −0.81 ppm and the proton multiplet at 4.10 (Fig. 3) allowed us to trace the resonances due to the inositol moiety in the ^{1}H-^{1}H correlation spectrum. Comparison of proton and phosphorus resonance intensities and analysis of ^{1}H-^{13}C-correlated HMQC spectra permitted the identification of di-2-*O*-β-mannosyl-di-*myo*-inositol-phosphate (Fig. 4). The position of the glycosidic bond, between the hydroxyl group of carbon 1 of mannose and carbon 2 of *myo*-inositol, was derived from a clear connectivity between H_1 of mannose and H_2 of inositol observed by nuclear Overhauser effect spectroscopy. The configuration of the anomeric carbon was also derived from the pattern of nuclear Overhauser effect connectivities between the anomeric proton of mannose and protons H_2, H_3, and H_5 in the mannose ring (not shown).

The molecular configuration corresponding to di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate has a C_2 symmetry axis, and therefore it is expected that equivalent carbon atoms in the two moieties should originate a single resonance; however, if this compound were derived from di-*myo*-inositol-1,3′-phosphate, the symmetry would be broken by the attachment of the mannose moieties, and distinct resonances could occur. In all spectral data, only one set of resonances was observed for both moieties of the molecule connected by the phosphate bridge, and therefore we propose the configuration designated di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate for the new compound. The carbon and proton chemical shift values of the identified phosphodiester compounds are summarized in Table 1, and a conformation of di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate is shown in Fig. 5. This compound will be designated throughout the text simply as di-mannosyl-di-*myo*-inositol-phosphate.

TABLE 1. NMR parameters of the phosphorous-containing solutes identified in *Thermotoga maritima* and in *Thermotoga neapolitana*

Moiety	Chemical shift(s) (ppm) of[a]:					
	DMDIP		DIP		$DIP_{1,3}$	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
Inositol						
C-1	d 76.4 J_{PC} = 6.1 Hz	4.10	d 76.9 J_{PC} = 6.6 Hz	4.03	d 77.0 J_{PC} = 6.6 Hz	4.02
C-2	80.0	4.46	71.6	4.28	71.6	4.26
C-3	70.5	3.54	71.0	3.55	71.0	3.55
C-4	73.0	3.66	72.6	3.64	72.6	3.64
C-5	74.3	3.32	74.3	3.33	74.2	3.33
C-6	d 72.0 J_{PC} = 6.1 Hz	3.81	d 71.9 J_{PC} = 6.6 Hz	3.76	d 71.9 J_{PC} = 6.6 Hz	3.76
Mannose						
C-1	101.1	4.93				
C-2	70.9	4.15				
C-3	73.1	3.69				
C-4	67.2	3.56				
C-5	76.6	3.42				
C-6	61.4	3.92, 3.73				

[a] DMDIP, di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate; DIP, di-*myo*-inositol-1,1′(3,3′)-phosphate; $DIP_{1,3}$, di-*myo*-inositol-1,3′-phosphate.

Organic solutes in other members of the order *Thermotogales*. The growth of *Thermotoga neapolitana* at the optimum temperature and salinity led to the accumulation of the same organic solutes detected in *Thermotoga maritima*, except for α-glutamate and β-glutamate, which were not present in the latter strain (Table 2). Intracellular organic solutes could not be detected in *Thermotoga thermarum*, grown at 70°C in medium containing 0.4% (0.07 M) NaCl, or *F. islandicum*, grown at 70°C in medium without NaCl. In *P. miotherma*, β-mannosylglycerate, trehalose, and glycine betaine were the predominant organic solutes during growth at 55°C in medium containing 3.0% (0.51 M) NaCl. The major organic solutes in *Thermosipho africanus* grown at 75°C in 1.9% (0.33 M) NaCl were proline, α-glutamate, and an as yet unidentified compound.

The effect of salinity and growth temperature on the accumulation of organic solutes in *Thermotoga neapolitana*. Since the cultures of *Thermotoga neapolitana* had higher growth rates and final yields than *Thermotoga maritima*, the former organism was used for the experiments on the effect of growth temperature and salinity on the accumulation of organic solutes. The optimum salinity for the growth of *Thermotoga neapolitana* was approximately 2.7% (0.46 M) NaCl, and the optimum growth temperature was about 80°C (growth rate of 0.24 H^{-1}). Alterations in the growth temperature and the salinity of the medium led to a decrease in the specific growth rate, but growth was possible at temperatures ranging between 65 and 88°C and salinities between 1.5 and 4.7% (0.26 and 0.80 M) NaCl.

At the lowest salinity for growth, the total organic solutes were present in low concentrations that did not exceed 0.3 μmol · mg of protein^{-1} (Fig. 6). At this salinity di-*myo*-inositol-1,1′(3,3′)-phosphate, di-*myo*-inositol-1,3′-phosphate, di-2-*O*-β-mannosyl-di-*myo*-inositol-phosphate, and α-glutamate were present in low but roughly equal amounts, while β-glutamate was undetectable. The increase in the salinity of the growth medium resulted in a progressive increase in the concentration of all of the solutes but was more pronounced with β-glutamate, which increased fourfold in medium containing 4.7% (0.80 M) NaCl compared with the level in optimal salinity conditions. However, di-myo-inositol-1,1′(3,3′)-phosphate was the major organic solute at high salinities, accounting for 38% of the total solute pool in medium containing 4.7% (0.80 M) NaCl.

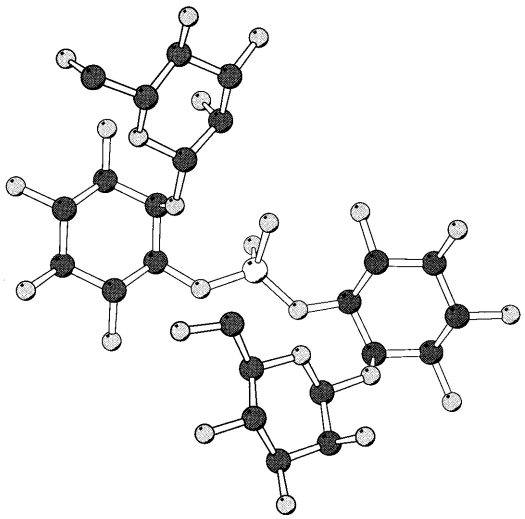
The growth temperature also exerted a profound effect on the concentration of organic solutes in *Thermotoga neapolitana*. The total pool of organic solutes increased about sevenfold in the growth temperature range tested (Fig. 6). The concentration of di-*myo*-inositol-1,1′(3,3′)-phosphate increased 7.6-fold between 65 and 88°C, but no significant increase was observed above the optimal growth temperature. In contrast, di-*myo*-inositol-1,3′-phosphate and di-mannosyl-di-*myo*-inositol-phosphate, not detected during growth at the lowest temperature, increased dramatically as the growth temperature was raised; in fact, they became the major solutes at supraoptimal growth temperatures and increased seven- and sixfold, respectively, when the temperature was raised by 8°C above the optimum for growth. On the other hand, β-glutamate, the major solute at 65°C, decreased to undetectable levels as the growth temperature was increased, while α-glutamate remained roughly constant throughout the growth temperature range.

Potassium levels were determined in intact cells grown at 80 and at 88°C in medium containing the optimum level of NaCl for growth. At these temperatures the K^+ levels reached 3.4 and 2.9 μmol · mg of protein^{-1}, respectively.

DISCUSSION

NMR led to the identification of the new solutes di-*myo*-inositol-1,3′-phosphate and di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate in *Thermotoga maritima* and *Thermotoga neapolitana*. These solutes, together with the previously described di-*myo*-inositol-1,1′(3,3′)-phosphate, belong to a class of organic solutes derived from *myo*-inositol-phosphate. Thus far, di-*myo*-inositol-1,1′(3,3′)-phosphate has been detected only in hyperthermophilic members of the *Archaea* (8, 24, 32), and this is therefore the first report on the occurrence of di-*myo*-inositol-1,1′(3,3′)-phosphate in members of the domain *Bacteria*. Moreover, this compound was found only in the two hyperthermophilic *Thermotoga* species and was also recently detected in *A. pyrophilus*, the only other hyperthermophilic bacterium described thus far (23).

This work reveals an important contribution of di-*myo*-inositol-1,1′(3,3′)-phosphate, di-*myo*-inositol-1,3′-phosphate, and di-mannosyl-di-*myo*-inositol-phosphate to the organic solute pool of hyperthermophilic *Thermotogales* species when subjected to temperature or salt stress. In fact, this family of



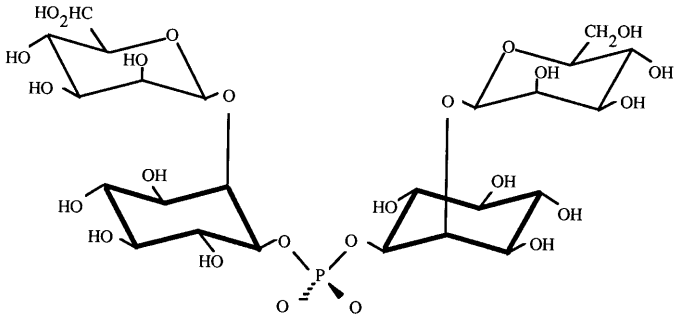


FIG. 5. A molecular representation (20) (left) and a stick structure (right) of di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate.

compounds accounts for 90 and 70% of the total solute pool of *Thermotoga neapolitana* at high growth temperature (88°C) or high salinity (4.7% NaCl), respectively.

A clear correlation between the accumulation of di-*myo*-inositol-1,1′(3,3′)-phosphate and temperature stress was demonstrated in *M. igneus* (8) and *P. furiosus* (24). On the other hand, the accumulation of di-*myo*-inositol-1,1′(3,3′)-phosphate at supraoptimal growth temperatures was replaced, in *Thermotoga neapolitana*, by the dramatic increase of di-*myo*-inositol-1,3′-phosphate and di-mannosyl-di-*myo*-inositol-phosphate, suggesting that this family of compounds has a role in thermoprotection of cellular components in hyperthermophilic species.

The unusual amino acid β-glutamate, previously found to serve as a compatible solute only in methanogens (7, 21, 31), was found to serve as a prominent osmolyte in *Thermotoga neapolitana*, increasing in concentration concomitantly with the NaCl concentration of the growth medium.

It is noteworthy that all compounds that accumulate in the hyperthermophilic *Thermotogales* species in response to either salt or temperature stress are charged while mesophilic bacteria accumulate primarily neutral compatible solutes (4, 9, 17). Moreover, the charged compound mannosylglycerate was detected in large amounts in the thermophilic species of the genera *Rhodothermus* and *Thermus* in response to saline stress. Also, the major organic solutes detected in hyperthermophilic members of the *Archaea* (7, 8, 13, 24, 31, 32) are charged, indicating further that a relationship may exist between the accumulation of charged compounds and high growth temperatures. In *Thermotoga neapolitana*, potassium is present in high intracellular levels exceeding the total negatively charged organic solutes and therefore can serve as a counterion for these compounds. This cation is also a counterion for negatively charged organic solutes in other halotolerant and halophilic members of the *Bacteria* and *Archaea* (9, 24, 25, 33).

The accumulation of phosphocompounds by microorganisms in stress responses deserves further comment; major organic phosphorus-containing compounds, such as phytic acid, can be used as phosphorus reserves in eukaryotes (22); 2-methyl-1,2,3,4-tetrahydroxybutane-2,4-cyclo-PP_i was found in *Desulfovibrio gigas* (36) and in *Brevibacterium ammoniagenes* (26), in which it was suggested to play a role as a bacterial oxidative antistress agent. Glycerol 1,2-cyclic phosphate has been found in high concentrations in centric diatoms, but its physiological role has not yet been elucidated (2). The potassium salt of cyclic-2,3-bisphosphoglycerate accumulates in thermophilic methanogens as the temperature is raised above the optimum for growth and could play a role in the thermostabilization of enzymes (13), but this compound has been reported to be an intermediate in an unusual branch of gluconeogenesis (12) and has also been detected in mesophilic methanogens (35). The occurrence in the order *Thermotogales* of high concentrations of phosphorus-containing solutes in response to a high temperature stress may therefore not be that unusual.

The two species *Thermotoga neapolitana* and *Thermotoga maritima* have the highest growth temperatures within the order *Thermotogales* and share the same organic solutes, probably because of their very close phylogenetic relationship (18). Different solutes were found in the other species examined: the

TABLE 2. Intracellular concentrations of organic solutes at the optimum growth temperature and salinity determined by ^{1}H NMR in the species of the *Thermotogales*

Species	Intracellular concn (μmol · mg of protein^{-1})[a] of:								
	DMDIP	DIP	$DIP_{1,3}$	α-Glutamate	β-Glutamate	Proline	β-Mannosylglycerate	Trehalose	Glycine betaine
Thermotoga maritima	0.16 (40)	0.48 (120)	0.20 (50)	—	—	—	—	—	—
Thermotoga neapolitana	0.09 (23)	0.33 (83)	0.12 (30)	0.19 (48)	0.05 (13)	—	—	—	—
Thermotoga thermarum	—	—	—	—	—	—	—	—	—
Thermosipho africanus	—	—	—	0.06 (15)	—	0.04 (10)	—	—	—
P. miotherma	—	—	—	—	—	—	1.23 (308)	1.18 (295)	0.92 (230)
F. islandicum	—	—	—	—	—	—	—	—	—

[a] Cell protein content in the samples for the determination of organic solutes ranged from 11.8 mg for *F. islandicum* to 32.9 mg for *Thermotoga neapolitana.* A crude estimation of the intracellular concentrations of solutes (millimolar) based on a cell volume of 2.2 μl · mg of dry mass^{-1} for *Escherichia coli* (5) is given in parentheses. The protein consent was assumed to be 55% of the cell dry mass. DMDIP, di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate; DIP, di-*myo*-inositol-1,1′(3,3′)-phosphate; $DIP_{1,3}$, di-*myo*-inositol-1,3′-phosphate; —, not detected.

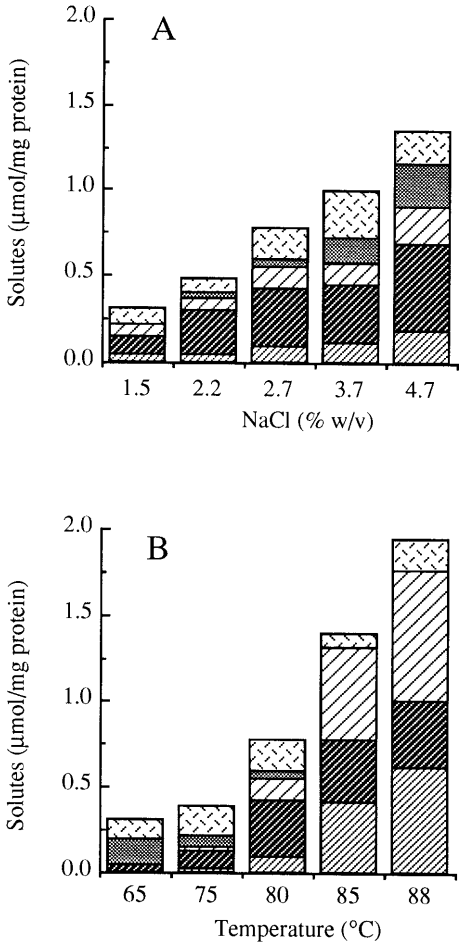


FIG. 6. Effect of the NaCl concentration in the growth medium at 80°C (A) and of the growth temperature in medium containing 2.7% NaCl (B) on the accumulation of di-2-*O*-β-mannosyl-di-*myo*-inositol-1,1′(3,3′)-phosphate (▨), di-*myo*-inositol-1,1′(3,3′)-phosphate (▨), di-myo-inositol-1,3′-phosphate (▨), α-glutamate (▨), and β-glutamate (▨) by *Thermotoga neapolitana* cells during the late exponential phase of growth. The growth rates at the optimum temperature (80°C) were 0.21 h^{-1} in 1.5% NaCl, 0.24 h^{-1} in 2.7% NaCl, and 0.15 h^{-1} in 4.7% NaCl. The growth rates at the optimum salinity (2.7% NaCl) were 0.09 h^{-1} at 65°C, 0.24 h^{-1} at 80°C, and less than 0.10 h^{-1} at 88°C.

predominant organic solute in the slightly thermophilic *P. miotherma* was β-mannosylglycerate. This solute has also previously been shown to serve as an osmolyte in thermophilic members of the *Bacteria* (25) and hyperthermophilic members of the *Archaea* (24). On the other hand, α-glutamate, proline, glycine betaine, and trehalose detected in *Thermotoga neapolitana*, *Thermotoga maritima*, *Thermosipho africanus*, and *P. miotherma* serve as compatible solutes in a wide range of unrelated mesophilic and moderately thermophilic members of the *Archaea* and *Bacteria* (9, 17, 25, 31, 33).

Our results indicate that the accumulation of solutes in response to either salt stress or supraoptimal temperatures is restricted, in the order *Thermotogales*, to the organisms that grow in saline media containing over 0.4% (0.07 M) NaCl, since *Thermotoga thermarum* and *F. islandicum*, with low salinity optima, do not accumulate organic solutes in detectable levels. It is possible therefore that a correlation exists between the accumulation of solutes and organisms that require saline conditions for growth. The data thus far available in the literature on organic solutes in thermophilic organisms suggest that temperature constrains the variety of solutes accumulated by these organisms (8, 24, 25, 32). This is conceivable, since some solutes could be better suited than others to stabilize cellular processes at high growth temperatures, but factors other than temperature may also be relevant.

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