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ORIGINAL PAPER

Organic solutes in *Rubrobacter xylanophilus*: the first example of di-*myo*-inositol-phosphate in a thermophile

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Abstract The thermophilic and halotolerant nature of *Rubrobacter xylanophilus* led us to investigate the accumulation of compatible solutes in this member of the deepest lineage of the Phylum *Actinobacteria*. Trehalose and mannosylglycerate (MG) were the major compounds accumulated under all conditions examined, including those for optimal growth. The addition of NaCl to a complex medium and a defined medium had a slight or negligible effect on the accumulation of these compatible solutes. Glycine betaine, di-*myo*-inositol-phosphate (DIP), a new phosphodiester compound, identified as di-*N*-acetylglucosamine phosphate and glutamate were also detected but in low or trace levels. DIP was always present, except at the highest salinity examined (5% NaCl) and at the lowest temperature tested (43°C). Nevertheless, the levels of DIP increased with the growth temperature. This is the first report of MG and DIP in an actinobacterium and includes the identification of the new solute di-*N*-acetylglucosamine phosphate.

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Introduction

Some microorganisms have developed specific adaptations to extraordinarily inhospitable environments; however, most organisms appear to react to stresses, within inherent limits, by mobilizing available resources crucial for their survival. Hyperosmotic shock, for example, induces the accumulation of small organic molecules, designated compatible solutes (Brown 1976). More rarely, potassium chloride is accumulated and in some cases potassium and negatively charged compatible solutes are both involved in osmotic adjustment (da Costa et al. 1998). Increasing evidence supports an additional talent of compatible solutes in the stabilization of the native folding of enzymes (Lamosa et al. 2000). Some compatible solutes, namely trehalose are also involved in the response to other stress conditions induced by temperature, desiccation, freezing, oxygen deprivation, nutrient starvation, toxic compounds and oxidative agents, and are viewed as general stress protectants (Hoelzle and Streeter 1990; da Costa et al. 1998; Santos and da Costa 2002; Elbein et al. 2003).

Trehalose, glycine betaine and glutamate are widespread in nature while other organic solutes are restricted to a few groups of organisms. Cyclic-2,3-bisphosphoglycerate (cBPG), for example, has been found only in methanogenic archaea (Roberts 2004); diglycerol phosphate (DGP) is restricted to *Archaeoglobus* spp. and di-*myo*-inositol-phosphate (DIP) has only been identified in hyperthermophilic archaea and bacteria which suggests a role in thermoprotection (Santos and da Costa 2002). Mannosylglycerate (MG), on the other hand, was initially discovered

in a group of red algae and later in several thermophilic bacteria and hyperthermophilic archaea where it behaves like a typical compatible solute, leading to the hypothesis that MG is an archetypal solute of prokaryotes living at high growth temperatures (Bouveng et al. 1955; Santos and da Costa 2002). However, the accumulation of MG in marine red algae and the discovery of genes for the synthesis of MG in mesophilic bacteria and in archaeal metagenomes obtained from low temperature environments argue against the hypothesis of a role of MG exclusively in the adaptation to thermal environments (Empadinhas and da Costa 2006).

The bacteria of the genus *Rubrobacter* represent the most ancient lineage of the phylum *Actinobacteria* (High G + C Gram-positive bacteria) known and are assigned to three known species, namely *R. radiotolerans*, *R. taiwanensis* and *R. xylanophilus* with optimum growth temperatures of 45, 55, and 60°C, respectively (Carreto et al. 1996; Chen et al. 2004; Suzuki et al. 1988). All strains examined of *Rubrobacter* are gamma-radiation resistant and *R. xylanophilus* is, not only the most thermophilic actinobacterium known, but also the only true extremely radiation-resistant thermophile (Suzuki et al. 1988; Ferreira et al. 1999; Chen et al. 2004). *R. xylanophilus* is also halotolerant growing in medium containing up to 6% NaCl. Because it is not common to find an organism with these characteristics, it was deemed important to investigate the effect of salinity and temperature on the diversity and on levels of compatible solutes.

Materials and methods

Strains and culture conditions

The type strain of *R. xylanophilus* PRD-1^T (=DSM 9941^T) was from our collection. The organism was grown aerobically in *Thermus* medium (Williams and da Costa 1992) containing tryptone (1.0 g L^{-1}) and yeast extract (1.0 g L^{-1}), and in a minimal medium (M medium) containing basal salts of Degryse medium 162 (Degryse et al. 1978), yeast extract (0.1 g L^{-1}), glucose (5.0 g L^{-1}) and NH_4Cl (2.0 g L^{-1}); the pH was adjusted to 8.2. To examine the effect of osmotic stress on the accumulation of intracellular solutes, cells were grown at 60°C in medium with 2.5, 4.0 or 5.0% NaCl (w/v), or without NaCl. Cultures were also grown at 43, 50, 60 and 67°C in medium without NaCl to examine the effect of the growth temperature on the accumulation of intracellular solutes. Growth of the organisms was performed in 1-liter metal-capped Erlenmeyer flasks containing 200 ml of medium in a reciprocal-water bath shaker (120 rpm) and monitored by measuring the turbidity of the cultures at 600 nm. Cells were harvested during the mid-exponential phase of growth by centrifugation (8,000 × *g*, 4°C for 10 min) and washed twice with a NaCl solution identical in concentration to that of the medium in which the cells were grown.

Extraction of organic solutes

Cell pellets were extracted twice with boiling 80% ethanol and the extracts were freeze-dried as described previously (Silva et al. 1999). The dry weight of cells was determined by filtering cell suspensions under vacuum, through dried pre-weighted 0.22 µm pore size filters (Gelman). The filters were dried for 24 h at 70°C and weighted again.

Purification of the new phosphorylated sugar compound

Ethanol extracts of *R. xylanophilus* cells containing the unidentified compound were loaded on to a QAE-Sephadex column (Pharmacia, Uppsala, Sweden) equilibrated at pH 9.8 with 5 mM sodium carbonate buffer. The sample was eluted applying a linear gradient between 5 mM and 1 M of $NaHCO_3$ buffer. Fractions were collected and analyzed by NMR. The fractions containing the new compound were pooled, the buffer removed in an activated Dowex 50 W-X8 column and freeze dried. The purest sample was dissolved in a mixture of dichloromethane: methanol: 25% ammonia (6:10:5, v/v/v), centrifuged and the supernatant applied to a silica gel column equilibrated with the same solvent. After removing the solvent by evaporation, the fractions were analyzed by NMR.

NMR spectroscopy

Freeze-dried extracts were analyzed by nuclear magnetic resonance (NMR) as described previously (Silva et al. 1999). The quantification of solutes was determined by comparing the intensities of resonances of the compounds in the sample with the one from a concentration standard, in our case formate. All spectra were acquired on a Bruker DRX500 spectrometer. For quantification purposes, ^{1}H-NMR spectra were acquired with water presaturation, 6 µs pulse width (60° flip angle) and a repetition delay of 60 s. Chemical shifts were relative to 3-(trimethylsilyl)propanesulfonic acid (sodium salt). Formate was added as an internal concentration standard. ^{1}H-NMR spectra were also acquired with selective irradiation of the phosphorous signal at –2.73 ppm.

^{31}P-NMR spectra were recorded at 202.45 MHz using a broadband inverse detection 5-mm probe head. Broadband proton decoupling was applied during the acquisition time only, using the wideband alternating-phase low-power technique for zero-residue splitting sequence (WALTZ);

for selective irradiation, spectra were acquired with continuous irradiation of the desired proton signal.

Two-dimensional spectra were performed using standard Bruker pulse programs. Proton-homonuclear shift correlation spectroscopy (COSY) and total-correlation spectroscopy (TOCSY) were acquired collecting $4096(t_2) \times 512(t_1)$ data points; while in ^{1}H-^{13}C and ^{1}H-^{31}P heteronuclear multiple quantum coherence spectra (HMQC) $4096(t_2) \times 256(t_1)$ data points were collected (Bax and Summers 1986). Delays of 3.5 ms and 65 ms were used for evolution of $^{1}J_{XH}$ in the carbon and phosphorous correlation spectra, respectively.

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 at –33 V in 50% methanol/0.1% formic acid.

High-performance liquid chromatography (HPLC)

Amino acids were analyzed in a Gilson-ASTED system according to the manufacturer's instructions. The amino acid derivatives resulting from the pre-column derivatization with orthophthaldeyde/2-mercaptoethanol were separated on a Spherisorb ODS column (particle size, 5 μm; 150 mm long, 4.6 mm ID), at a flow rate of 2.5 mL/min, using a ternary solvent system: buffer A (250 mM sodium phosphate, 15%; 200 mM propionic acid, 20%; acetonitrile, 7%; DMSO, 3%; pH 6.2); buffer B (acetonitrile, 40%; methanol, 33%; DMSO, 7%); and buffer C (250 mM sodium phosphate, 25%; 250 mM propionic acid, 20%; acetonitrile, 7%; DMSO, 3%; pH 5.5). The effluent was monitored by a fluorescent detector (Gilson, model 121; excitation and emission wavelengths at 340 and 410 nm, respectively). The Gilson system software performed the integration of the amino acid peak area and further calculations, and quantification was allowed by running standard amino acids solutions in the same conditions.

Results

Identification and quantification of the organic solutes

^{1}H-NMR spectra of the cell extracts were recorded and the signals compared with those from the literature. This analysis allowed the detection of mannosylglycerate (MG), and trehalose, as major compounds, as well as lower amounts of glycine betaine, di-*myo*-inositol-phosphate (DIP) and very low levels of an unidentified compound with a signal in the region characteristic of the anomeric protons of hexoses. The analyses of the ^{31}P-NMR spectrum revealed the presence of a resonance assigned to DIP and another signal at –2.73 ppm that could not be immediately assigned and was therefore suspected to belong to the same unidentified compound. Due to the small relative amount of the new solute, we resorted to chromatography to purify it. The first chromatographic step gave origin to two partially purified fractions, one containing the new solute and DIP, and another one with the new compound, as well as DIP and MG. Since the purification was followed by NMR it was possible to observe that the relative intensities of the anomeric signal in the ^{1}H and the unidentified ^{31}P resonance was constant in all the samples, strongly supporting the view that the new sugar compound was phosphorylated. This assumption was confirmed by the acquisition of a ^{1}H-^{31}P correlation HMQC spectrum that revealed a strong signal between the phosphorous and the anomeric signal at 5.46 ppm, and a lesser signal at the same phosphorous resonance to a proton signal at 3.99 ppm, interpreted as the proton at position 2 of the sugar moiety. This interpretation was confirmed by a homonuclear proton correlation spectrum (COSY) (Fig. 1). The structure of the phosphorous signal is that of a triplet of triplets with coupling constants of J_{PH} 7.44 and 3.10 Hz, indicating that it is split by two groups of two protons. Since the HMQC showed connectivities only to positions 1 and 2 of the sugar moiety, there has to be two sugar moieties splitting the phosphorous resonance i.e. the molecule is a symmetric di-sugar phosphodiester. This deduction was confirmed by the acquisition of phosphorous spectra with selective irradiation of the sugar protons at positions 1 and 2 giving rise to distinct triplets, and by the fact that the proton/phosphorous relative intensities of the new compound were the same as DIP (a phosphodiester).

The fraction containing only the new solute and DIP was applied to a silica gel column that separated the two compounds. The ^{1}H spectrum of this sample revealed a singlet at 2.06 ppm with a 3:1 intensity ratio to the anomeric signal, which led us to suspect the presence of an *N*-acetyl substituent. To confirm this hypothesis, the solvent in the NMR sample was exchanged from 100% D_2O to 10% D_2O leading to the appearance of one extra proton resonance in the amide region of the spectrum. The COSY spectrum (Fig. 1) permitted to assign all proton resonances of the hexose and showed that the acetyl group was bound to position 2. The acquisition of a ^{1}H-^{13}C HMQC allowed the assignment of the carbon resonances (Table 1). Analysis of the coupling constants (Table 1) revealed that the hexose was glucose in the α-pyranosyl configuration, therefore, establishing the structure of the new compound as di-*N*-acetyl-glucosamine phosphate (Fig. 1). This identification was further confirmed by mass spectrometry. The

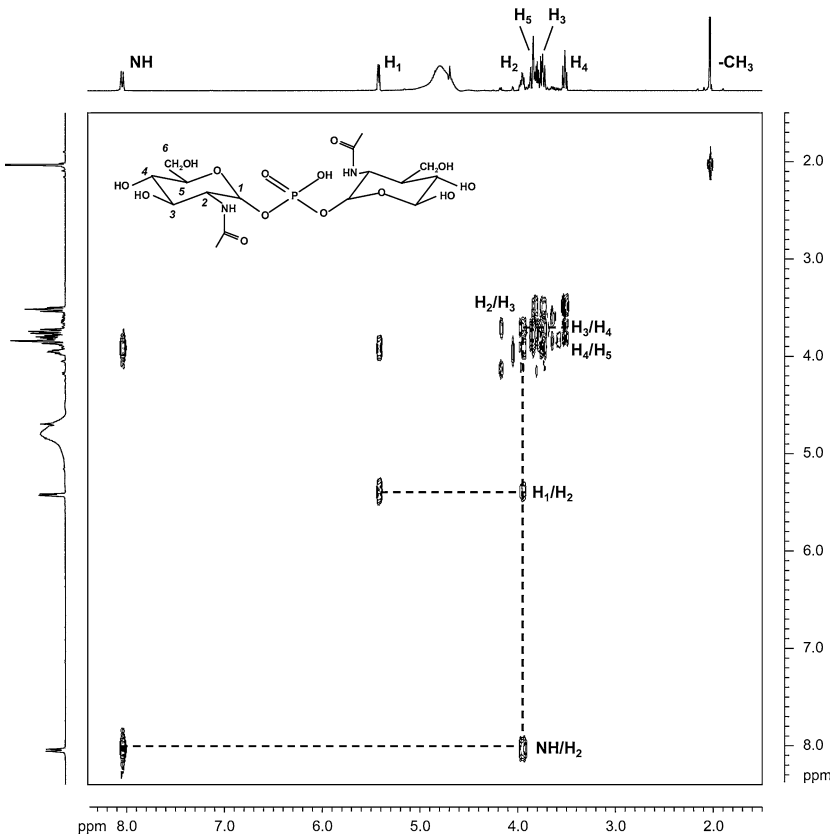

Fig. 1 Proton homonuclear correlation spectrum (COSY) of the new compound in 90% H_2O. A schematic representation of the new phosphodiester compound, di-*N*-acetyl-glucosamine phosphate (DAGAP), is depicted as an *inset*

Table 1 NMR parameters of di-*N*-acetyl-glucosamine phosphate

Moiety	^{13}C-NMR	^{1}H-NMR		^{31}P-NMR	
	δ (ppm)	δ (ppm)	$^{3}J_{HH}$ (Hz)	δ (ppm)	$^{3}J_{PH}$ (Hz)
Glucosyl					
C_1	100.38	5.46	$^{3}J_{1,2}$ = 3.42		$^{3}J_{H1}$ = 7.44
C_2	59.87	3.99	$^{3}J_{2,3}$ = 9.54		$^{3}J_{H2}$ = 3.10
C_3	76.81	3.77	$^{3}J_{3,4}$ = 10.03		
C_4	75.83	3.55	$^{3}J_{4,5}$ = 9.29		
C_5	79.27	3.86	ND		
C_6	66.74	3.88; 3.81	ND		
Amine		8.09	$^{3}J_{NH,2}$ = 9.05		
Methyl	28.20	2.06			
Phosphorous				–2.73	

ND not determined

mass spectrum of the pure compound revealed a signal with a m/z of 503.9 corresponding to the expected mass of di-*N*-acetyl-glucosamine phosphate.

Effect of salinity and temperature on the growth rate and on the accumulation of intracellular organic solutes

The organism was halotolerant, with an optimal growth rate in medium without added NaCl, but was able to maintain consistent growth in media containing up to 5% NaCl. The organism had an optimum growth around 60°C, growing at temperatures as low as 43°C and as high as 67°C. In the M medium the growth rates were slightly lower than those obtained in *Thermus* medium and decreased more rapidly at supra-optimal salinity and temperature (Fig. 2). Moreover, growth could not be measured in M medium at 43°C (Fig. 2d).

The total intracellular solute pools in cells grown in M medium were higher than those measured in cells grown in *Thermus* medium, under all conditions tested, but

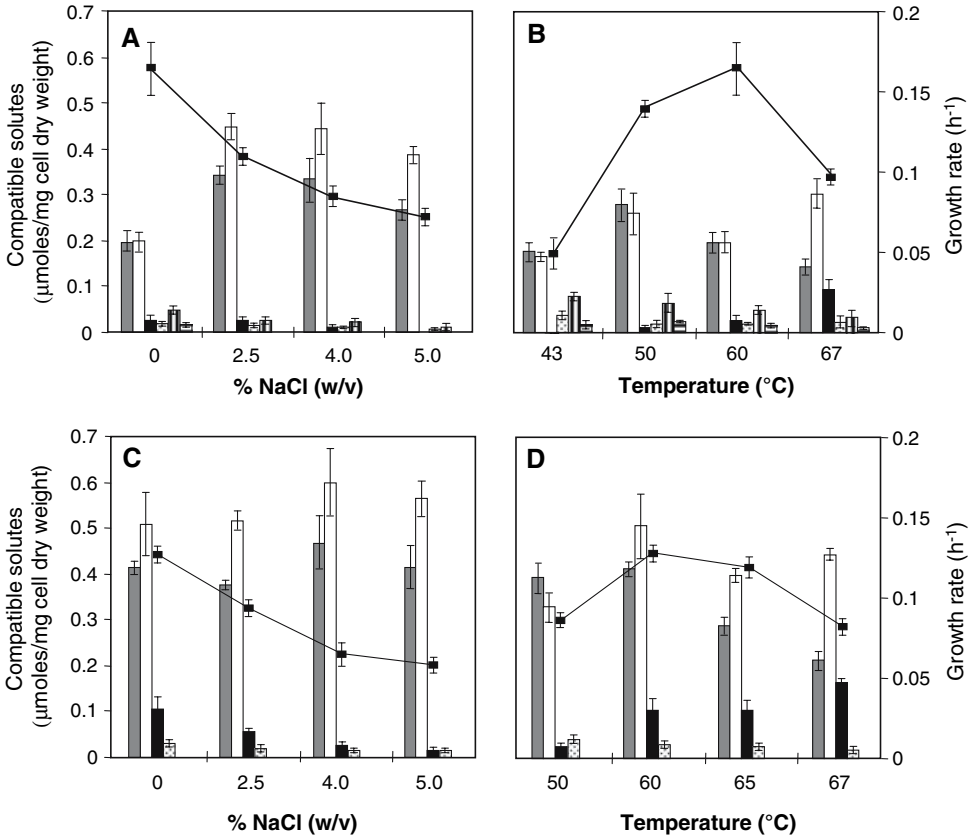

Fig. 2 Growth rate of *R. xylanophilus* (*filled square with line*) in *Thermus* medium (**a**, **b**) and M medium (**c**, **d**) and the effect of NaCl concentration (**a**, **c**) and temperature (**b**, **d**) on the accumulation of compatible solutes in cells harvested during mid-exponential phase of growth. Concentration of trehalose (*open rectangle*), mannosylglycerate (*light shaded rectangle*), di-*myo*-inositol-phosphate (*filled rectangle*), glycine betaine (*rectangle with vertical lines*), and di-*N*-acetyl-glucosamine phosphate (*rectangle with* dots) were determined by NMR; glutamate (*rectangle with horizontal lines*) concentration was determined by HPLC

otherwise the levels of each solute varied little with salinity of the medium or growth temperature (Fig. 2). High solute pools were found under optimal growth conditions in M medium and *Thermus* medium without added NaCl. The major organic solutes were, under all conditions examined, trehalose and MG. In *Thermus* medium, the increase in the salinity from 0 to 2.5% NaCl resulted in an increase in the concentration of trehalose and MG, which remained fairly constant when the concentration of NaCl was raised to 4 and 5% (Fig. 2a). In M medium, trehalose and MG concentrations did not change with salinity, but the concentration of DIP decreased significantly (Fig. 2c). DIP was not detected during growth at 43°C in *Thermus* medium, but otherwise increased with growth temperature, in both media, never reaching levels as high as trehalose and MG (Fig. 2b and d). Amino acids, over 90% of which were glutamate, were always present in very low amounts that did not exceed 0.023 µmol per mg of cell dry weight (Fig. 2a). The concentration of the newly identified solute, di-*N*-acetyl-glucosamine phosphate, was always low and remained constant under all conditions tested. Glycine betaine was detected only in cells grown in *Thermus* medium and the highest concentration was at the lowest temperature tested (Fig 2b).

Discussion

The organic solutes of *R. xylanophilus* were always found in high levels in media without NaCl changing little during growth under salt and temperature stress, and thus making it difficult to clearly attribute a role to their accumulation. These solutes, must, by virtue of the intracellular levels, confer osmotic protection to the organism under salt stress. However, some Low G + C Gram-Positive Bacteria also accumulate high levels of compatible solutes, primarily amino acids, in medium containing low levels of NaCl (Poolman and Glasker 1998). Generally, glutamate, proline and ectoine are the most abundant compatible solutes of those Bacteria, while a few high G + C Gram-Positive Bacteria (Phylum *Actinobacteria*) studied, namely corynebacteria and streptomycetes accumulate trehalose, proline, glutamine and alanine under osmotic stress (Shimakata and Minatogawa 2000; Wolf et al. 2003). These organisms as well as mycobacteria accumulate a low steady-state trehalose pool, and in some *Streptomyces* sp., trehalose is also involved in spore germination being implicated in their heat and desiccation resistance (McBride and Ensign 1987; Woodruff et al. 2004). However, the accumulation of high concentrations of sugar-based organic solutes in *R. xylanophilus* under

non-stressing conditions and upon salt stress is, to our knowledge, unprecedented. The utilization of a minimal medium devoid of trehalose, demonstrates that *R. xylanophilus*, like *Corynebacterium* spp. and *Mycobacterium* spp. can synthesize this solute (De Smet et al. 2000; Wolf et al. 2003). The accumulation of MG by *R. xylanophilus* came somewhat as a surprise because this organism has a lower growth range than other thermophilic bacteria, namely *T. thermophilus* and *Rhodothermus marinus*, where MG serves as a compatible solute. In fact, we detected MG in cells from cultures of *R. xylanophilus* grown at 43°C which is the lowest growth temperature of any known bacterium that accumulates this compatible solute.

Glycine betaine (GB), a common compatible solute of mesophilic prokaryotes, was a minor solute in *R. xylanophilus* grown in *Thermus* medium, indicating that it was taken up, along with glutamate, from the yeast extract and tryptone. This solute cannot, however, have a role in osmotic adjustment in this organism because of the minor amounts accumulated. Moreover, betaine is not detected in (hyper)thermophilic bacteria or archaea, indicating that it is not used for osmotic adjustment in these organisms (Santos and da Costa 2002).

Interestingly, the pool of organic solutes in *R. xylanophilus* comprises two symmetrical phosphodiesters: the novel compound, di-*N*-acetyl-glucosamine phosphate (DAGAP), which was detected in minor amounts under all conditions examined, and di-*myo*-inositol phosphate, a polyol-phosphodiester widespread in hyperthermophilic archaea and bacteria, but never encountered in organisms with optimal growth temperatures below 80°C (Santos and da Costa 2002). The occurrence of DIP in *R. xylanophilus*, a thermophile with $T_{opt} = 60$°C was, therefore, unexpected. Moreover, the levels of DIP increased clearly with the growth temperature, a trend also observed in the hyperthermophiles examined (Santos and da Costa 2002).

It is noteworthy that all the phosphodiester solutes of hyperthermophiles reported to date, such as diglycerol phosphate, glycerol-phospho-*myo*-inositol, and di-mannosyl-di-*myo*-inositol phosphate are polyol derivatives, whereas the new solute identified here comprises a phosphate group linking two sugar moieties (Santos and da Costa 2002; Lamosa et al. 2006). The finding that the thermophilic bacterium *R. xylanophilus* accumulates DIP and MG leads us to speculate that the growth temperature range for the accumulation of these compatible solutes is more flexible than initially suspected.

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