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ORIGINAL PAPER

Di-*myo*-inositol phosphate and novel UDP-sugars accumulate in the extreme hyperthermophile *Pyrolobus fumarii*

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Abstract The archaeon *Pyrolobus fumarii*, one of the most extreme members of hyperthermophiles known thus far, is able to grow at temperatures up to 113°C. Over a decade after the description of this organism our knowledge about the structures and strategies underlying its remarkable thermal resistance remains incipient. The accumulation of a restricted number of charged organic solutes is a common response to heat stress in hyperthermophilic organisms and accordingly their role in thermoprotection has been often postulated. In this work, the organic solute pool of *P. fumarii* was characterized using ^{1}H, ^{13}C, and ^{31}P NMR. Di-*myo*-inositol phosphate was the major solute (0.21 μmol/mg protein), reinforcing the correlation between the occurrence of this solute and hyperthermophily; in addition, UDP-sugars (total concentration 0.11 μmol/mg protein) were present. The structures of the two major UDP-sugars were identified as UDP-α-GlcNAc3NAc and UDP-α-GlcNAc3NAc-(4 ← 1)-β-Glc*p*NAc3NAc. Interestingly, the latter compound appears to be derived from the first one by addition of a 2,3-*N*-acetylglucoronic acid unit, suggesting that these UDP-sugars are intermediates of an *N*-linked glycosylation pathway. To our knowledge the UDP-disaccharide has not been reported elsewhere. The physiological roles of these organic solutes are discussed.

Keywords Compatible solutes · Hyperthermophiles · *Pyrolobus fumarii* · *N*-Glycosylation · Di-*myo*-inositol phosphate

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Introduction

Nearly a decade ago, the scientific community was excited by the discovery of *Pyrolobus fumarii*, an archaeon able to grow at temperatures up to 113°C (Blöchl et al. 1997). This extreme hyperthermophile, isolated from the walls of a black smoker at the Mid-Atlantic Ridge, thrives at temperatures of superheated water (T_{op} 106°C) and will not grow below 90°C. The position of *P. fumarii* as the most extreme hyperthermophile known was contested by the report about an archaeon growing at up to 121°C (Kashefi and Lovley 2003). Nevertheless, *P. fumarii* remains the archetypal hyperthermophile living at the upper temperature and pressure limits for life.

The structural and metabolic features that allow extreme hyperthermophiles to grow under such aggressive conditions are not satisfactorily understood. There is no doubt that the vast majority of proteins from hyperthermophiles have enhanced intrinsic stability when compared to their mesophilic counterparts (Sterner and Liebl 2001; Vieille and Zeikus 2001). However, a growing body of evidence suggests that extrinsic factors have a role in the

stabilization of cellular components (Phipps et al. 1991; Takai et al. 1997; Ladenstein and Antranikian 1998; Daniel and Cowan 2000; Santos et al. 2007a). In particular, it has been shown that marine hyperthermophiles accumulate unusual organic solutes that are believed to be part of their strategy for adaptation to hot environments. Moreover, it appears that solutes of hyperthermophiles have specialized roles, i.e., while solutes such as mannosylglycerate and diglycerol phosphate are primarily involved in osmoprotection, di-*myo*-inositol phosphate (DIP) and derivatives are consistently associated with the heat stress response, and, therefore, are probably involved in thermoprotection.

We have examined the solute pool of several thermophiles and hyperthermophiles in order to investigate the chemical diversity and physiological roles of compatible solutes of organisms adapted to high temperature (Santos et al 2007a). However, progress is often limited by the poor growth of many hyperthermophiles and the need for peculiar culture conditions. Since *P. fumarii* is one of the most extreme hyperthermophiles known, we were particularly interested in determining the nature of the organic solutes accumulating in this organism.

Materials and methods

Culture conditions, extraction and quantification of intracellular solutes

P. fumarii cells were grown as previously described at 106°C in medium containing 1.7% NaCl (Blöchl et al. 1997). Cell mass was extracted twice with boiling 80% ethanol by the method of Reed et al. (1984) modified as previously described by Martins and Santos (1995). The freeze-dried extracts were dissolved in D_2O for NMR analysis. The protein content of the cells was determined by the Bradford assay (Bradford 1976).

Partial purification of the novel NDP-sugars

The NDP-sugars were purified from *P. fumarii* cell extracts by anion exchange chromatography. The sample was loaded onto a QAE-Sephadex column (Pharmacia, Uppsala, Sweden) equilibrated with 5 mM potassium carbonate (pH 8.2) and developed with a linear gradient of ammonium carbonate (5 mM to 1 M). The target compounds were detected by UV absorption at 280 nm taking advantage of the absorption band displayed by the NDP moieties. Fractions displaying UV absorption were analysed by silica TLC, using a mixture of chloroform:methanol:acetic acid:water (7.5:12.5:2:16). NDP-sugars were present in three of the fractions; these were desalted in an activated Dowex 50W-X8 column, freeze-dried, and dissolved in D_2O for later NMR analysis.

Nuclear magnetic resonance spectroscopy

All spectra were acquired on a Bruker DRX500 spectrometer. ^{1}H NMR spectra were acquired with water presaturation. Chemical shifts were relative to 3-(trimethylsilyl)propanesulfonic acid (sodium salt). For quantification purposes formate was added as an internal concentration standard and a repetition delay of 60 s was used for spectra acquisition. The resonance of proton 6 in the uracyl moiety was used for quantification of UDP-sugars and that of proton 5 in the inositol units was used for quantification of DIP. The results were confirmed by ^{31}P NMR using as concentration standard methylphosphonate contained in a capillary tube.

^{13}C and ^{31}P NMR spectra were recorded at 125.77 and 202.45 MHz, respectively, using 5 mm selective probe heads. In both cases proton decoupling was applied during the acquisition time only. Chemical shifts were referenced with respect to (trimethylsilyl)propanesulfonic acid for ^{13}C and external 85% H_3PO_4 for ^{31}P.

Two-dimensional spectra (COSY, NOESY, TOCSY, HMQC, and HMBC) were recorded using standard Bruker pulse programs. In the heteronuclear multiple quantum coherence spectra (HMQC), a delay of 3.5 ms was used for the evolution of $^{1}J_{CH}$ and in the heteronuclear multiple bond connectivity spectrum (HMBC) a delay of 73.5 ms was used for the evolution of long range couplings.

Mass spectrometry

Mass spectra were acquired on a LCQ advantage ion trap mass spectrometer from ThermoFinnigan (San Jose, CA, USA) equipped with an electrospray ionization interface operated in the negative mode. Samples were injected at 300°C and −33 V in 50% methanol/0.1% formic acid.

Results

^{1}H, ^{13}C and ^{31}P NMR spectral analysis of *P. fumarii* extracts revealed strong resonances immediately assigned to DIP by comparison with the spectra of DIP extracted from other sources (Rodrigues et al. 2007). Several unidentified compounds that displayed NMR features consistent with NDP-sugars were also present (Fig. 1). Quantification of organic solutes showed that DIP was the major solute (210 nmol/mg protein), while the total amount of NDP-sugars was 106 nmol/mg protein (Fig. 1). We

were unable to identify the unknown NDP-sugars by spiking the sample with common NDP-sugars or by resorting to NMR databases. Therefore, the sample was subjected to chromatographic steps to facilitate the structural characterization of the compounds.

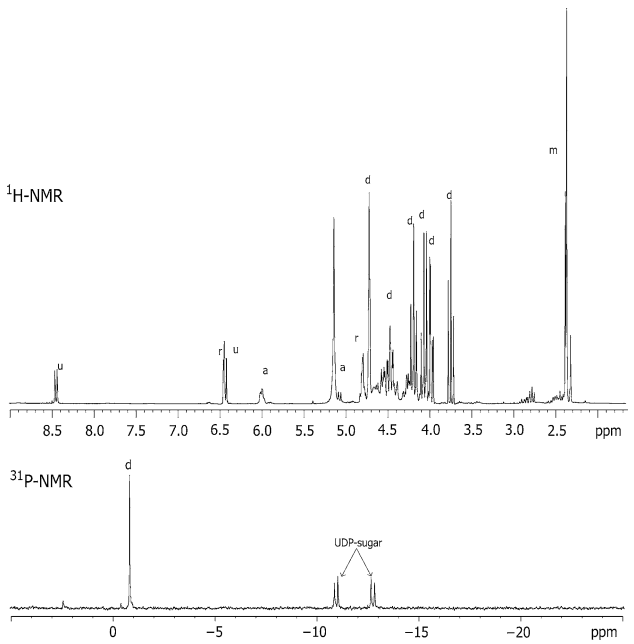

Fig. 1 ^{1}H and ^{31}P NMR spectra of an ethanolic extract of *Pyrolobus fumarii* in D_2O. The resonances assigned to di-*myo*-inositol phosphate are labelled with *d* resonances due to UDP-sugars are labelled with, *u* uracyl moieties, *r* ribosyl moieties, *a* anomeric protons of the glucosyl moieties, and *m* methyl groups

The two fractions derived from ion-exchange chromatography displayed identical ^{31}P NMR spectra with two doublet resonances centred at −10.9 and −12.7 ppm ($^{2}J_{PP}$ = 19 Hz) characteristic of diphosphodiesters. However, ^{1}H NMR showed that the chromatographic steps enabled the separation of two major compounds, both containing uridyl groups. In addition to all the NMR spectral features typical of UDP-sugars, the compounds originated extra resonances consistent with the presence of *N*-acetyl groups linked to the sugar unit. To prove the occurrence of these groups and establish their position, the solvent in the NMR samples was exchanged to 90% H_2O/10% D_2O; this led to the appearance of two extra doublet resonances in the amide region of the spectrum (7.95 and 8.15 ppm) obtained with the fraction eluted at the lowest ionic strength (compound A) and four extra similar resonances in the spectrum of the other fraction (compound B) (Fig. 2).

The structure of the molecules was established from sets of 2D-NMR spectra (COSY, TOCSY, ^{13}C–^{1}H HMQC, NOESY and ^{13}C–^{1}H HMBC). The spectra of compound A showed that the anomeric carbon at 94.9 ppm belonged to a hexose, while the anomeric carbon at 89 ppm belonged to the ribosyl moiety (Table 1). Analysis of the phosphorus–proton coupling pattern showed that carbon 5 of ribose and carbon 1 of the hexose were bound to the phosphate groups of the diphosphodiester bridge. The NOESY and ^{13}C–^{1}H HMBC experiments allowed identifying the positions of the two *N*-acetyl groups that are linked to C2 and C3 of the hexose moiety. Furthermore, the pattern of coupling constants (J_{HH}) and (J_{CH}) of the glycosyl moiety was characteristic of an α-glucopyranose configuration. Putting together all the NMR evidence, compound A was

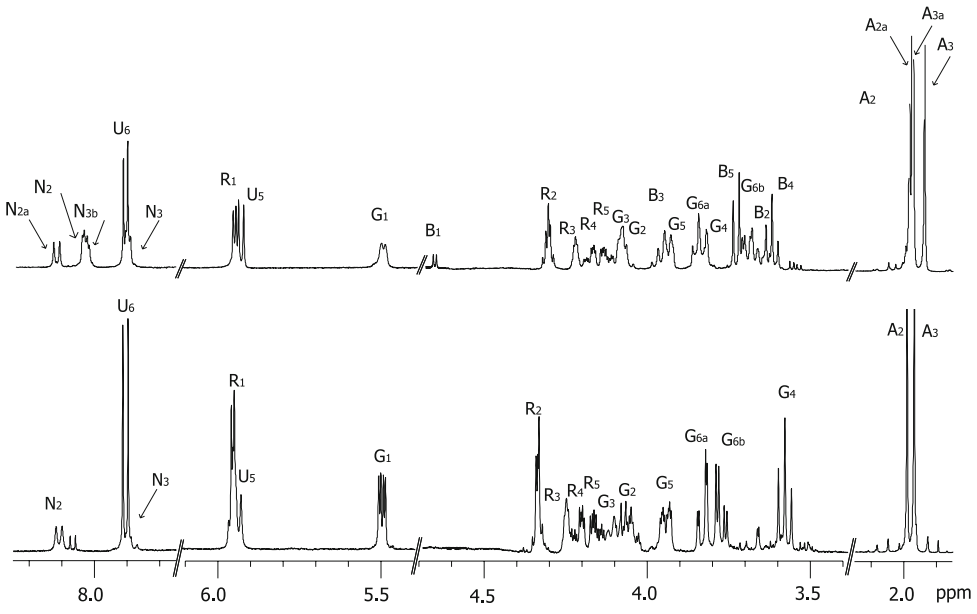

Fig. 2 ^{1}H NMR spectra of the two isolated UDP-sugars in 90% H_2O/10% D_2O. Peaks due to α-glucosyl, β-glucoronyl, ribosyl, uridyl, amide and acetyl groups are labelled with G, B, R, U, N and A, respectively. The subscripts refer to proton numbering

Table 1 NMR parameters of the two UDP-sugars identified in *Pyrolobus fumarii*

Moiety	UDP-α-GlcNAc3NAc			UDP-α-GlcNAc3Nac-(4 ← 1)-β-Glc*p*NAc3NAc		
	^{13}C NMR	^{1}H NMR		^{13}C NMR	^{1}H NMR	
	δ (ppm)	δ (ppm)	$^{n}J_{HH}$ (Hz)	δ (ppm)	δ (ppm)	$^{n}J_{HH}$ (Hz)
Uridyl						
U_5	103.06	5.93	$^{3}J_{5,6} = 8.0$	103.06	6.02	$^{3}J_{5,6} = 8.0$
U_6	142.15	7.92		142.15	7.92	
Ribosyl						
R_1	88.98	5.95	n.d.	88.75	6.04	n.d.
R_2	74.30	4.39	n.d.	74.05	4.42	n.d.
R_3	70.11	4.35	n.d.	70.23	4.42	n.d.
R_4	83.62	4.26	n.d.	83.67	4.33	n.d.
R_5	65.9	4.2, 4.18	n.d.	65.5	4.31, 4.22	n.d.
α-Glucosyl						
G_1	94.95	5.50	$^{3}J_{1,2} = 3.08$	94.04	5.56	$^{3}J_{1,2} = 2.46$
G_2	52.39	4.09	$^{3}J_{2,3} = 11.84$	52.22	4.17	$^{3}J_{2,3} = 9.87$
G_3	53.01	4.18	$^{3}J_{3,4} = 9.31$	51.15	4.18	$^{3}J_{3,4} = 9.18$
G_4	67.87	3.59	$^{3}J_{4,5} = 9.84$	75.27	3.92	$^{3}J_{4,5} = 10.92$
G_5	73.70	3.95	$^{3}J_{5,6a} = 2.46$	72.83	4.01	$^{3}J_{5,6a} = 2.46$
G_{6a}	60.88	3.85	$^{2}J_{6a,6b} = 12.46$	60.01	3.89	$^{2}J_{6a,6b} = 14.00$
G_{6b}	60.88	3.79	$^{3}J_{6b,5} = 4.31$	60.01	3.74	$^{3}J_{6b,5} = 4.11$
2-CH_3	22.20	1.98		22.20	1.98	
3-CH_3	22.54	1.96		22.54	1.96	
2-NH		8.16	$^{3}J_{NH} = 9.84$		8.07	$^{3}J_{NH} = 9.11$
3-NH		7.95	$^{3}J_{NH} = 9.84$		7.90	$^{3}J_{NH} = 8.83$
β-Glucoronyl						
B_1				102.15	4.67	$^{3}J_{1,2} = 8.18$
B_2				54.43	3.72	$^{3}J_{2,3} = 2.31$
B_3				55.04	4.03	$^{3}J_{3,4} = 9.73$
B_4				70.39	3.66	$^{3}J_{4,5} = 9.73$
B_5				78.33	3.79	
B_6				175.33		
2-CH_3				22.20	2.06	
3-CH_3				22.54	2.03	
2-NH					8.17	$^{3}J_{NH} = 9.74$
3-NH					7.95	$^{3}J_{NH} = 9.11$

n.d. not determined

identified as UDP-*N*-2,3-diacetamino-2,3-dideoxy-D-α-glucopyranose, from now on abbreviated as UDP-α-GlcNAc3NAc (Fig. 3).

A similar strategy was followed to identify compound B. Besides the anomeric ribosyl resonance (at 6.04 ppm) two other resonances were apparent in the region characteristic of anomeric protons (5.56 and 4.67 ppm). Moreover, the intensity of each of these three anomeric resonances was identical to that of the uracyl resonances, indicating that the activated sugar moiety in compound B was a disaccharide. The resonance at 5.56 ppm belonged to a spin system very similar to that observed for compound A (assigned to a glucosyl residue with two *N*-acetyl groups). The resonance at 4.67 ppm belonged to a spin system with five resonances in the aliphatic region. ^{13}C–^{1}H HMQC and HMBC experiments showed that the second sugar is a hexose with a carboxyl group at position 6, i.e., a glucoronyl residue, with two *N*-acetyl groups at positions 2 and 3. The HMBC spectrum also showed that the anomeric carbon of the glucoronyl moiety was linked to carbon 4 of the glucosyl moiety (Fig. 4). The analysis of the ^{1}H–^{1}H coupling constants revealed that both hexoses had the pyranose configuration, but the glucosyl unit had an α-pyranose configuration while the glucoronyl unit displayed a β-pyranose conformation. Finally, compound B was identified as UDP-*N*-2,3-diacetamino-2,3-dideoxy-D-α-glucopyranose-(4 ← 1)-β-2,3-diacetamido-2,3-dideoxy-β-glucoronic acid (Fig. 3), from

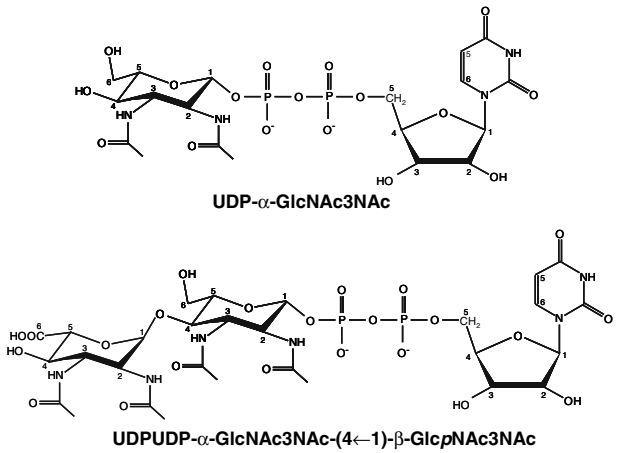

Fig. 3 Structures of the two UDP-sugars identified in *Pyrolobus fumarii*

now on abbreviated as UDP-α-GlcNAc3NAc-(4 ← 1)-*β*-Glc*p*NAc3NAc.

The molecular mass of compounds A and B were determined by mass spectrometry. Each sample originated only a major signal with an *m/z* value of 647.2 and 905.2, respectively, which are in excellent agreement with the structures proposed: the expected mass of UDP-α-GlcNAc3NAc is 647 g/mol and for the UDP-α-GlcNAc3NAc-(4 ← 1)-*β*-Glc*p*NAc3NAc is 905 g/mol.

The ratio of peak intensities in the spectra of the cell extract lead to the conclusion that compounds A and B appear in a relative proportion of 1.75:1.

Discussion

DIP is the major organic solute found in *P. fumarii*, one of the most extreme hyperthermophilic organisms known thus

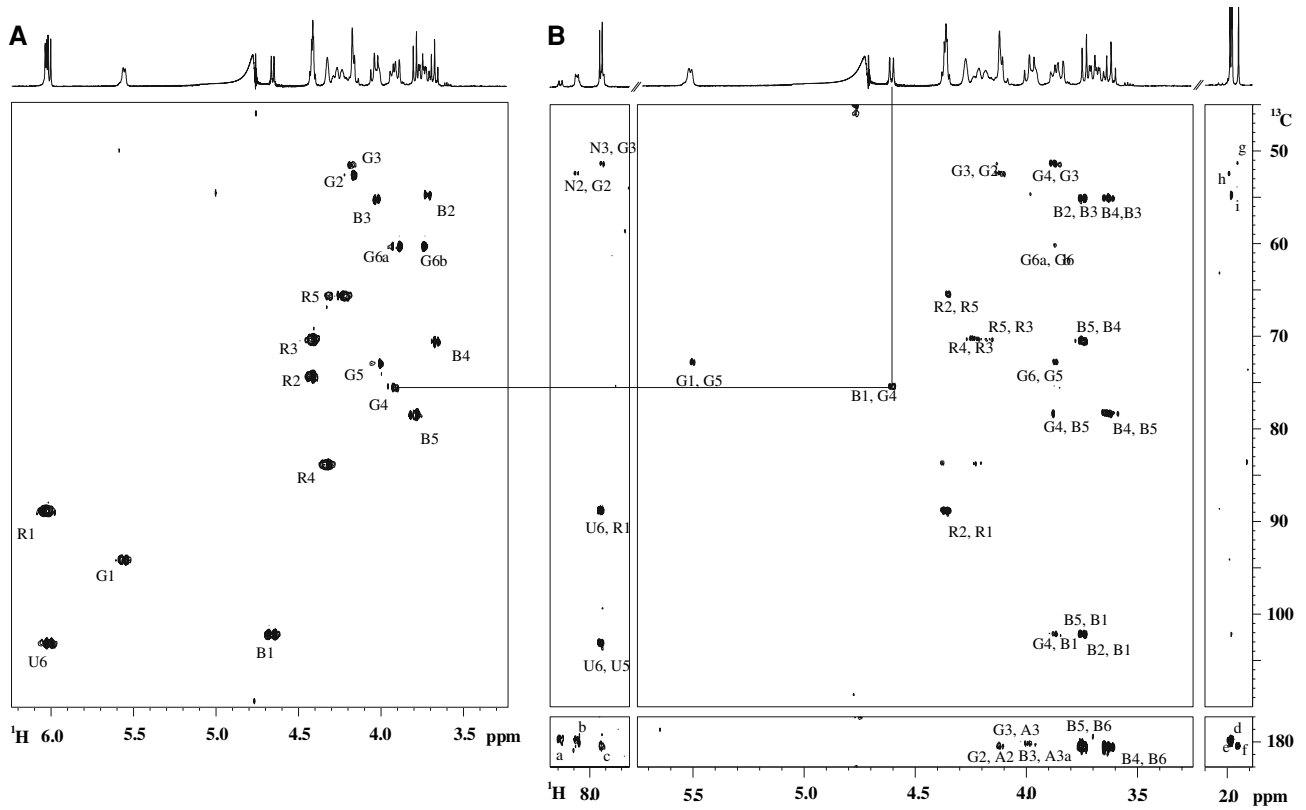

Fig. 4 A highlight of the ^{13}C–^{1}H HMBC (**a**) and the ^{13}C–^{1}H HMQC (**b**) of the UDP-α-GlcNAc3NAc-(4 ← 1)-*β*-Glc*p*NAc3NAc in 90% H_2O/10% D_2O. Peaks due to the α-glucosyl, *β*-glucoronyl, ribosyl, uridyl, amide and acetyl moieties are labelled with G, B, R, U, N, and A, respectively. A′ designates the methyl group in the *N*-acetyl moieties. The numbers refer to proton numbering in the molecule as in Fig. 3. *Cross-peaks* are labelled with the designations of the specific carbon and proton nuclei involved in the correlation. For example (*B1*, *G4*) represents the connectivity between proton 1 in the *β*-glucoronyl moiety and carbon 4 in the α-glucosyl moiety. *a* The 2-bond correlation between NH and the carbonyl of the acetyl group at position 2 of the B moiety, *b*, *c* the corresponding connectivity in the acetyl groups at positions 2 and 3 of the G moiety, respectively, *d* 2-bond connectivity between the methyl protons and the carbonyl of the *N*-acetyl group at position 2 of the B moiety, *e*, *f* 2-bond connectivities between the methyl protons and the carbonyl in the *N*-acetyl groups at positions 2, and 3 of the G moiety, respectively, *g*–*i* designate 4-bond connectivities between methyl protons and carbons G3, G2 and B3

far. The strong correlation between DIP and hyperthermophiles has been widely illustrated (Santos and da Costa 2002; Santos et al. 2007a). In fact, DIP is present in the large majority of marine hyperthermophiles, whether bacteria (Martins et al. 1996; Ramakrishnan et al. 1997a, b; Lamosa et al. 2006; Santos et al. 2007a) or archaea (Scholz et al. 1992; Ciulla et al. 1994; Martins and Santos 1995; Martins et al. 1997; Lamosa et al. 1998; Gonçalves et al. 2003; Santos et al. 2007a); on the other hand, it has never been found in organisms with optimal growth temperature below 60°C.

Hyperthermophiles appear to accumulate different solutes in response to different stresses: while mannosylglycerate, diglycerol phosphate and amino acids are preferentially accumulated in salt stress conditions, the level of DIP and DIP-derivatives respond to supra-optimal growth temperatures (Martins and Santos 1995; Martins et al 1997; Santos et al. 2007a). Moreover, in all organisms known to accumulate DIP, the level of this solute increases consistently in response to heat stress (Martins and Santos 1995; Martins et al. 1996; Martins et al. 1997; Ramakrishnan et al. 1997a; Gonçalves et al. 2003; Lamosa et al. 2006). Therefore, the presence of DIP as the major solute in *P. fumarii*, one of the most extreme hyperthermophiles described, further reinforces the view that DIP may play an important role in the adaptation of organisms to hot environments. At least in vitro, the ability of DIP to protect proteins against heat denaturation has been confirmed in several examples (Scholz et al. 1992; our unpublished results); in addition, the potential of DIP to prevent protein aggregation and fibril formation was demonstrated recently (Santos et al. 2007b).

Unexpectedly, relatively high amounts of UDP-sugars were also found in cell extracts of *P. fumarii*, but it is unlikely that these compounds play a role in osmo or thermo adaptation. Other hyperthermophilic archaea, e.g., *Pyrococcus furiosus* (Ramakrishnan et al. 1997a), *Methanothermus fervidus* (Hartmann and König 1989) and *Methanobacterium thermoautotrophicum* (Hartmann et al. 1990) accumulate substantial amounts of UDP-sugars, which are the precursors in the synthesis of different glycosylated structures, namely peptidoglycan, *S*-layer proteins and exopolysaccharides (Hartmann and König 1990; König et al. 1994; Parolis et al. 1999).

N-glycosylation is one of the glycosylation pathways present in archaea. This pathway starts with the activation of a monosaccharide by UTP. To the resulting UDP-sugar, new units are sequentially added to form a nucleotide activated oligosaccharide. The final oligosaccharide (that can comprise a varying number of monosaccharides) is transferred to a lipid carrier, dolicholphosphate or dolicholpyrophosphate, and finally to the NH group of an amino acid acceptor (Hartmann and König 1990; Eichler and Adams 2005). With this in mind, the simultaneous presence of an activated monosaccharide, UDP-α-GlcNAc3NAc, and the derivative, UDP-α-GlcNAc3NAc-(4 ← 1)-β-Glc*p*NAc3NAc, strongly suggests that these are intermediates of an *N*-glycosylation pathway in *P. fumarii*. The cell wall of *P. fumarii* is composed of a surface layer (*S*-layer) of tetrameric protein complexes (Blöchl et al. 1997), a common target for *N*-glycosylation in archaea (Claus et al. 2002, 2005). Therefore, it is tempting to speculate that the *S*-layer is the final target of this hypothetical pathway.

A curious feature of the two UDP-sugars characterized in this study is the presence of two acetamido groups, instead of the single one commonly found in living systems. To our knowledge, the activated disaccharide UDP-α-GlcNAc3NAc-(4 ← 1)-β-Glc*p*NAc3NAc has not been reported elsewhere. The hexose unit in UDP-α-GlcNAc3NAc is also very unusual, having been observed only in the hyperthermophilic bacterium *Aquifex pyrophilus* as a constituent of lipid A (Plötz et al. 2000). The second hexose moiety, β-Glc*p*NAc3NAc, although rare is more common, having been found in lipopolysaccharides of a few bacterial species (Shashkov et al. 1995; Wenzel et al. 2005), in an exopolysaccharide of *Haloferax denitrificans* (Parolis et al. 1999) and in a trisaccharide of *Methanococcus voltae* flagellin (Voisin et al. 2005).

In conclusion, this work shows that DIP is the major solute accumulating in the most hyperthermophilic organism described thus far; moreover the structures of two unusual UDP-sugars, probably involved in *N*-glycosylation pathways of this organism were characterized. A major research effort is needed to understand the mechanisms behind the amazing ability of *P. fumarii* to live on the edge.

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