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Restoration of the di-myo-inositol-phosphate pathway in the piezo-hyperthermophilic archaeon *Thermococcus barophilus*

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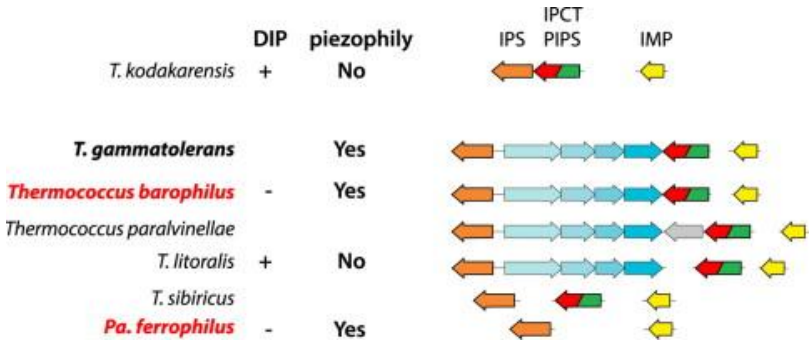
Abstract :

Most *Thermococcales* accumulate di-myo-inositol-phosphate (DIP) as an organic solute as a response to heat stress. We have studied the accumulation of this osmolyte in the high-hydrostatic pressure adapted hyperthermophile *Thermococcus barophilus*. We found no accumulation of DIP under any of the stress conditions tested, although this archaeon harbors the 3 DIP synthesis genes. Lack of synthesis is due to the lack of expression of TERMP_01135 coding for the second step of DIP synthesis. In contrast to other species, the *T. barophilus* synthesis operon is interrupted by a four gene locus, in reverse orientation. Restoring an operon like structure at the DIP locus restored DIP synthesis, but did not have an impact on growth characteristics, suggesting that other mechanisms have evolved in this organism to cope with heat stress.

Graphical abstract



Highlights

► *T. barophilus* lacks the accumulation of the thermal osmolyte di-myo-1,1′-inositol phospahte (DIP). ► Lack of DIP synthesis is due to the lack of expression of the IMPCT/PDIPS gene. ► Mannosylglycerate compensates for the lack of DIP synthesis during thermal stress. ► Restoring the ability to synthesize DIP did not impact the thermal stress response.

Keywords : High hydrostatic pressure, Heat stress, di-myo-inositol-phosphate, *Thermococcus barophilus*

1. INTRODUCTION

Hydrothermal vents are characterized by large temperature fluctuations from fluid temperatures as high as 400°C (Cayman Trough, Western Caribbean Sea) at the heart of the vent, to 2°C, the average temperature of the surrounding deep ocean waters [1]. Due to this extremely steep gradient and the fluctuating environment, it is expected that microorganisms from hydrothermal vents express a strong thermal stress response [8-10]. In a simplistic view, the effects of heat stress on cells and cell structures can be explained by a single factor, e.g. the reduced activity of water, inside or in the vicinity of the cells. Under reduced water activity, improper folding of protein occur and reduce or abolish protein activity, which has numerous cellular consequences [11]. A common strategy among microorganisms to cope with thermal stress involves the accumulation of low-molecular-mass organic compounds. These are named compatible solutes, because they do not interfere with cellular metabolism [2-4]. Compatible solutes of hyperthermophiles are similar to those used in mesophiles, i.e. sugars, amino acids, polyols. Hyperthermophiles also accumulate specific solutes, little or never encountered in mesophiles, such as mannosylglycerate (MG) [6, 12], di-*myo*-1,1'-inositol phosphate (DIP) [13, 14], diglycerol phosphate and derivatives of these compounds [15, 16]. While MG is accumulated mostly in response to high salinity [10, 17], DIP is usually accumulated in response to high temperatures [5, 6, 18]. Osmolytes, such as MG or DIP, help maintain proper protein folding and protein function [19] and permit normal enzyme activity, i.e. prevent protein denaturation, in response to osmotic and heat stress [4, 16, 20]. MG has been shown to preserve protein folding through an increase of protein rigidity [21, 22].

In the deepest parts of the oceans, hydrothermal vent ecosystems are also submitted to extremely high hydrostatic pressures (HHP), which can reach 110 MPa, e.g. 1100 times the atmospheric pressure, at the bottom of the Marianna Trench [23, 24]. HHP is known to impact cellular components [25, 26]. Physically the impact of pressure bears resemblance to both a

lowering of temperature, since it will reinforce the structure of some molecules, such as membrane lipids, and an increase in temperature, since it will as well destabilize other structures, such as proteins [26]. In a simplistic approach, the impact of HHP may be reduced to the Le Chatelier general law of chemical equilibrium, which implies that an increase in pressure will favor the smallest state in a chemical system [27]. Thus, if the volume of the native protein is smaller than the volume of the unfolded protein, this protein will be stabilized by pressure, and conversely. Moderate pressure often increases thermal stability of proteins [25, 28], and consequently protein efficiency is often reported [28]. Several pressure-adapted, i.e. piezophilic, microorganisms have been isolated from various deep-sea hydrothermal vents [29-31], which grow optimally at hydrostatic pressures higher than 0.1 MPa. In piezophiles, some proteins show a better tolerance at high pressure than their homologs from piezosensitive isolates [32-34]. In the SSB protein of *Photobacterium profundum* this enhanced tolerance has been linked to an increase in Proline content leading to increased rigidity of the protein structure [35], a mechanism essentially similar to that observed for the stabilization of proteins to osmotic and thermal stresses by the osmolytes MG and DIP [21, 22]. Knowing the antagonistic or emphasizing effects of HHP and temperature on macromolecular structures in the cells, it was interesting to characterize the nature of the cell response to heat stress in piezophilic hyperthermophiles.

One of the first piezophilic isolate was *Thermococcus barophilus* strain MP, isolated from the Snake Pit hydrothermal vent system on the Mid-Atlantic Ridge, which grows optimally at 40MPa, 85°C and 3% salinity [30]. Most of the hyperthermophilic piezophiles belong to the same family, the *Thermococcales* [26]. In *Thermococcales* such as *T. celer* [5] or *P. furiosus* [6], DIP is accumulated in amounts above 1 µmol/mg of protein. DIP is synthesized from glucose-6-phosphate in three steps, encoded by three genes: an Inositol-1-phosphate synthase (IPS), a bifunctional CTP:Inositol-1-phosphate cytidylyltransferase/Phospho-di-inositol-1-phosphate synthase

(IPCT/PIPS) and an Inositol-1-monophosphatase (IMP). In most *Thermococcus*, the IPCT/PIPS and the IPS genes form a 2-gene operon-like structure, while the IMP gene is located in another genomic location (Figure 1) [36-38]. The three genes being present in *T. barophilus* strain MP is consistent with this strain expressing a heat stress response similar to that of other *Thermococcales*.

We have investigated the heat stress response of the piezophilic archaeon *Thermococcus barophilus* by monitoring the accumulation of DIP as a function of temperature in the wild-type strain MP. We show that this strain does not accumulate detectable levels of DIP under any temperature conditions tested. We show that this inability originates from the lack of expression of the IPCT/PIPS gene. Restoring the IPS/IPCT/PIPS operon restored DIP synthesis in *T. barophilus* mutants, but did not affect growth characteristics.

2. MATERIALS AND METHODS

2.1 Microorganisms and growth conditions

Thermococcus barophilus strain MP was grown in *Thermococcales* Rich Medium (TRM) [31]. Cultivation at low pressure was performed in sealed serum vials while cultures under HHP were performed in sterile syringes as previously described [30]. Cultures were inoculated with 0.5% (v,v) of a glycerol stock, stored anaerobically at -80°C at a starting cell concentration of 5.10^5 cells per milliliter. Cell growth was monitored by direct cell counts in a Thomas chamber (0.01 mm depth). Experiments were performed at least in triplicate.

2.2 Construction of DIP synthesis restoration mutants

Deletion mutants of the 4-gene putative sugar transporter encoded by genes TERMP_01131-TERMP_01134 were obtained as described in Thiel *et al.* (2014) [39] using a knockout plasmid containing the IPS (TERMP_01130) and IPCT/PIPS (TERMP_01135) genes, in which the TGA

codon of IPCT/PIPS was fused to base -63 upstream of the ATG of the IPS ORF. Proper deletion was confirmed by PCR amplification and sequencing using primers located on either sides of the deletion (Table 1). *T. barophilus* mutant derivative TbΔPST1 is available from strain collection of the University of Britany (Souchothèque de Bretagne, http://www.univ-brest.fr/souchotheque/) under the strain number UBOCC 3259.

2.3 RNA extraction, reverse transcription and quantitative PCR

Total RNAs were extracted from mid-exponential phase cultures following a single step RNA extraction procedure adapted from *P. furiosus* [40, 41]. The RNAs were treated with DNAse and further purified with the RNeasy kit from Qiagen according to the manufacturer's instructions. The absence of residual genomic DNA was verified by direct PCR amplification using gene targets listed in table 1. Total RNA were reverse transcribed using the RevertAid™ H Minus Reverse Transcriptase kit (Fermentas, Lituany). The resulting cDNA was used as a template for target specific PCR amplification using primer pairs for each DIP genes (Table 1). RT-qPCR assays were performed on a Mx3000 QPCR system (Agilent Technologies, Santa Clara, CA, USA) using the Brilliant II Ultra-Fast SYBR® Green QPCR master mix (Stratagene, La Jolla, CA, USA). The 20 µl reactions contained 1 µl of target cDNA (1 ng.µl^{-1}), 0.3 µl of 1/500 diluted reference dye and 1 µmol.l^{-1} of each forward and reverse primers. Negative controls without template were included in each run. A specific cloned reference was used for each target gene. Transcripts levels were normalized to 16S rRNA genes. Values are reported as a ratio of expression levels relative to growth conditions under optimal temperature at atmospheric pressure.

2.4 Extraction of intracellular solutesT. barophilus cells (Total of 10^{10}) were harvested in mid-exponential phase by centrifugation (5000g, 15 min, 4°C), washed once with an isotonic NaCl solution, and extracted by the method of Reed [42] with the exception that the extraction was performed for 30 min in boiling 80% ethanol. Cells were removed by centrifugation 10 min at

12000g at 4°C. Supernatants were transferred to a clean tube and lyophilized (Alpha 1-2 LDplus, Martin Christ, Germany). The dried residue was dissolved in D_2O for NMR analyzes.

2.5 NMR spectroscopy

All spectra were acquired on a Bruker DRX 500 spectrometer at room temperature and 30° pulse. ^{13}C-NMR spectra were acquired at 125.8 MHz using a TCI ^{1}H/^{13}C/^{15}N 5mm probehead. ^{31}P-NMR spectra were acquired at 202.5 MHz using a 5 mm TBI ^{1}H/{BB}/^{13}C probe. NMR studies were performed at the Institut des Sciences Analytiques of the CNRS (ISA, Villeurbane, France).

3. RESULTS AND DISCUSSION

3.1 Characterization of the heat stress response in T. barophilus.

T. barophilus strain MP grows from ca. 65°C to 95°C at atmospheric pressure, and up to 100°C at 40MPa, with an optimum at 85°C at both pressures [9]. To optimize thermal stress conditions, *T. barophilus* was grown in TRM [31] at varying temperatures ranging form 65°C to 98°C under optimal pressure and salinity conditions, e.g 40 MPa and 3% NaCl. As expected, the highest growth rate and largest cell yields were obtained for cultures at 85°C. Growth yields below 75°C and above 95°C were severely affected and could not yield enough cellular material for further osmolyte extraction. Temperatures between 80°C and 90°C showed little impact on growth parameters, inducing only a short, ca. 2h, growth lag, while a longer growth retardation (ca. 6-8h) and reduced growth yield (0.3 log) was observed for growth at 95°C. Based on these results, 80°C was chosen as the sub-optimal temperature growth condition and 90°C and 95°C were chosen as supra-optimal temperatures. Atmospheric pressure was chosen as the low pressure. The supra-optimal pressure condition was fixed at 70MPa, which was previously shown to match growth parameters for strain MP at 0.1MPa [31].

Organic solutes were extracted from *T. barophilus* cells grown under these 4 temperature, and 3 pressure conditions yielding 12 different pressure x temperature conditions. DIP extracted from *P. furiosus* grown under optimal temperature conditions (100 °C, 0.1 MPa) was used as a positive control for extraction and analysis. The ethanol extracts were examined by natural abundance ^{1}H- and ^{13}C-NMR for the presence of DIP. Surprisingly, no peaks clearly associated with DIP could be seen in the ^{1}H- and ^{13}C-NMR spectra under any temperature in *T. barophilus* cell extracts (data not shown). Moreover, DIP, or its derivatives, could not be identified when acquiring specific ^{31}P-NMR spectra (Figure 2, right panel). In the control experiments performed on *P. furiosus* grown at 100°C, the specific signal for DIP was clearly visible in both ^{1}H- and ^{13}C-NMR (data not shown), as well as ^{31}P-NMR spectra (Figure 2, right panel), which clearly demonstrate that if DIP was indeed produced during growth of *T. barophilus*, we should be able to detect it in the NMR spectra. To test whether our stress conditions were sufficient to induce DIP accumulation in *T. barophilus* we analyzed samples extracted from cells grown at 98°C under high hydrostatic pressure (40 MPa/98°C, and 70 MPa/98°C), since this temperature does not support growth at atmospheric pressure. Even under these conditions we could not detect DIP production in *T. barophilus* strain MP. Thus far, the absence of DIP synthesis had been observed only in one other *Thermococcales* species, *Palaeococcus ferrophilus*, which instead of DIP accumulates increasing amounts of the salt-specific osmolyte, mannosylglycerate (MG), with increasing thermal stress [10]. The lack of DIP synthesis in *P. ferrophilus* can be attributed to the loss of one of the DIP synthesis genes, e.g. the IPCT/PIPS gene, which is responsible for the conversion of myo-inositol-phosphate, into the DIP precursor di-myo-inositol-di-phosphate (unpublished genome data, available from the JGI, http://genome.jgi.doe.gov). However, the lack of DIP accumulation in *T. barophilus* cells contrasts with that of *P. ferrophilus*, since the *T. barophilus* genome harbors a complete set of DIP synthesis genes.

3.2 The IPCT/PIPS gene is not expressed in T. barophilus

To test whether the lack of DIP synthesis could originate from poor expression of the DIP synthesis genes in *T. barophilus*, total RNAs were prepared from cells grown under optimal temperature conditions (85°C) and under thermal stress (90°, 95°C) at two pressure conditions (0.1MPa and 40MPa). Interestingly, we detected the expression of only two of the three DIP synthesis genes in strain MP whichever the temperature considered (Table 2). The mRNA of TERMP_01046, which could encode the inositol-1-monophosphatase (IMP), the last enzyme of the DIP pathway, is the gene expressed at the highest levels, but did not seem to be regulated by temperature. Similarly, the expression of TERMP_01135 which could encode IPS, the first enzyme of the pathway, is slightly up regulated at 90°C and 95°C, although this increase is modest (Table 2). High hydrostatic pressure did not impact the expression levels of these two genes. In contrast the expression of TERMP_01130, which could encode IPCT/PIPS, the second step of the pathway, was not detected (Table 2). These results show that the lack of expression of the IPCT/PIPS gene is responsible for the lack of DIP synthesis in *T. barophilus*. It is interesting to note that the same gene is responsible for the lack of DIP synthesis in *P. ferrophilus*, although the overlaying mechanism differs [10]. In the cell, IPS and IMP are responsible for the synthesis of myo-inositol from D-glucose-6-phosphate in two steps via myo-inositol-monophosphate. Myo-inositol and myo-inositol-phosphate are central cellular metabolites involved in essential metabolic pathways. Indeed, myo-inositol-monophosphate is the constituent of phospholipids and inositol polyphosphates. Myo-inositol-monophosphate and CDP:archaeol are the substrate of the archaetidylinositol-1-phosphate synthase to form archaetidylinositol-phosphate, the precursor of archaeal lipids containing a phosphatidylinositol (PI) polar head group [43]. Inositol phospholipids are common in Archaea and ubiquitous in the *Thermococcales* [44]. All known archaeal genomes present a putative archaetidylinositol-1-phosphate synthase, which for example could be encoded by TERMP_01226

in *T. barophilus* strain MP [45]. To date, there are no known alternative myo-inositol synthesis pathways or homologues of the IPS or IMP genes in the *Thermococcales*, which may explain why the IPCT/PIPS gene might be the only one dispensable in the DIP synthesis pathway.

3.3 Restoration of DIP synthesis in T. barophilus.

The IPCT/PIPS-IPS synthesis operon structure is highly conserved among *Thermococcales* with six notable exceptions, *T. barophilus, T. gammatolerans, T. paralvinellae, T. nautilus, T. litoralis* and *T. sibiricus* (Table 3) [38]. In the later two strains, the IPCT/PIPS and IPS genes are not linked (Table 3), while in the first three species the two genes are separated by a 4- or 5-gene insertion. This insertion could code for a putative sugar transporter (PST) and be expressed in reverse orientation to the IPCT/PIPS-IPS genes. The same PST is found upstream of the IPS gene in *T. litoralis* but not in *T. sibiricus*. In the last species, *T. nautilus*, the two genes are separated by a transposase gene which is in the same orientation as the DIP synthesis genes. In all other species, the intergenic region between IPCT/PIPS and IPS is extremely conserved at the sequence level (Figure 3). To explore whether the 4-gene insertion could be responsible for the lack of DIP synthesis in *T. barophilus*, we engineered its removal and restored an operon-like structure at the IPCT/PIPS - IPS locus in *T. barophilus*. 63 bases upstream of the IPS ORF that were conserved in the *T. litoralis* and *T. sibiricus* genomes were kept in this construct (Figure 3) and fused to the TGA codon of the TERMP_01135 ORF. This 63 pb artificial intergenic region shares extensive similarities with the canonical intergenic sequence observed in other *Thermococcus* species (Figure 3). Although short, it contains motifs sharing similarity with the promoter sequence of *Thermococcales* (TATA box) and is properly spaced to function as a promoter to drive the expression of IPS [46, 47]. Two independent deletion mutants, TbΔPST1 and TbΔPST2, were produced and confirmed by sequencing. As summarized in Table 4, these deletion did not have a significant impact on growth

parameters of the 2 mutants, at optimal or supra optimal growth temperatures. However, the accumulation of DIP could be observed in the mutants under thermal stress, e.g. at 95°C, but not under optimal temperature conditions (Figure 3, left panel). Furthermore, transcripts corresponding to the IPCT/PIPS gene were detected in the two mutant derivatives under optimal and thermal stress conditions under atmospheric or high hydrostatic pressure conditions (Table 3). There is no evidence of temperature-dependent gene regulation of the IPCT/PIPS gene, although the production and accumulation of DIP only occurs under thermal stress (Table 2), which supports the occurrence of post transcriptional regulation of the accumulation of osmolytes to respond to stress in *T. barophilus*. These results clearly demonstrate that the lack of DIP synthesis in the piezo-hyperthermophile *T. barophilus* could be attributed to the presence of the 4-gene PST cluster into the IPCT/PIPS-IPS operon. The mechanism by which this inhibition occurs has not been addressed. These results also strongly suggest that the thermal stress response in *T. barophilus* has evolved to implicate other mechanisms. Different alternative thermal stress responses in the *Thermococcales* have been described. Aspartate and glutamate have been shown to accumulate in *T. kodakarensis* mutants in which the synthesis of DIP had been deleted [49]. In *T. barophilus*, we found no evidence for the accumulation of these two amino acids under thermal stress. In the wild-type strain MP, apartate and glutamate were estimated to represent 0.07 and 0.14, and 0.04 and 0.12 µmol/mg of protein at 85°C and 95°C respectively. MG has also been shown to substitute to DIP as a thermal stress osmolyte in *P. ferrophilus* [10]. The potential for this osmolyte to substitute for DIP was later confirmed in the heat adaptation of *P. furiosus* [48]. In *T. barophilus,* we notice asimilar significant increase in the accumulation of MG from 0.1 µmol/mg of protein at 85°C to 0.54 µmol/mg of protein at 95°C. This observation suggests that in *T. barophilus* the absence of DIP synthesis is compensanted during thermal stress by the accumulation of MG, similarly to what has been observed in the other piezophilic species, *P. ferrophilus* [10].

3.4 Evolution of DIP synthesis in the piezophilic Thermococcales.

As mentionned above, *T. barophilus* is the first example of *Thermococcales* which is unable to accumulate the osmolyte DIP as a response to thermal stress but possesses a complete set of DIP synthesis genes. It is only the second species with *P. ferrophilus* to be unable to accumulate DIP. Thus far, DIP was shown to accumulate in greater quantities under increased thermal stress in all strains harboring the DIP synthesis genes. All *Thermococcales* have been isolated from black smoker-like hydrothermal fluids or chimney fragments, and exhibit very similar physico-chemical growth requirements. However, in contrast to the other *Thermococcales* species knwon to produce DIP, the *T. barophilus* and *P. ferrophilus* species are piezophilic, harboring growth pressure optima of 40 MPa and 30 MPa respectively [10, 30, 50], which raises questions whether this trait might be linked to their adaptation to high hydrostatic pressure. In this study we show that the lack of DIP accumulation of DIP in *T. barophilus* is linked to the presence of the putative sugar transporter locus between the IPS and IPCT/PIPS genes. A survey of the proteins involved in the synthesis of DIP performed on the sequences of the two genes (IPCT/PIPS and IPS) as well as on the putative sugar transporter genes present upstream of IPS in 6 *Thermococcus* species clearly shows a congruence between the phylogeny of this locus (Figure S1) and the core genome (Figure 4) and 16S phylogenies [51] with one exception. All topologies demonstrate a well supported group containing 5 of the 6 species harboring the PST locus, comprising *T. barophilus*, *T. litoralis*, *T. sibiricus*, *T. sp.* strain PK and *T. paralvinellae* (Figure S1), which supports a common origin of the IPCT/PIPS-PST-IPS cluster in the ancestor of these archaeal species (Figure 4). Horizontal gene transfer might explain the origin of this cluster of genes in the *T. gammatolerans* background. Within the *T. barophilus* cluster, *T. litoralis* is the only species originating from the surface as well as the only species known to accumulate DIP. In this species, the IPCT/PIPS and IPS genes have become genetically unlinked, which might have restored the expression of the IPCT/PIPS gene. Together

these results support the existence of a possible link between the adaptation to HHP and the lack of DIP synthesis in the *Thermococcales*. No matter how tempting this hypothesis, we are still lacking a rationale to explain why the lack of DIP, the canonical thermal stress response molecule would give a growth or selective advantage to piezophilic *Thermococcales*. Furthermore, the accumulation of DIP in the other species of this cluster or in *T. gammatolerans* has not yet been investigated, leaving open the possibility that only *T. barophilus* and *P. ferrophilus* lack DIP synthesis. Thus, additional experiments on *T. gammatolerans*, which shares the same genetic structure at the IPCT/PIPS-IPS locus as *T. barophilus*, would be required to establish a link between the adaptation to high hydrostatic pressure and thermal stress response.

4. CONCLUSIONS

The present study demonstrate that the lack of DIP synthesis and accumulation as a function of heat stress in *T. barophilus* is due to the presence of a 4-gene putative sugar transporter located in reverse orientation between the IPCT/PIPS and IPS genes. Since the second gene of the putative operon is expressed in the wild type strain, but not the first gene, the rationale for this inhibition is elusive. However, the restoration of the *Thermococcus*-like two-gene operon restored DIP gene expression and DIP synthesis in *T. barophilus*. To our knowledge, *T. barophilus* is only the second example of a *Thermococcales* which is unable to synthesize DIP as a function of thermal stress, both of which are also adapted to growth under high hydrostatic pressure.

Competing Interests

The authors declare no competing interests.

296 **Author contributions**

297 PO, AC, MJ conceived and designed the experiments. MJ, AT created the DIP mutants.
298 AC, AM, PO performed the experiments and analyzed the date. PO, AC, MJ wrote the manuscript.

299

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307

RT-PCR					
gene tag	gene	primer	orientation	forward	gene position
TERMP_01046	IMP	1046F	Forward	TGGAGTTAGCCCCAGCGGAGA	96-116
		1046R	Reverse	AGCACTCGCTGCTATGTCGGT	615-595
TERMP_1130	IPCT/ PIPS	1130F	Forward	AGGGCAGTGATTCTTGCGGCT	13-33
		1130R	Reverse	CGTCTTCATGCTCGCACGTGC	924-904
TERMP_1135	IPS	1135F	Forward	TTCCATTGGCAAATGAGCTGCCA	98-120
		1135R	Reverse	TGCCCTTCTCTGCTGGTCCTGG	1084-1063
16S rRNA		Arch344F	Forward	ACGGGGYGCAGCAGGCGCGA	
		Arch910R	Reverse	GCTCCCCCGCCAATTC	
PST deletion verification primers					
	IPCT/ PIPS	PST_up	Forward	TAGCCGGGCAAATAAAAGCTCTCTTT	1187-1212
	IPS	PST_down	Reverse	CCCATAATAGCCGAGATCTCCTC	74-96

308

309 **Table 1:** List of primers used in this study for PCR and RT-PCR.

310

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312

		MP			PST1		PST2	
		IPS	IMPCT /PDIPS	IMP	IPS	IMPCT /PDIPS	IPS	IMPCT /PDIPS
	85°C	1	nd	1	1.10	1	1.21	1.12
0.1 MPa	90°C	1.38	nd	0.9	1.31	1.14	1.13	0.90
	95°C	1.27	nd	1.2	1.0	1.12	1.1	1.1
	85°C	0.92	nd		1.20	0.78		
40 MPa	90°C	1.21	nd		0.99	0.58		
	95°C	1.23	nd		1.43	0.68		

313

314 **Table 2:** RT-qPCR analysis of DIP synthesis genes in the wild type (MP) and the DIP restored
315 mutants (TbΔPST1, TbΔPST2). Expression levels have been normalized to *16S rRNA*
316 expression level. Expression levels are reported relative to expression levels at 85°C. Values
317 are averages of duplicate experiments. nd, not detected.
318

319

Species	Strain	size (bp)			neighboring gene(s) to IPS[b]	DIP[c]
		IGS[a]	IPCT/PIPS[a]	IPS[a]		
T. barophilus	MP	4938	1287	1152	4-gene locus Sugar transporter	ND
T. paralvinellae	ES1	6701	1314	1152	5-gene locus Sugar transporter	
T. nautilus	30.1	1488	1305	1149	1 gene Transposase	
T. gammatolerans	EJ3	4778	1284	1149	4-gene locus Sugar transporter	
T. litoralis	NS-C	unlinked	1284	1152	NA	up to 0.37 µmol/mg protein
T. sibiricus	MM379	unlinked	1278	1149	NA	
T. kodakarensis	KOD1	43	1299	1149	IPCT/PIPS	up to 0.14 µmol/mg protein
T. cleftensis	CL1	42	1299	1149	IPCT/PIPS	
T. sp.	4557	43	1299	1149	IPCT/PIPS	
T. guaymasensis	TYS	44	1284	1149	IPCT/PIPS	
T. onnurineus	NA2	61	1278	1149	IPCT/PIPS	
T. eurythermalis	A501	59	1284	1149	IPCT/PIPS	
T. sp.	AM4	44	1299	1149	IPCT/PIPS	
T. stetteri	K3	-[d]	-	-	NA	up to 0.45 µmol/mg protein
T. celer	Vu13	-[d]	-	-	NA	up to 1.2 µmol/mg protein
T. zilligii	AN1	-[d]	-	-	NA	ND

320

321 **Table 3 :** Structure of the IPCT/PIPS-IPS locus in the genus *Thermococcus*. a) size in bp of

322 the IPCT/PIPS and IPS genes and the Intergenic sequence (IGS) between the two genes. b)

323 genomic structure of the IGS sequence; c) quantification of DIP accumulation [5, 49]. d)

324 Genome sequence not known. ND: not detected. NA, not applicable.

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	MP	PST1	PST2
85°C	0.77 ± 0.02	0.77 ± 0.02	0.78 ± 0.03
90°C	0.60 ± 0.01	0.61 ± 0.02	0.62 ± 0.01
95°C	0.53 ± 0.03	0.53 ± 0.08	0.53 ± 0.03
40MPa - 85°C	1.24 ± 0.03	1.24 ± 0.02	1.25 ± 0.04

327

328 **Table 4:** Growth rate for the wild type (MP) and the two DIP mutant clones (TbΔPST1,
329 TbΔPST2) of strain MP. The values are average from four independent experiments.

330

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Figure captions

Figure 1: DIP synthesis pathway (A) and genetic organization (B) in *Thermococcus barophilus* strain MP.

Figure 2: Extracts from ^{31}P NMR spectra of partially purified osmolytes from the wild type (MP) and one of the DIP restoration mutant of *T. barophilus* (TbΔPST2) grown under optimal temperature (85°C) or under heat stress (95°C) at atmospheric pressure. Osmolytes purified from *Pyrococcus furiosus* grown at 100°C (Pf 100) were used as a positive control to assign the specific peak for DIP (-1.1 ppm).

Figure 3: Detailed alignment of the sequence upstream of the IPS start codon in the *Thermococcus* genus.

Figure 4: Genetic organization of the DIP synthesis loci in the *Thermococcales*. The phylogenetic tree of the *Thermococcales* has been calculated on the concatenate protein sequence of the strict core genome (544 families) with PhyML on 1000 bootsptrap replicates.

Figure S1: Phylogenetic trees of the IPCT/PIPS, IPS and putative sugar transporter in the *Thermococcales*. All genes have been aligned with MUSCLE. Alignements were optimized by hand before the calculation of phylogenetic trees with PhyML on 1000 bootsrap replicates. Trees have been rooted with the IPCT/PIPS, IPS and PST loci of *P. horikoshii*.

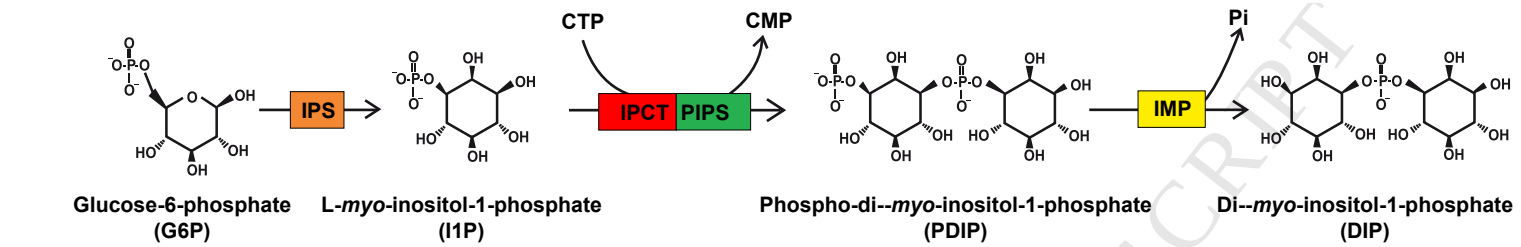
Literature

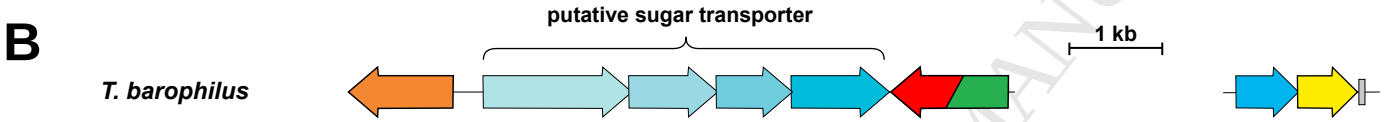
1. Prieur, D., *Microbiology of deep-sea hydrothermal vents.* Trends in Biotechnology, 1997. **15**(7): p. 242-244.
2. Brown, A.D., *Microbial water stress.* Bacteriological Reviews, 1976. **40**(4): p. 803-846.
3. Galinski, E.A., *Osmoadaptation in bacteria.* Advances in microbial physiology, 1995. **37**: p. 272-328.
4. Ventosa, A., J.J. Nieto, and A. Oren, *Biology of moderately halophilic aerobic bacteria.* Microbiology and Molecular Biology Reviews, 1998. **62**(2): p. 504-+.
5. Lamosa, P., et al., *Effects of temperature, salinity, and medium composition on compatible solute accumulation by Thermococcus spp.* Applied and Environmental Microbiology, 1998. **64**(10): p. 3591-3598.
6. Martins, L.O. and H. Santos, *Accumulation of mannosylglycerate and di-myo-inosytol-phosphate by Pyrococcus furiosus in response to salinity and temperature.* Applied and Environmental Microbiology, 1995. **61**(9): p. 3299-3303.
7. Vannier, P., et al., *Complete genome sequence of the hyperthermophilic piezophilic, heterotrophic and carboxydotrophic archaeon Thermococcus barophilus MP.* Journal of Bacteriology, 2011. **193**: p. 1481–1482.
8. Holden, J.F. and J.A. Baross, *Enhanced thermotolerance and temperature-induced changes in protein-composition in the hyperthermophilic archaeon ES4.* Journal of Bacteriology, 1993. **175**(10): p. 2839-2843.
9. Marteinsson, V.T., et al., *Physiological responses to stress conditions and barophilic behavior of the hyperthermophilic vent archaeon Pyrococcus abyssi.* Applied and Environmental Microbiology, 1997. **63**(4): p. 1230-1236.
10. Neves, C., M.S. da Costa, and H. Santos, *Compatible solutes of the hyperthermophile Palaeococcus ferrophilus: Osmoadaptation and thermoadaptation in the order Thermococcales.* Applied and Environmental Microbiology, 2005. **71**(12): p. 8091-8098.
11. Ooi, T., *Thermodynamics of protein-folding - effects of hydration and electrostatic interactions.* Advances in Biophysics, Vol 30, 1994, 1994. **30**: p. 105-154.
12. Nunes, O.C., et al., *Compatible solutes in the thermophilic bacteria Rhodothermus marinus and Thermus thermophilus.* Applied and Environmental Microbiology, 1995. **61**(6): p. 2351-2357.
13. Scholz, S., et al., *Di-myo-inositol-1,1'-phosphate - a new inositol phosphate isolated from Pyrococcus woesei.* FEBS Letters, 1992. **306**(2-3): p. 239-242.
14. Martins, L.O., et al., *New compatible solutes related to di-myo-inositol-phosphate in members of the order Thermotogales.* Journal of Bacteriology, 1996. **178**(19): p. 5644-5651.
15. da Costa, M.S., H. Santos, and E.A. Galinski, *An overview of the role and diversity of compatible solutes in Bacteria and Archaea.* Advances in biochemical engineering/biotechnology, 1998. **61**: p. 117-53.
16. Santos, H. and M.S. da Costa, *Organic solutes from thermophiles and hyperthermophiles.* Hyperthermophilic Enzymes, Pt C, 2001. **334**: p. 302-315.
17. Empadinhas, N., et al., *Pathway for the synthesis of mannosylglycerate in the hyperthermophilic archaeon Pyrococcus horikoshii - Biochemical and genetic characterization of key enzymes.* Journal of Biological Chemistry, 2001. **276**(47): p. 43580-43588.
18. Silva, Z., et al., *Combined effect of the growth temperature and salinity of the medium*

on the accumulation of compatible solutes by Rhodothermus marinus and Rhodothermus obamensis. Extremophiles, 1999. **3**(2): p. 163-172.
19. Lamosa, P., et al., *Protein stabilization by compatible solutes - Effect of diglycerol phosphate on the dynamics of Desulfovibrio gigas rubredoxin studied by NMR.* European Journal of Biochemistry, 2003. **270**(23): p. 4606-4614.
20. Yancey, P.H., *Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses.* Journal of Experimental Biology, 2005. **208**(15): p. 2819-2830.
21. Borges, N., et al., *Comparative study of the thermostabilizing properties of mannosylglycerate and other compatible solutes on model enzymes.* Extremophiles, 2002. **6**(3): p. 209-216.
22. Pais, T.M., et al., *Relationship between protein stabilization and protein rigidification induced by mannosylglycerate.* Journal of Molecular Biology, 2009. **394**(2): p. 237-250.
23. Yayanos, A.A., *Microbiology to 10,500 metters in the deep-sea.* Annual Review of Microbiology, 1995. **49**: p. 777-805.
24. Zobell, C.E., *Bacterial life at the bottom of the Philippine trench.* Science, 1952. **115**(2993): p. 507-508.
25. Michels, P.C. and D.S. Clark, *Pressure-dependence of enzyme catalysis*, in *Biocatalysis at Extreme Temperatures*, M.W.W. Adams and R.M. Kelly, Editors. 1992, American Chemical Society: Washington, DC. p. 108-121.
26. Oger, P. and M. Jebbar, *The many ways of coping with pressure.* Research in Microbiology, 2010. **161**: p. 799-809.
27. Le Chatelier, H.L., *Sur un énoncé général des lois d'équilibres chimiques.* Comptes-rendus de l'Académie des sciences, 1884. **99**: p. 786-789.
28. Eisenmenger, M.J. and J.I. Reyes de Corcuera, *High pressure enhancement of enzymes: A review.* Enzyme and Microbial Technology, 2009. **45**(5): p. 331-347.
29. Jannasch, H.W. and M.J. Mottl, *Geomicrobiology of deep-sea hydrothermal vents.* Science, 1985. **229**(4715): p. 717-725.
30. Marteinsson, V.T., et al., *Thermococcus barophilus sp. nov., a new barophilic and hyperthermophilic archaeon isolated under high hydrostatic pressure from a deep-sea hydrothermal vent.* International Journal of Systematic Bacteriology, 1999. **49**: p. 351-359.
31. Zeng, S., et al., *Pyrococcus CH1, an obligate piezophilic hyperthermophile : extending the upper pressure-temperature limits for life.* ISME Journal, 2009. **3**(7): p. 873-876.
32. Kasahara, R., et al., *Piezo-adapted 3-isopropylmalate dehydrogenase of the obligate piezophile Shewanella benthica DB21MT-2 isolated from the 11,000-m depth of the Mariana trench.* Bioscience Biotechnology and Biochemistry, 2009. **73**(11): p. 2541-2543.
33. Morita, T., *Structure-based analysis of high pressure adaptation of alpha-actin.* Journal of Biological Chemistry, 2003. **278**(30): p. 28060-28066.
34. Nishiguchi, Y., T. Miwa, and F. Abe, *Pressure-adaptive differences in lactate dehydrogenases of three hagfishes: Eptatretus burgeri, Paramyxine atami and Eptatretus okinoseanus.* Extremophiles, 2008. **12**(3): p. 477-480.
35. Chilukuri, L.N., D.H. Bartlett, and P.A.G. Fortes, *Comparison of high pressure-induced dissociation of single-stranded DNA. binding protein (SSB) from high pressure-sensitive and high pressure-adapted marine Shewanella species.* Extremophiles, 2002. **6**(5): p. 377-383.

36. Rodionov, D.A., et al., *Genomic identification and in vitro reconstitution of a complete biosynthetic pathway for the osmolyte di-myo-inositol-phosphate.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(11): p. 4279-4284.
37. Rodrigues, M.V., et al., *A unique beta-1,2-mannosyltransferase of Thermotoga maritima that uses di-myo-inositol phosphate as the mannosyl acceptor.* Journal of Bacteriology, 2009. **191**(19): p. 6105-6115.
38. Goncalves, L.G., et al., *Evolution of the biosynthesis of di-myo-inositol phosphate, a marker of adaptation to hot marine environments.* Environmental Microbiology, 2012. **14**(3): p. 691-701.
39. Thiel, A., et al., *Genetic Manipulations of the Hyperthermophilic Piezophilic Archaeon Thermococcus barophilus.* Applied and Environmental Microbiology, 2014. **80**(7): p. 2299-2306.
40. Chomczynski, P. and N. Sacchi, *The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on.* Nature Protocols, 2006. **1**(2): p. 581-585.
41. Voorhorst, W.G.B., et al., *Characterization of the celB gene coding for beta-glucosidase from the hyperthermophilic archaeon Pyrococcus furiosus and its expression and site-directed mutation in Escherichia coli.* Journal of Bacteriology, 1995. **177**(24): p. 7105-7111.
42. Reed, R.H., et al., *Carbohydrate accumulation and osmotic-stress in cyanobacteria.* Journal of General Microbiology, 1984. **130**(JAN): p. 1-4.
43. Morii, H., et al., *Ubiquitous distribution of phosphatidylinositol phosphate synthase and archaetidylinositol phosphate synthase in Bacteria and Archaea, which contain inositol phospholipid.* Biochemical and Biophysical Research Communications, 2014. **443**(1): p. 86-90.
44. Oger, P. and A. Cario, *Adaptation of the archaeal membrane.* Biophysical Chemistry, 2013. **15**: p. 42-56.
45. Vannier, P., et al., *Complete Genome Sequence of the Hyperthermophilic, Piezophilic, Heterotrophic, and Carboxydotrophic Archaeon Thermococcus barophilus MP.* Journal of Bacteriology, 2011. **193**(6): p. 1481-1482.
46. Thomm, M. and G. Wich, *An archaebacterial promoter element for stable RNA genes with homology to the TATA box of higher eukaryotes.* Nucleic Acids Research, 1988. **16**(1): p. 151-163.
47. Reiter, W.D., U. Hudepohl, and W. Zillig, *Mutational analysis of an archaebacterial promoter - Essential role of a TATA box for transcription efficiency and start-site selection in vitro.* Proceedings of the National Academy of Sciences of the United States of America, 1990. **87**(24): p. 9509-9513.
48. Esteves, A.M., et al., *Mannosylglycerate and Di-myo-Inositol Phosphate Have Interchangeable Roles during Adaptation of Pyrococcus furiosus to Heat Stress.* Applied and Environmental Microbiology, 2014. **80**(14): p. 4226-4233.
49. Borges, N., et al., *Thermococcus kodakarensis mutants deficient in di-myo-inositol phosphate use aspartate to cope with heat stress.* Journal of Bacteriology, 2010. **192**(1): p. 191-197.
50. Takai, K., et al., *Palaeococcus ferrophilus gen. nov., sp nov., a barophilic, hyperthermophilic archaeon from a deep-sea hydrothermal vent chimney.* International Journal of Systematic and Evolutionary Microbiology, 2000. **50**: p. 489-500.
51. Hensley, S., et al., *Thermococcus paralvinellae sp. nov. and Thermococcus cleftensis sp. nov. of hyperthermophilic heterotrophs from deep-sea hydrothermal vents.*

501 International Journal of Systematic and Evolutionary Microbiology, 2014. **64**(11): p.
502 3655-3659.





Cario et al. Restoration of DIP synthesis in T. barophilus
Figure 1

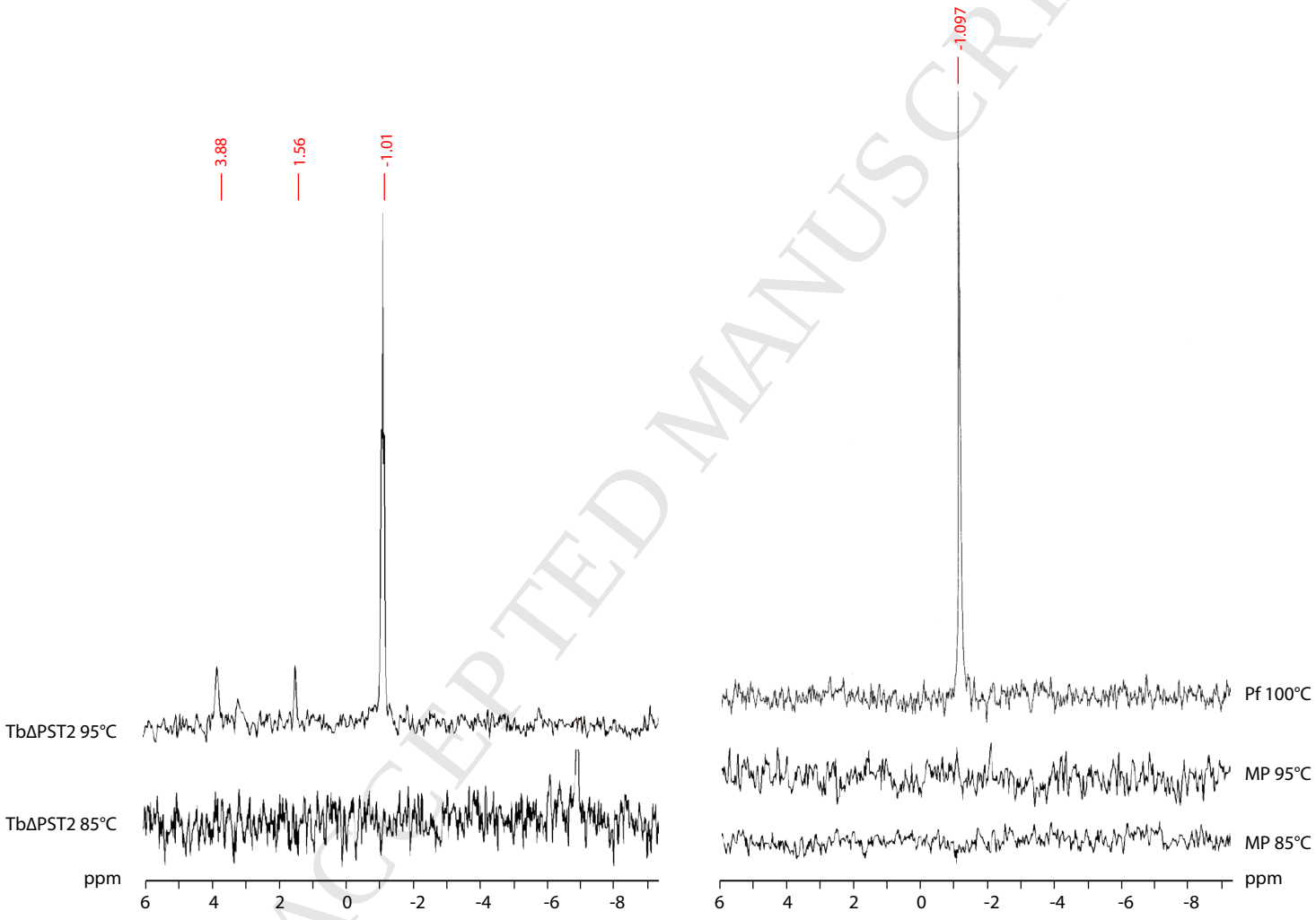


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Figure 2

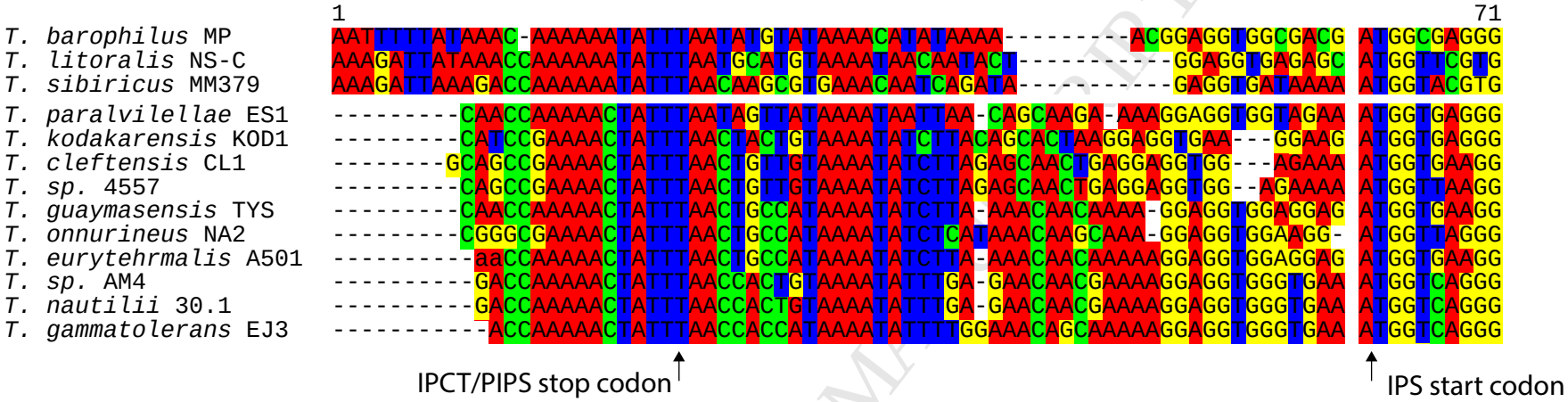

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Figure 3

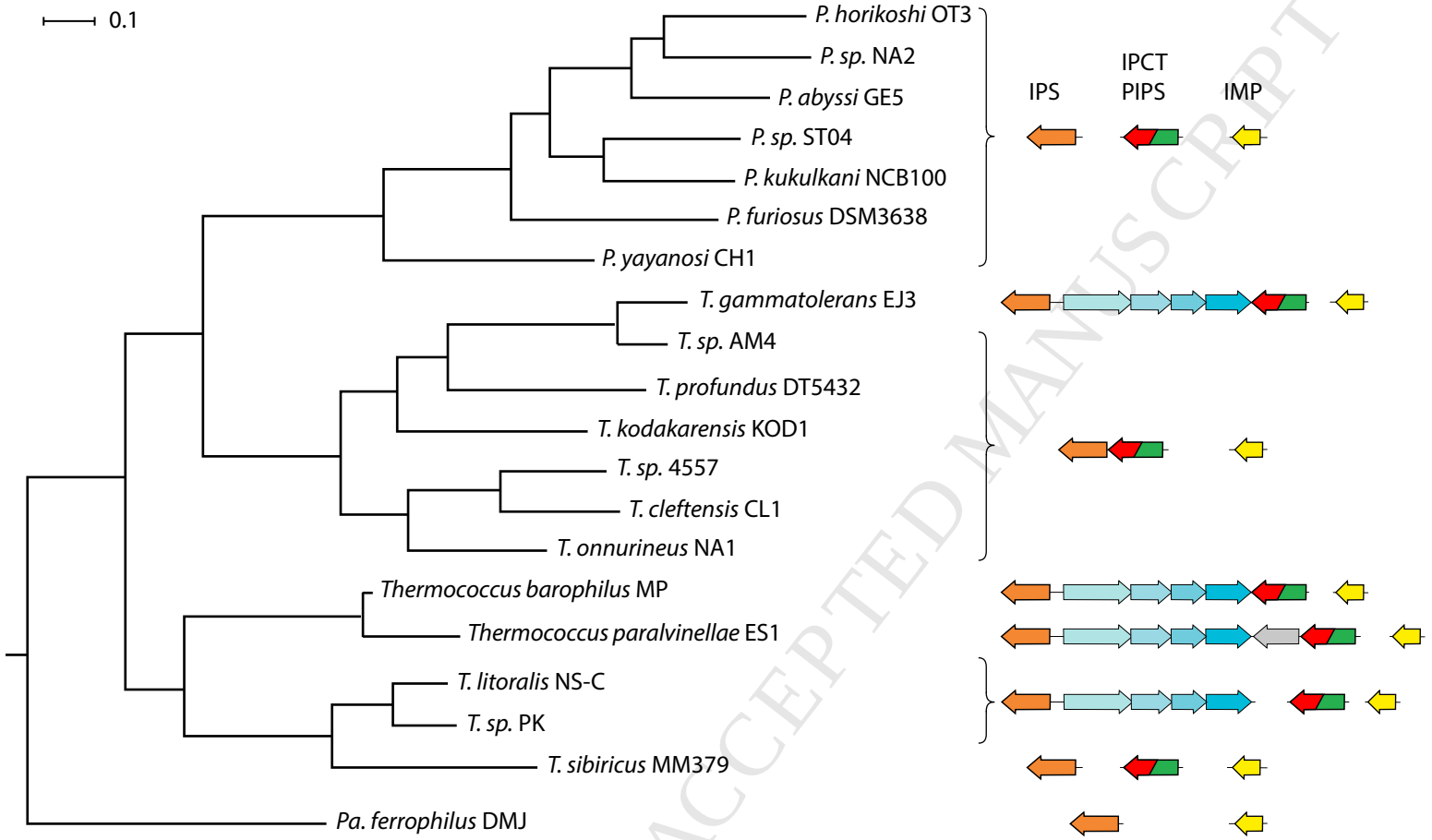

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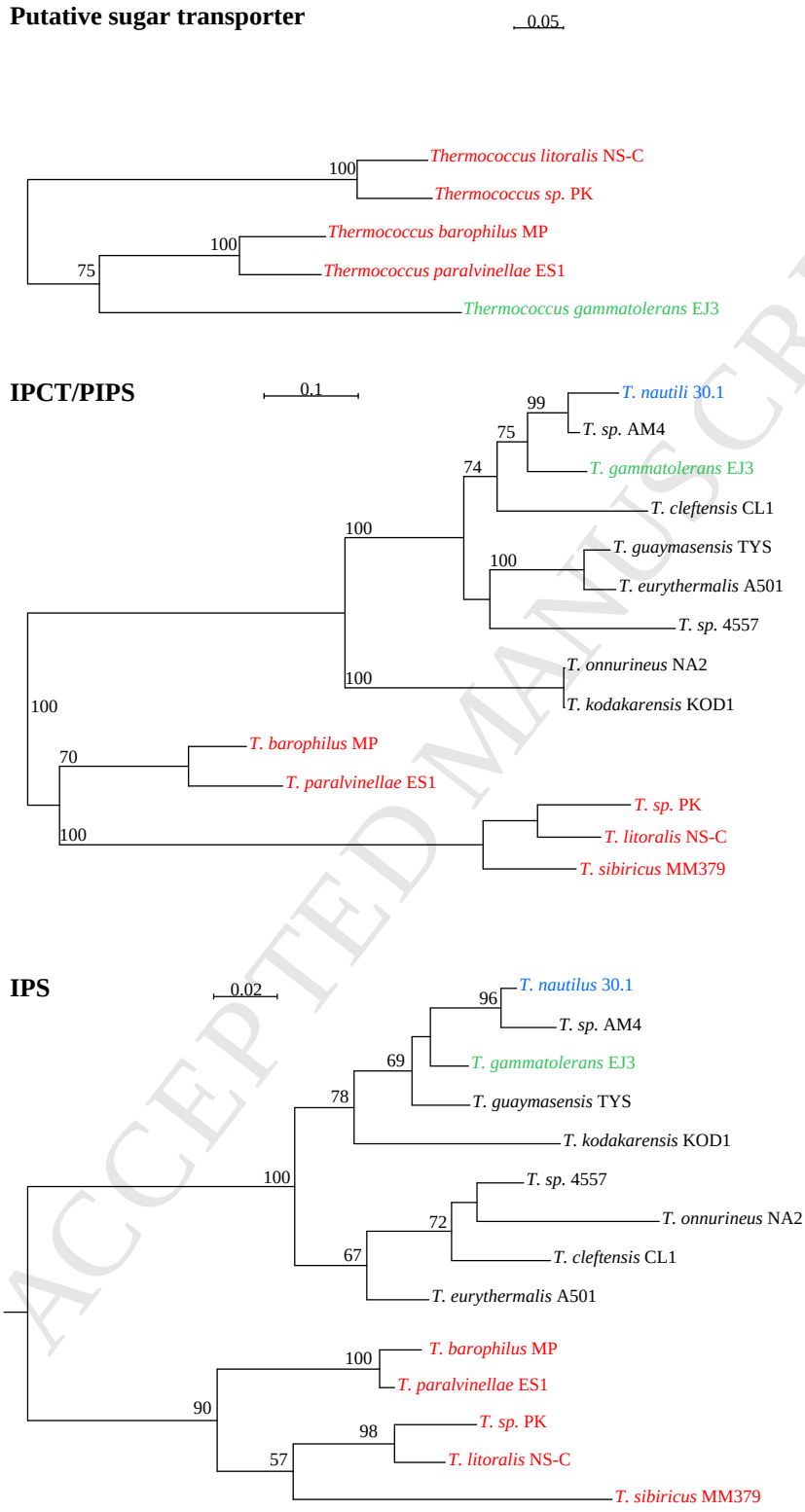
Figure 4



Cario et al. Restoration of DIP synthesis in T. barophilus
Figure S1