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A novel pathway for the synthesis of inositol phospholipids uses cytidine diphosphate (CDP)-inositol as donor of the polar head group

Carla D. Jorge, Nuno Borges and Helena Santos*
Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. da República-EAN, Apartado 127, Oeiras 2780-157, Portugal.

Summary

We describe a novel biosynthetic pathway for glycerophosphoinositides in *Rhodothermus marinus* in which inositol is activated by cytidine triphosphate (CTP); this is unlike all known pathways that involve activation of the lipid group instead. This work was motivated by the detection in the *R. marinus* genome of a gene with high similarity to CTP:L-*myo*-inositol-1-phosphate cytidylyltransferase, the enzyme that synthesizes cytidine diphosphate (CDP)-inositol, a metabolite only known in the synthesis of di-*myo*-inositol phosphate. However, this solute is absent in *R. marinus*. The fate of radiolabelled CDP-inositol was investigated in cell extracts to reveal that radioactive inositol was incorporated into the chloroform-soluble fraction. Mass spectrometry showed that the major lipid product has a molecular mass of 810 Da and contains inositol phosphate and alkyl chains attached to glycerol by ether bonds. The occurrence of ether-linked lipids is rare in bacteria and has not been described previously in *R. marinus*. The relevant synthase was identified by functional expression of the candidate gene in *Escherichia coli*. The enzyme catalyses the transfer of L-*myo*-inositol-1-phosphate from CDP-inositol to dialkylether glycerol yielding dialkylether glycerophosphoinositol. Database searching showed homologous proteins in two bacterial classes, *Sphingobacteria* and *Alphaproteobacteria*. This is the first report of the involvement of CDP-inositol in phospholipid synthesis.

Received 17 October, 2014; revised 26 November, 2014; accepted 27 November, 2014. *For correspondence. E-mail santos@itqb.unl.pt; Tel. +351 214 469 541; Fax +351 214 469 543.

Introduction

Inositol and its derivatives constitute a large family of compounds present in the three domains of life. *Myo*-inositol, the most prevalent form in nature, holds a central position in inositol metabolism and is the precursor of a variety of metabolites, such as inositol-containing phospholipids, inositol (poly)phosphates and inositol phosphodiesters (Michell, 2008; 2011; Santos *et al*., 2011). A variety of physiological roles has been assigned to inositol-derived compounds. In eukaryotes inositol, (poly)phosphates and phosphoinositides participate in cell signalling, in cell regulation and in protein anchoring at the cell surface (for reviews, see Michell, 2008; 2011). Some members of *Eukarya*, *Archaea* and *Bacteria* are able to cope with environmental stress by accumulating inositol derivatives as compatible solutes (Michell, 2011; Santos *et al*., 2011). The ionic solute, di-*myo*-inositol phosphate is the most prominent example, accumulating in hyper/thermophilic *Bacteria* and *Archaea* primarily in response to heat stress, whereas free *myo*-inositol and methyl-inositols play a role in osmoadaptation of *Eukarya* (Yancey, 2005; Michell, 2008; Santos *et al*., 2011). Phosphatidylinositol and archaetidylinositol are used as structural components of cell membranes in *Eukarya* and most *Archaea* respectively. In contrast, phosphatidylinositols and derivatives are rare in bacterial membranes, but they are essential for cell viability in some pathogenic bacteria, such as *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*, highlighting the respective biosynthetic enzymes as promising targets for the development of novel antimicrobial drugs (Jackson *et al*., 2000; Crellin *et al*., 2013).

To date, three pathways have been described for the synthesis of inositol phospholipids (Morii *et al*., 2014) (Fig. 1). In *Eukarya*, phosphatidylinositol is synthesized from inositol and cytidine diphosphate (CDP)-diacylglycerol by the action of phosphatidylinositol synthase (PIS) (Daniels and Palmer, 1980; Carman and Fischl, 1992; Antonsson, 1994). This single-step pathway is distinct from the phosphorylating pathway (two-step pathway), used in *Archaea* and *Bacteria*. In these

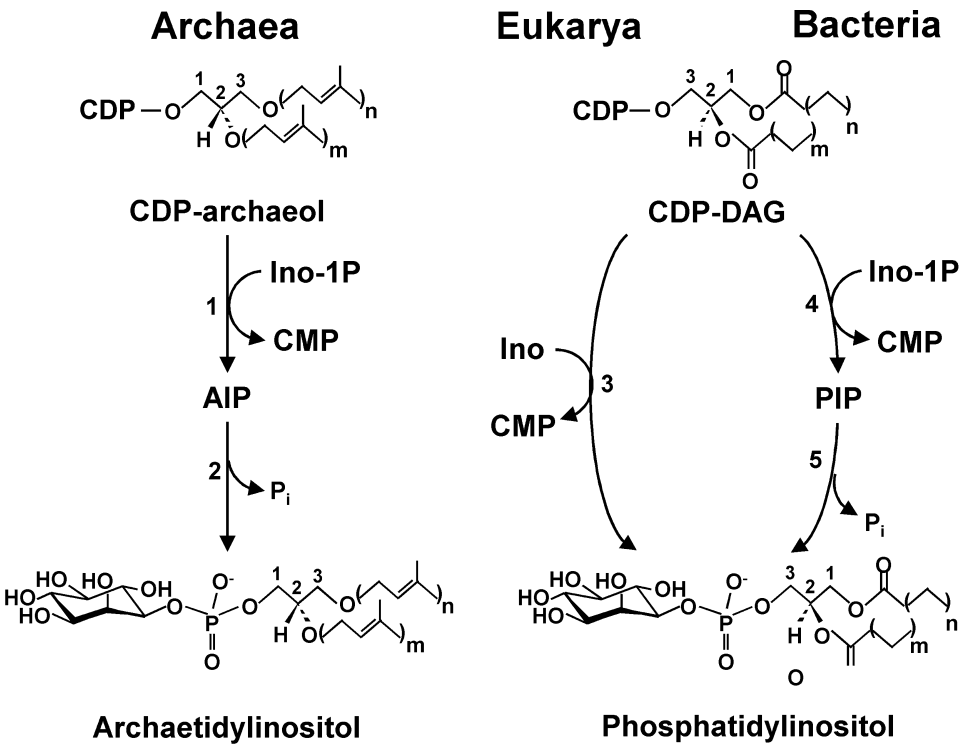

Fig. 1. The three pathways known for the synthesis of inositol glycerophospholipids in the three domains of life. Enzymes: 1, archaetidylinositol phosphate synthase; 2, archaetidylinositol phosphate phosphatase; 3, phosphatidylinositol synthase; 4, phosphatidylinositol phosphate synthase; 5, phosphatidylinositol phosphate phosphatase. Ino, *myo*-inositol; Ino-1P, L-*myo*-inositol-1-phosphate; AIP, archaetidylinositol phosphate; PIP, phosphatidylinositol phosphate; CMP, cytidine monophosphate; Pi, inorganic phosphate.

domains of life, CDP-archaeol/CDP-diacylglycerol reacts with inositol-1-phosphate to form archaetidylinositol phosphate or phosphatidylinositol phosphate as intermediates, which upon dephosphorylation yield archaetidylinositol and phosphatidylinositol respectively. Archaetidylinositol phosphate synthase (AIPS) and phosphatidylinositol phosphate synthase (PIPS) are the key enzymes of the phosphorylating pathways. The activity of AIPS was initially detected in *Methanothermobacter thermoautotrophicus* and *Pyrococcus furiosus* (Morii *et al*., 2009), and the activity of PIPS was first demonstrated in mycobacteria (Morii *et al*., 2010). Recently, it was recognized that AIPS and PIPS are ubiquitously distributed, respectively, in *Archaea* and *Bacteria* that contain inositol phospholipids (Morii *et al*., 2014).

In this work, we describe a novel pathway for the synthesis of inositol phospholipids. The relevant synthase catalyses the transfer of the inositol-1-phosphate group from CDP-inositol to dialkylether glycerol, producing the corresponding ether-linked glycerophosphoinositol. The enzyme of the thermophilic bacterium *Rhodothermus marinus* was characterized and BLAST searches revealed homologues in members of *Sphingobacteria* and *Alphaproteobacteria*, but not in *Archaea* or *Eukarya*. A phylogenetic analysis of the enzymes responsible for the synthesis of inositol phospholipids is presented.

Results

The fate of CDP-inositol in R. marinus *cell extracts*

CDP-inositol is the alcohol donor for the synthesis of di-*myo*-inositol phosphate, a compatible solute closely restricted to hyper/thermophilic *Bacteria* and *Archaea* (Borges *et al*., 2006). The analysis of the *R. marinus* genome highlighted a gene (Rmar_1196) with 28–32% of sequence identity with the genes known to encode L-*myo*-inositol-1-phosphate cytidylyltransferase (IPCT), the enzyme that synthesizes CDP-inositol from L-*myo*-inositol-1-phosphate and cytidine triphosphate (CTP). This function was confirmed by expression in *E. coli*. Thus far, CDP-inositol is known as a precursor for the synthesis of di-*myo*-inositol phosphate, and no other function has yet been ascribed to that metabolite (Rodrigues *et al*., 2007). Compatible solute accumulation in *R. marinus* has been extensively examined, but di-*myo*-inositol phosphate was never detected in the solute pool of this bacterium (Nunes *et al*., 1995; Silva *et al*., 1999). Accordingly, homologues of di-*myo*-inositol phosphate synthase are not found in the *R. marinus* genome. These observations

indicate a role for CDP-inositol other than the synthesis of di-*myo*-inositol phosphate. Therefore, we set out to investigate the fate of CDP-inositol in this thermophilic bacterium. To this end, radiolabelled CDP-inositol was produced and provided to *R. marinus* cell extracts (soluble and membrane fractions). Incorporation of radioactive inositol was detected in the lipidic phase (Fig. 2, lane 1), but not in the soluble protein fraction (data not shown).

Characterization of inositol-containing lipid product

To gain insight into the structure of the lipid product, the *R. marinus* membrane fraction was incubated with radiolabelled CDP-inositol in the presence or absence of DAG (1,2–dipalmitoyl-*sn*-glycerol), the most common glycerolipid in bacteria. Thin-layer chromatography (TLC) analyses of the reaction products showed radioactive spots with identical migration in the presence or absence of DAG (Fig. 2, lanes 1 and 2); they migrated differently from 1,2-dioleoyl-*sn*-glycero-3-phosphoinositol (phosphatidylinositol), used as standard (Fig. 2, lane 10). We concluded that the acceptor of the inositol phosphate moiety from CDP-inositol is intrinsically present in the membrane fraction and is distinct from DAG. The reaction was also performed with non-labelled CDP-inositol to obtain the amount of product needed for structural analysis. The phospholipid product was extracted from the TLC plate and analysed by liquid chromatography-mass spectrometry (LC-MS), which revealed the presence of five inositol phospholipids (see Fig. S1). The major component gave the following signals: m/z 809.6 (M-H), m/z 629.7 (M-H-180) and m/z 647.7 (M-H-162), which are assigned to the ionized molecule, and fragments resulting from loss of inositol. Signals m/z 259.1 and m/z 241.1 in Fig. 3 are characteristics of the inositol phosphate head group (Hsu and Turk, 2000). The lack of signals due to fatty acid fragments suggests an ether-linked phospholipid, i.e., the hydrocarbon chains are bound to the glycerophosphate backbone by ether linkages instead of the typical ester bonds. The nature of the ether-linkage was confirmed by incubating the recombinant lipid synthase with CDP-inositol plus ether glycerolipids extracted from *R. marinus* (see below for detailed description). The LC-MS data revealed that the major inositol phospholipid produced by *R. marinus* has the molecular formula $C_{43}H_{86}O_{11}P^-$ and a molecular mass of 810 Da. These findings indicate that the alkyl chains have a total of 34 carbon atoms. The other four minor inositol phospholipids (see Fig. S1) have molecular masses of 782, 796, 824 and 838 Da, and generated fragmentation patterns similar to that of the major product. The results suggest that the five inositol phospholipids differ by one methylene group (14 mass units). Altogether, the results demonstrate that *R. marinus* has a newly discovered activity, herein designated as Bacterial dialkylether glyceroPhosphoInositol synthase (BEPIS), which uses CDP-inositol and dialkylether glycerols to produce phosphoinositol ether lipids. Naturally, the next step is the identification of the encoding gene. We noticed that the IPCT gene was present in an operon-like structure together

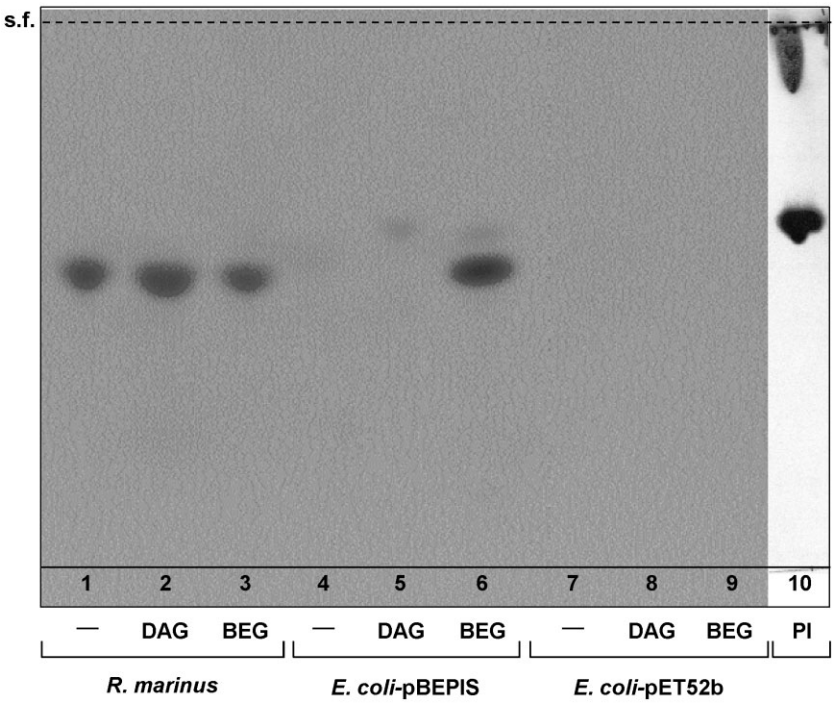

Fig. 2. Thin-layer chromatogram of radiolabelled inositol phospholipid products obtained from incubation of membrane fractions of *R. marinus*, *E. coli*-pBEPIS or *E. coli*-pET52b with radiolabelled CDP-inositol plus ether or ester glycerolipids. Each membrane fraction (200 μg) was incubated, at 65°C, during 1 h, with CDP-L-*myo*-[^{14}C]inositol in the absence (lanes 1, 4 and 7) or presence of exogenous DAG (lanes 2, 5 and 8) or exogenous bacterial dialkylether glycerols (BEG) (lanes 3, 6, and 9). Other constituents of the reaction mixture were 50 mM Tris-HCl (pH 8.0); 20 mM $MgCl_2$; 5 mM ß-mercaptoethanol, and 0.5% Triton X-100. After the enzymatic reaction, the lipid products were extracted, separated by TLC using a solvent system composed by chloroform/methanol/acetic acid/water (80:30:20:10 v/v), and the radiolabelled spots visualized by autoradiography. 1,2-Dioleoyl-*sn*-glycero-3-phosphoinositol (PI) was used as a phospholipid standard (lane 10); the commercial sample was subjected to TLC analysis using the same protocol except that spraying with molybdic acid reagent and charring at 120°C was used for spot visualization. A contaminant running near to the solvent front was also detected. s.f., solvent front.

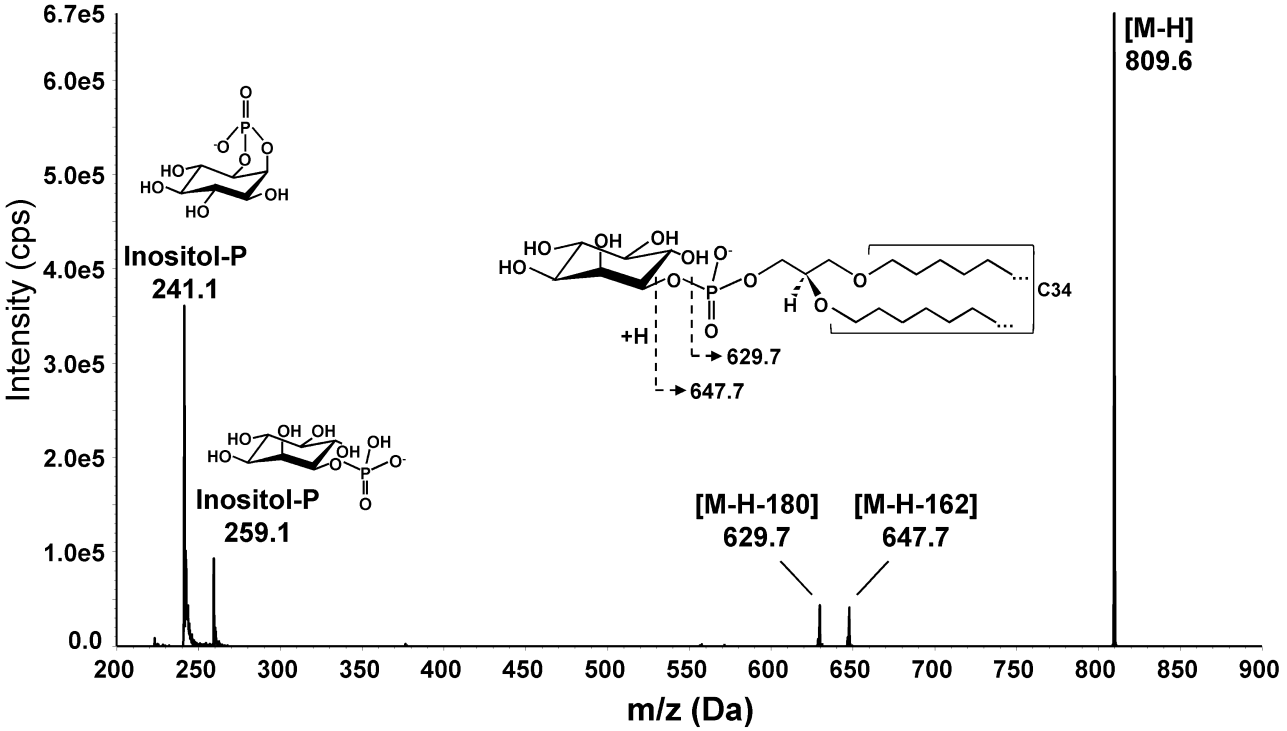

Fig. 3. Negative ion liquid chromatography-mass spectrometry (LC-MS) spectrum of the reaction product synthesized by *Rhodothermus marinus* membranes upon incubation with CDP-inositol. The signals are assigned to inositol-P (m/z = 259.1 Da), rearranged inositol-P (m/z = 241.1 Da) and fragments resulting from the loss of inositol (m/z = 629.7 and 647.7 Da), according to Hsu and Turk (2000).

with L-*myo*-inositol-1-phosphate synthase (IPS) and a gene annotated as CDP-alcohol phosphatidyltransferase (Fig. 4). Therefore, this seemed to be the most promising candidate. The activity of the recombinant protein was assessed in *E. coli* cell membranes to confirm that a preparation of dialkylether glycerols from *R. marinus* supplied with radiolabelled CDP-inositol produced as major product a compound identical to that detected in the native cell membranes (Fig. 2, lanes 1 and 6). Importantly, we found that this compound was present in *R. marinus* cells (Fig. S2). In addition, we verified that the formation of the lipidic product was dependent on CDP-inositol because it was not formed when CDP-[^{14}C]inositol was replaced by L-*myo*-[^{14}C]inositol-1-phosphate (Fig. S3). We confirmed that a membrane fraction of *E. coli* C43(DE3) harbouring the empty plasmid did not synthesize inositol phospholipids from CDP-inositol alone or combined with DAG or the dialkylether glycerol preparation derived from *R. marinus* (Fig. 2, lanes 7–9).

Sequence comparison

To explore the distribution of putative BEPIS homologues, a BLAST search using the *R. marinus* protein as query was performed in the NCBI protein database. Reliable hits (cut-off = 6e-35) were found in members of 10 bacterial genera: *Granulibacter*, *Gluconacetobacter*, *Acidomonas*, *Acidocella*, *Nitrospirillum*, *Kordiimonas*, *Novosphingobium*, *Sphingomonas*, *Erythrobacter* and *Sphingobacterium*. Significantly, BEPIS is present along with the genes for the synthesis of CDP-inositol and inositol-1-phosphate in operon-like structures (Fig. 4). The only exception to this rule is *Nitrospirillum amazonens* (45% identity with *R. marinus* BEPIS), which does not possess homologues of IPS and IPCT. Therefore, the physiological role of putative BEPIS in that organism is unknown.

Properties of recombinant enzymes BEPIS and IPCT

Incubation of recombinant BEPIS with CDP-L-*myo*-[^{14}C]inositol plus dialkylether glycerols, extracted from *R. marinus* cells, led to the production of 14 nmol of inositol phospholipids per milligram of protein. Diacylglycerol was examined as a potential substrate, but was not used by BEPIS. Inositol-1-phosphate was also evaluated as an alcohol acceptor, but it was not recognized. The presence of Mg^{2+} and detergents, namely Triton X-100 or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), was essential for enzyme activity (Table 1).

The recombinant IPCT was highly specific for CTP and inositol-1-phosphate. Other nucleotide donors [adenosine triphosphate (ATP), guanosine triphosphate

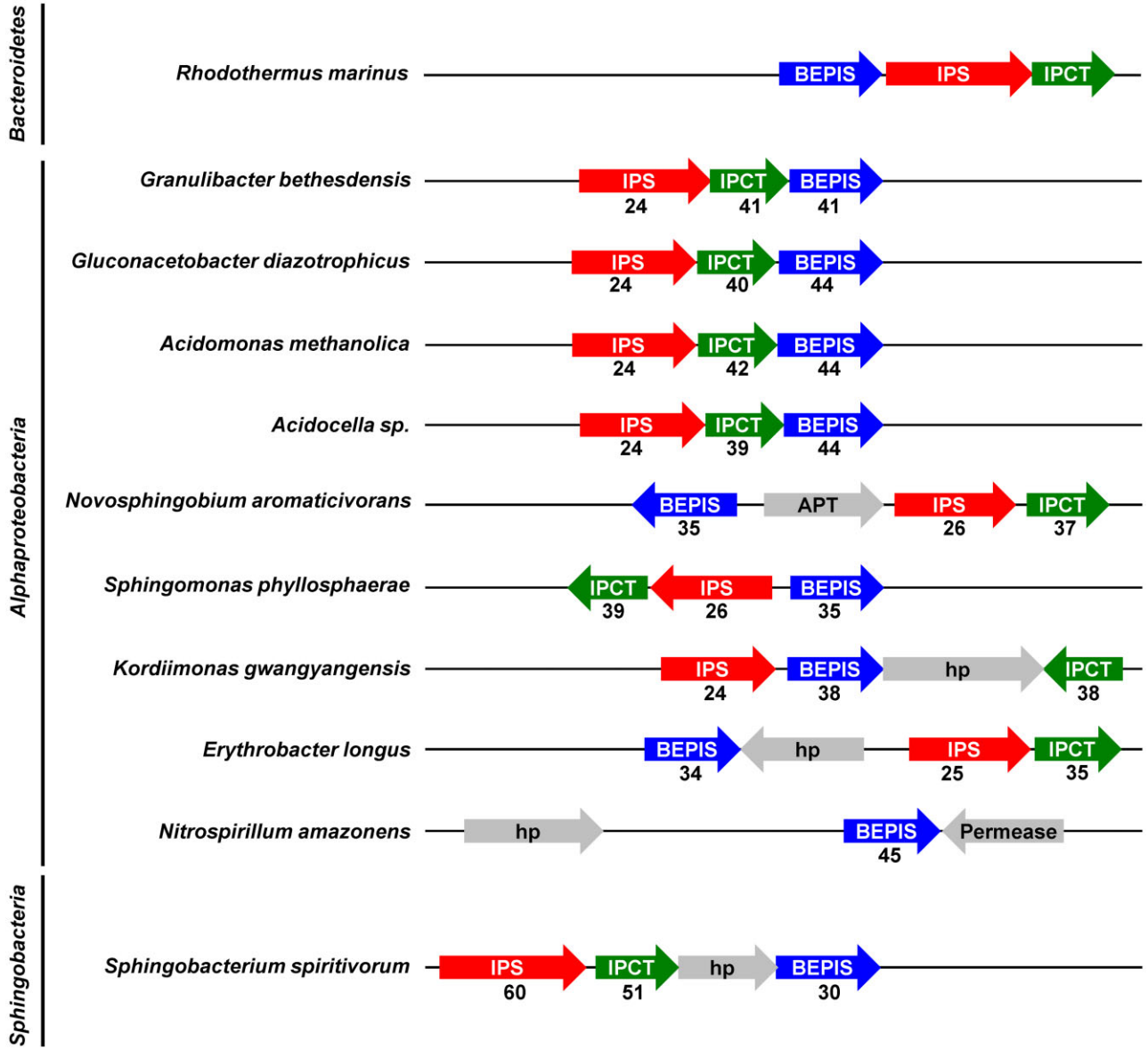

Fig. 4. Genomic organization of the genes encoding the enzymes involved in the synthesis of dialkylether glycerophosphoinositol in several bacteria: IPS, L-*myo*-inositol-1-phosphate synthase; IPCT, L-*myo*-inositol-1-phosphate cytidylyltransferase; and BEPIS, bacterial dialkylether glycerophosphoinositol synthase; hp, hypothetical protein; APT, CDP-alcohol phosphatidyltransferase; Permease, lipopolysaccharide export system permease protein. BLAST was used for sequence comparison. The numbers indicate sequence identity as percentage relative to *Rhodothermus marinus* proteins. Cover percentages in BLAST alignments are above 80% (BEPIS), 90% (IPCT), and 45% (IPS); cover percentage for IPCT of *S. spiritivorum* is 51%.

(GTP) and uridine triphosphate (UTP)] and other alcohol acceptors (*myo*-inositol, glycerol, DL-glycerol-phosphate, α-glucose-1-phosphate, β-glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1-phosphate and galactose-1-phosphate) were not substrates of the enzyme (Table S1).

The recombinant IPCT showed maximal activity in the presence of 20 mM Mg^{2+} and was strictly dependent on this cation; the activity was strongly reduced in the presence of 20 mM Co^{2+} (11%) and abolished when 20 mM Ni^{2+} was provided. The activity of IPCT at 75°C was 59.8 ± 4.0 μmol min^{-1} mg of $protein^{-1}$ and increased 3.5-fold at 95°C; no activity was detected at temperatures below 45°C. The enzyme exhibited typical Michaelis–Menten kinetics for the two substrates with apparent K_m values of 2.6 ± 0.5 mM and 1.8 ± 0.5 mM for inositol-1-phosphate and CTP, respectively, and a V_{max} of 75.7 ± 6.8 μmol min^{-1} mg of $protein^{-1}$ (Fig. S4). A half-life

Table 1. Activity of BEPIS under different reaction conditions.

Membrane fraction[a]	Reaction mixture[c]	Relative activity (%)[b]
E. coli-pBEPIS	CDP-[^{14}C]inositol + *R. marinus* ether glycerolipids + $MgCl_2$ + Triton X-100	100
	Without ether glycerolipids	0.7 ± 0.1
	Without $MgCl_2$	1.8 ± 0.2
	Without Triton X-100	41.3 ± 1.3
	With Triton X-100 replaced by CHAPS[d]	93.3 ± 3.8
	With ether glycerolipids replaced by DAG[e]	1.1 ± 0.0
E. coli-pET52b	CDP-[^{14}C]inositol + *R. marinus* ether glycerolipids + $MgCl_2$ + Triton X-100	0
R. marinus	CDP-[^{14}C]inositol + *R. marinus* ether glycerolipids + $MgCl_2$ + Triton X-100	18.6 ± 0.4
	With addition of DAG[e]	11.0 ± 1.2

a. 200 μg of protein from each membrane fraction was used per assay.
b. 100% corresponds to 14.0 nmol ± 1.2 ether-linked inositol phospholipid per mg of protein determined after 1 h of incubation.
c. Complete reaction mixture contained 26 μM CDP-L-*myo*-[^{14}C]inositol, 0.6 mM of unlabelled CDP-L-*myo*-inositol, ether glycerolipids from *R. marinus*, 20 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.5% (w/v) Triton X-100 and 50 mM Tris-HCl (pH 8.0).
d. Triton X-100 was replaced by 0.5% (w/v) CHAPS in the reaction mixture.
e. Exogenous ether glycerolipids were replaced by diacylglycerol (DAG).

of approximately 5 h at 75°C was exhibited by the enzyme. In addition, the residual activity of IPCT was determined after 10 min of incubation at temperatures between 75 and 90°C. The enzyme retained full activity at 75°C, but lost 20%, 47% and 88% after 10 min at 85°C, 87°C, and 90°C respectively.

Discussion

A large number of studies have been performed on the thermophilic bacterium *R. marinus*, especially in view of the potential biotechnological interest of its thermostable enzymes, such as mannanase, pullulanase, chitinase, cellulase, laminarinase and xylanase (Bjornsdottir *et al*., 2006). Our group investigated the profiles of solute accumulation in this organism and established the routes for biosynthesis (Martins *et al*., 1999; Silva *et al*., 1999). Mannosylglycerate and mannosylglyceramide are the major organic solutes, accumulating primarily in response to heat and osmotic stress respectively (Nunes *et al*., 1995; Silva *et al*., 1999). Di-*myo*-inositol phosphate, a canonical solute of hyper/thermophilic *Bacteria* and *Archaea*, has not been found in any of the *R. marinus* strains examined (Santos *et al*., 2011), but the present work shows that this bacterium possesses an enzyme that produces CDP-inositol, which is a precursor for the synthesis of di-*myo*-inositol phosphate (Fig. 4). Therefore, this finding immediately raises the question about the physiological role of CDP-inositol in *R. marinus*. In this study, we identified a novel biosynthetic pathway that uses CDP-inositol and dialkylether glycerol as substrates to produce dialkylether glycerophosphoinositol (Fig. 5). Hence, our work revealed a second metabolic fate for CDP-inositol: the synthesis of ether-linked glycerophospholipids in addition to the early described synthesis of di-*myo*-inositol phosphate (Fig. 5). Coincidentally, only two metabolic fates have been ascribed to the related metabolite, CDP-glycerol, i.e., the synthesis of the compatible solute glycero-phospho-inositol and the synthesis of poly(glycerol-phosphate), which is a precursor of teichoic acids (Borges *et al*., 2006; Schertzer and Brown, 2008).

The newly described pathway (BEPI pathway) uses CDP-inositol as the polyol donor plus non-activated glycerolipid, whereas the three routes described thus far for the synthesis of inositol phospholipids [i.e., 1,2-dioleoyl-*sn*-glycero-3-phosphoinositol (PI), AIP and PIP pathways] invariably use CTP-activated glycerolipids: CDP-archaeol for AIP synthesis and CDP–DAG for PI and PIP syntheses (Daniels and Palmer, 1980; Carman and Fischl, 1992; Antonsson, 1994; Morii *et al*., 2009; 2010). To our knowledge, this is the first report on the use of activated inositol in the synthesis of inositol phospholipids. However, activated polar heads are used by most eukaryotic cells for the synthesis of phosphatidylethanolamine and phosphatidylcholine via the Kennedy pathway, a route rarely found in *Bacteria* (Kent *et al*., 2004; Gibellini and Smith, 2010).

This study unequivocally demonstrates the presence of ether-linked glycerolipids in the membranes of the bacterium *R. marinus*. Lipids with glycerol-ether linkages constitute a characteristic specific to the domain *Archaea* and they are only sporadically observed within the domain *Bacteria*: ether-linked inositol phospholipids described in the myxobacterium *Stigmatella aurantiaca* (Caillon *et al*., 1983); ether-phospholipids with glycerol as head group were detected in *Stigmatella aurantiaca* and in *Leucobacter exalbidus* (Caillon *et al*., 1983; Ue, 2011); ether-linked phospholipids with ethanolamine, hexoses and aminopentanetetrol were observed in *Thermodesulfobacterium* spp. (Hamilton-Brehm *et al*., 2013); mixed ether/ester polar lipids have been detected in *Stigmatella aurantiaca*, in *Thermodesulfobacterium* spp., in the sulfate reducers *Desulfosarcina variabilis* and

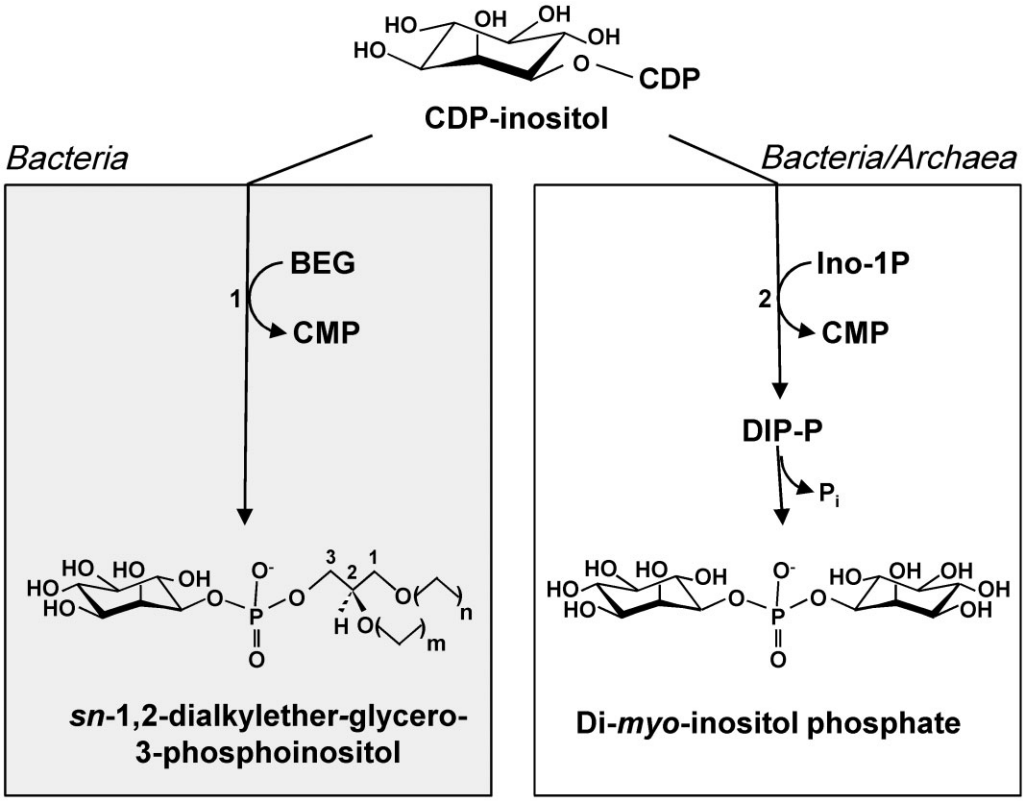

Fig. 5. The two pathways that use CDP-inositol. The novel pathway for inositol phospholipid synthesis (left panel) uses as substrates CDP-inositol and dialkylether glycerol (BEG), whereas the pathway for di-*myo*-inositol phosphate synthesis (right panel), uses CDP-inositol and inositol-1-phosphate. Enzymes: 1, dialkylether glycerophosphoinositol synthase (BEPIS); 2, di-*myo*-inositol phosphate phosphate synthase. Abbreviations: Ino-1P, L-*myo*-inositol-1-phosphate; DIP-P, phospho-di-*myo*-inositol phosphate; Pi, inorganic phosphate; CMP, cytidine monophosphate.

Desulforhabdus amnigenus, in members of the deep-branching hyperthermophilic genera *Aquifex* and *Thermotoga* and in the mesophile *Propionibacterium propionicum* (Huber *et al*., 1992; Rütters *et al*., 2001; Paściak *et al*., 2003; Damsté *et al*., 2007).

In addition to the prevalence of ether linkages in the phospholipids of *Archaea* and ester linkages in *Bacteria*, the stereo-configuration of the glycerophosphate moiety of glycerophospholipids is a characteristic distinctive of these two domains of life: *sn*-glycerol-1-phosphate is found in the core of archaeal glycerophospholipids, whereas the configuration *sn*-glycerol-3-phosphate is typical of the bacterial counterparts. The stereochemistry of glycerol-phosphate in the newly described dialkylether inositol phospholipid has not been investigated; however, we postulate that the alkyl chains are linked at positions 1 and 2 of glycerol-3-phosphate because the genome of *R. marinus* possesses a homologue of *sn*-glycerol-3-phosphate dehydrogenase, whereas *sn*-glycerol-1-phosphate dehydrogenase is not predicted. On the basis of this reasoning, the novel phospholipid of *R. marinus* should be designated *sn*-1,2-dialkylether-glycero-3-phosphoinositol.

Interestingly, the genes implicated in the synthesis of this inositol phospholipid of *R. marinus*, i.e., coding for IPS, IPCT and BEPIS, are predicted in the genome of a few pathogenic bacteria, namely *Granulibacter bethesdensis* and *Shingomonas spiritivorum*, known to cause a variety of diseases, such as bacteremia, peritonitis and chronic respiratory infections, in medically compromised patients (Tronel *et al*., 2003; Greenberg *et al*., 2006; Holland, 2010). Importantly, the massive utilization of antibiotics in recent decades has led to the emergence of bacterial resistance and the pressing need for novel antimicrobials. Because BEPIS is unrelated with human PIS, it may be a suitable target for the development of efficient antibiotics, provided that the enzyme is essential for pathogen viability.

The BEPIS protein has four predicted transmembrane segments (TMHMM SERVER V. 2.0) and contains the conserved motif [D-G-2(X)-A-R-8(X)-G-3(X)-D-3-(X)-D] that is characteristic of the CDP-alcohol phosphatidyltransferase family (Pfam 01066). Recently, the first three-dimensional structures of two members of this family were resolved by X-ray crystallography (Nogly *et al*., 2014; Sciara *et al*., 2014). Besides BEPIS, this large family includes other

synthases that use inositol-containing substrates, i.e., PIS, PIPS, AIPS and phospho-di-*myo*-inositol phosphate synthase (DIPPS). An unrooted phylogenetic tree (Fig. 6) showed a topology with five groups of sequences (bootstrap values greater than 70). The tree displays a clear division between enzymes involved in two-step pathways (AIPS, PIPS and DIPPS), and single step-pathways (BEPIS and PIS). In other words, there is a separation of the three enzyme clusters, which use inositol-1-phosphate as substrate, from the other two, which use inositol or CDP-inositol. It appears that the formation of a phosphorylated intermediate has been a determining factor during evolution. It is surprising that BEPIS homologues are restricted to the domain *Bacteria* given that the synthesis of the substrate CDP-inositol occurs in many representatives of hyperthermophilic archaea that accumulate di-*myo*-inositol phosphate. It is postulated that the BEPI pathway and di-*myo*-inositol phosphate synthesis have distinct evolutionary histories. Interestingly, a search in the genome databases showed no example of co-existence of the two biosynthetic pathways in a single organism.

In summary, this work describes a novel route for the synthesis of inositol phospholipids in which the polar head is CTP activated; this is unlike all known pathways that involve activation of the lipid group instead. Also, it shows that *R. marinus* synthesises inositol phospholipids with the glycerol backbone linked to the non-polar chains through ether bonds. The occurrence of inositol and ether linkages in the membrane components of bacteria is extremely rare, whereas these chemical traits are widespread in *Archaea*. *Rhodothermus marinus* is well adapted to high temperature (grows up to 77°C), but it is unlikely that these 'archaeal' features are related with the extremophilic character of this bacterium because homologues of the synthase are absent in the archaeal genomes currently available. A greater effort should be made to achieve a comprehensive characterization of membrane lipids, especially of prokaryotes, so that the biochemical and phylogenetic significance of the results reported here can be fully appreciated.

Experimental procedures

Materials

Glucose, glucose-6-phosphate, *myo*-inositol, L-*myo*-inositol-1-phosphate, α-glucose-1-phosphate, β-glucose-1-phosphate, fructose-6-phosphate, fructose-1-phosphate, galactose-1-phosphate, NAD^+, CTP, ATP, UTP, GTP, glycerol and DL-glycerol-phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Di-*myo*-inositol phosphate from *Pyrococcus woesei* was obtained from bitop AG (Witten, Germany). DAG and 1,2-dioleoyl-*sn*-glycero-3-phosphoinositol (PI) were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA). L-*myo*-[^{14}C]inositol-1-phosphate and CDP-L-*myo*-[^{14}C]inositol were synthesized enzymatically (this work) from [^{14}C(U)]glucose (3.7 MBq ml^{-1}) obtained from Perkin-Elmer Life Sciences.

*Preparation of L-*myo*-[^{14}C]inositol-1-phosphate and CDP-L-*myo*-[^{14}C]inositol*

CDP-L-*myo*-[^{14}C]inositol was produced from [^{14}C(U)]glucose by coupling the following enzymatic activities: hexokinase from *Thermoproteus tenax*, L-*myo*-IPS from *Archaeoglobus fulgidus* and L-*myo*-IPCT from *P. furiosus*. *Escherichia coli* cells harbouring the hexokinase or the *ips* genes were grown and induced as described previously (Rodrigues *et al*., 2007). Partial purification of recombinant hexokinase and IPS was carried out by heating the cell extracts for 30 min at 90°C and 60°C respectively. The *ipct* gene (PF_1058) was amplified by polymerase chain reaction (PCR) using Pfu polymerase (Fermentas) and cloned into the pET19b plasmid (Novagen), between the NdeI and BamHI sites. *Escherichia coli* C43(DE3) cells bearing this construct were grown in Luria–Bertani (LB) medium, at 37°C, with ampicillin (0.1 mg ml^{-1}) to an optical density at 600 nm of 0.6 and then protein production was induced with 1 mM IPTG for 5 h. Cells were harvested, suspended in Tris-HCl (20 mM; pH 7.6) containing 10 mM $MgCl_2$ and disrupted in a French press (100 mPa). Cell debris was removed by centrifugation (20 000 × *g*, 10 min, 4°C) and the cell extract was heated at 90°C for 10 min. Upon centrifugation (20 000 × *g*, 30 min, 4°C), the heat-treated cell extract was applied onto a His-Trap HP column (GE Healthcare BioScience AB, Uppsala, Sweden) and the IPCT was eluted with 500 mM imidazole. The final preparation of recombinant IPCT was judged pure by SDS-PAGE gel analysis.

The production of [^{14}C]glucose-6-phosphate was carried out in a reaction mixture (final volume of 1 ml) containing the recombinant hexokinase (2 mg), [^{14}C(U)]glucose (3.7 MBq 336 nmol^{-1}), 10 mM glucose, 5 mM ATP, 50 mM Tris-HCl (pH 7.6) and 10 mM $MgCl_2$. After 1 h of incubation at 70°C, the reaction mixture was centrifuged (10 000 × *g*, 10 min, 4°C). The resulting supernatant (800 µl) containing [^{14}C]glucose-6-phosphate was added to a reaction mixture (final volume of 1.2 ml) composed by the recombinant IPS (2.5 mg), 5 mM NAD^+ and 50 mM Tris-HCl (pH 7.6). After incubation at 85.5°C for 1 h, the reaction mixture was centrifuged (10 000 × *g*, 10 min, 4°C) and the resulting supernatant containing L-*myo*-[^{14}C]inositol-1-phosphate was mixed with activated charcoal to eliminate residual nucleotides. The partially purified L–*myo*-[^{14}C]inositol-1-phosphate (450 µl) was added to a reaction mixture (final volume of 825 µl) containing pure IPCT (60 µg), 5 mM CTP, 20 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.6). Upon incubation at 90°C for 30 min, the reaction mixture was filtered through a 10 kDa Omega Nanosep filter (Pall Life Sciences, Hampshire, UK) to remove proteins. The filtrate was treated with alkaline phosphatase (Roche) to dephosphorylate residual amounts of [^{14}C]glucose-6-phosphate and L-*myo*-[^{14}C]inositol-1-phosphate. The filtrate was analysed by TLC. This analysis revealed the presence of CDP-L-*myo*-[^{14}C]inositol as a major compound and also vestigial amounts of [^{14}C]glucose and L-*myo*-[^{14}C]inositol. The CDP-L-*myo*-[^{14}C]inositol produced

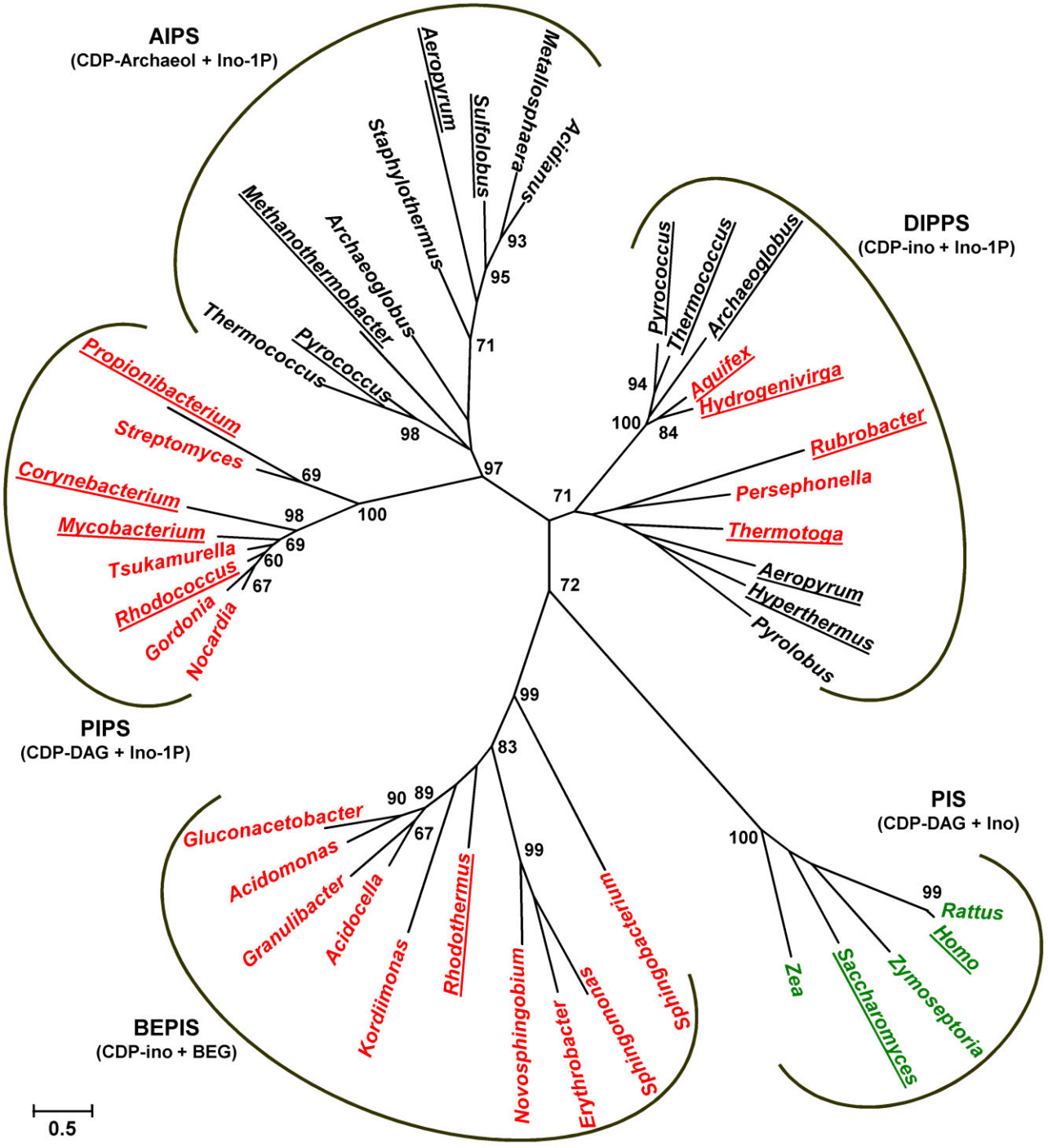

Fig. 6. Phylogenetic relationship of BEPIS and other members of the CDP–alcohol phosphatidyltransferase family that use inositol-containing substrates (i.e., AIPS, PIPS, PIS, and DIPPS proteins). MEGA6 software (Tamura *et al.*, 2013) was used for sequence alignment and to generate the phylogenetic tree using the maximum likelihood method based on the Tamura–Nei model (Tamura and Nei, 1993). Bootstrap values were calculated from 100 replicates. Only bootstrap values greater than 60 are shown. The scale bar represents the number of substitutions per site. *Bacteria* (red), *Archaea* (black) and *Eukarya* (green). Organisms possessing enzymes with the activity confirmed are underlined. The GenPept accession numbers are given in Table S2.

was quantified and used as a substrate of inositol phospholipid synthase enzyme.

Bacterial strain and growth conditions

Rhodothermus marinus strain DSM 4252^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Cultures of *R. marinus* were grown at 65°C, on Degryse 162 medium, as modified by Nunes and colleagues (1992), containing per litre, 2.5 g of tryptone, 2.5 g of yeast extract and 10 g of NaCl. Cell growth was monitored by measuring the turbidity at 600 nm. *Pyrococcus furiosus* strain COM1 was grown in medium as described in Esteves *et al*., (2014).

Extraction of R. marinus *phospholipids and preparation of ether-linked glycerolipids*

Total lipids of *R. marinus* were extracted using the trichloroacetic acid Bligh–Dyer extraction method described by Nishihara and Koga (1987). Briefly, frozen *R. marinus* cells (20 g, dry weight) were thawed in 20 ml of buffer A (50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$ and 5 mM β-mercaptoethanol) and disrupted by sonication (5 × 1 min pulses, with 1 min of cooling intervals between pulses). Cell debris was removed by centrifugation (10 000 × *g*, 10 min, 4°C) and the membrane fraction was obtained from centrifugation of the supernatant at 100 000 × *g* for 2 h at 4°C. Membranes were re-suspended in 20 ml of buffer A and mixed with 200 ml of $CHCl_3$/methanol/5% trichloroacetic acid (1:2:0.8, v/v). This mixture was stirred for 2 h at room temperature followed by addition of 200 ml of $CHCl_3$/water (1:1, v/v) to promote phase separation. The lower chloroform phase was removed and washed several times with 1.9 volumes of methanol/water (1:0.8; v/v) to eliminate trichloroacetic acid. The extracted lipids were dried under a stream of nitrogen. Ether-linked glycerolipids were recovered after acid methanolysis; the extracted lipids were mixed with 5% HCl-methanol and stirred for 3 h at 100°C (Nishihara and Koga, 1987). After cooling, the ether-linked glycerolipids were extracted as above described, except that the 5% trichloroacetic acid in the aqueous phase was replaced by water. The dried ether glycerolipids were re-suspended in 5% Triton X-100, stored at –20°C and used as substrate for inositol phospholipid synthase enzyme.

Cloning and expression of ipct and bepis genes

Chromosomal DNA was extracted from *R. marinus* with phenol-chloroform-isoamyl alcohol (Marmur, 1961). The *ipct* (YP_003290474.1) and *bepis* (YP_003290472.1) genes from *R. marinus* were amplified by PCR using *Pfu* DNA polymerase (Biolabs). The *ipct* gene was cloned into pET23a (Novagen) plasmid between NdeI and XhoI sites, and the *bepis* gene was cloned in the pET52b (Novagen) plasmid between NcoI and KpnI sites, yielding pTRm and pBEPIS, respectively. *Escherichia coli* BL21(DE3) and *E. coli* C43(DE3) were transformed with pTRm and pBEPIS, respectively, and transformants were selected on LB agar plates with ampicillin (0.1 mg ml^{-1}). *Escherichia coli* cells harbouring each construct were grown at 37°C in LB medium with ampicillin (0.1 mg ml^{-1}) to an optical density at 600 nm of 0.6, and protein synthesis was induced by addition of 1 mM IPTG (Roche Molecular Biochemicals). After induction, growth was allowed to continue for further 5 h. Cells were harvested by centrifugation and suspended in Tris-HCl (20 mM, pH 7.6) containing 10 mM $MgCl_2$, and disrupted twice by French press (100 mPa). Cell debris was removed by centrifugation (20 000 × *g*, 10 min, 4°C) and the cell free extracts were used for protein purification and/or characterization.

Purification of recombinant IPCT

The recombinant IPCT was purified from heat-treated cell free extracts (10 min at 80°C) by fast protein liquid chromatography (Amersham Biosciences). The heat-treated extract was applied to a Resource Q column (equilibrated with 20 mM Tris-HCl, pH 7.6) and eluted with a linear NaCl gradient (0.0–1.0 M) in the same buffer. IPCT activity was detected by ^{31}P-NMR spectroscopy (see next section for details), in the fractions eluted between 180 and 250 mM NaCl. These fractions were pooled, concentrated in a 10-kDa cut-off Centricon (Amicon) and loaded onto a Mono Q column (20 mM Tris-HCl, pH 7.6). IPCT activity was eluted at about 260 mM NaCl. The active samples were applied to a Superdex 200 gel-filtration column equilibrated with 20 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and elution was performed with this buffer. Protein concentration was estimated by the Bradford method (Bradford, 1976) and the purity evaluated by SDS-PAGE.

Biochemical characterization of recombinant IPCT

The biochemical properties of recombinant IPCT were assessed in reaction mixtures (400 µl) containing 50 mM Bis-Tris-Propane (pH 8.0), 20 mM $MgCl_2$, 8.8 mM CTP and 10 mM L-*myo*-inositol-1-phosphate. The mixtures were pre-incubated for 2 min at 75°C and the reactions initiated by addition of recombinant IPCT (5 µg) and stopped at different time points by immersion in liquid nitrogen. Subsequently, 12.5 µl of EDTA (0.5 M; pH 8.0) and 200 µl of ^2H_2O were added to the reaction mixture. ^{31}P-NMR spectroscopy was used to identify and quantify the reaction products. A known amount of di-glycerol-phosphate was added and used as an internal concentration standard for quantification of compounds. Spectra were acquired on a Bruker DRX500 spectrometer. The effect of divalent cations, Mg^{2+}, Ni^{2+} and Co^{2+}, on IPCT activity was determined. The temperature profile for activity was investigated between 45°C and 95°C at pH 8.0. IPCT thermostability was assessed at 75°C, 85°C, 87°C and 90°C by incubating the IPCT in 50 mM Bis-Tris-Propane (pH 8.0). The kinetic parameters (K_m and V_{max}) were assessed at 75°C in reaction mixtures containing 8.8 mM CTP and 0–10 mM L-*myo*-inositol-1-phosphate or 10 mM L-*myo*-inositol-1-phosphate and 0–8.8 mM CTP. The concentrations of the stock solutions of CTP and inositol-1-phosphate were also determined by ^{31}P-NMR. At appropriate times, samples were withdrawn and immediately examined for residual IPCT activity at 75°C. The kinetic parameters were obtained by fitting the curves to a hyperbolic function using the ORIGIN 5.0 PROFESSIONAL software (Origin Labs). The

substrate specificity of IPCT was studied in assays using CTP (8.8 mM) and each of the following substrates (10 mM): *myo*-inositol; *myo*-inositol-1-phosphate; glycerol; DL-glycerol-phosphate; α-glucose-1-phosphate; β-glucose-1-phosphate; glucose-6-phosphate; fructose-6-phosphate; fructose-1-phosphate, galactose-1-phosphate. ATP, GTP and UTP (10 mM) were also investigated as putative nucleotidyl donors in combination with inositol-1-phosphate (10 mM).

Preparation of membrane fractions containing native and recombinant BEPIS

Frozen *R. marinus* cells (approximately 4 g) and frozen *E. coli*-pBEPIS cells (approximately 4 g) were thawed in 5 ml of buffer A (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol) and disrupted by sonication (5×60 s pulses with 60 s cooling intervals between pulses). Intact cells and debris were separated by centrifugation ($10\,000 \times g$, 10 min, 4°C) and membrane fractions were obtained by centrifugation of the supernatant at $100\,000 \times g$ for 2 h at 4°C. The membrane fractions were re-suspended in 0.5 ml of buffer A and frozen at −20°C until use.

Assessment of BEPIS activity

The ability of BEPIS to produce inositol phospholipids was evaluated. The reaction mixtures (final volume, 200 µl) contained *R. marinus* or *E. coli*-pBEPIS membrane fractions (20 µg), 26 µM (5 nmol) CDP-L-*myo*-[^{14}C]inositol, 0.6 mM (128 nmol) of unlabelled CDP-L-*myo*-inositol, 0.6 mM DAG (or *R. marinus* ether glycerolipids), 20 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.5% (v/v) Triton X-100 and 50 mM Tris-HCl (pH 8.0). The reaction mixtures were incubated at 65°C during 1 h and stopped by addition of 1 ml of 0.1 M HCl in methanol. The mixtures were placed in glass tubes containing 1.5 ml of 0.1 M HCl in methanol and 2.5 ml of $CHCl_3$. The partition of the mixtures into aqueous and organic layers was performed with addition of 2.15 ml of 1 M $MgCl_2$. The organic layer was removed and washed twice with 0.1 M HCl, methanol 1 M $MgCl_2^{-1}$ (1:0.8, v/v). The radiolabelled inositol phospholipids produced were separated by TLC and quantified using a liquid scintillation counter (Beckman LS 6500). This assay was also carried out at 90°C with the membrane fraction of *P. furiosus*, but no activity was detected.

The ability of BEPIS to synthesize polyol-phosphodiesters (di-*myo*-inositol phosphate phosphate or glycero-phospho-*myo*-inositol phosphate) was also studied in reaction mixtures (0.4 ml) containing crude cell extract of *R. marinus* or *E. coli*-pBEPIS (around 2 mg), 10 mM $MgCl_2$, 5 mM of substrates and 20 mM Tris-HCl pH 7.6. The substrates tested were L-*myo*-inositol-1-phosphate; glycerol and DL-glycerol-3-phosphate as alcohol acceptors and CDP-inositol and CDP-glycerol as alcohol donors. The reactions were incubated at 65°C during 1 h and the products were analysed by ^{31}P-NMR spectroscopy.

Thin-layer chromatography

TLC analyses were performed in Silica Gel 60 plates, (Merck, Tokyo, Japan). The TLC of radiolabelled CDP-L-*myo*-inositol was performed with the solvent system composed by *n*-propanol/acetic acid (25%) (50:75; v/v), whereas the TLC of phospholipids was carried out with chloroform/methanol/acetic acid/water (80:30:20:10; v/v). Radioactive compounds were visualized using a Molecular Dynamics PhosphorImager (Laser Scanner), whereas non-radiolabelled compounds were visualized by spraying with molybdic acid reagent and heating.

Identification of the BEPIS reaction products

To obtain structural information of the product synthesized by BEPIS, a reaction of 5 ml was performed with membrane fractions of *R. marinus* and also with membrane fractions of *E. coli*-pBEPIS. The final concentration of the substrates remained unchanged, but the incubation time was increased to 6 h. Exogenous lipids were not added to the reaction mixture containing the membrane fraction of *R. marinus* (native BEPIS); in contrast, ether glycerolipids obtained from *R. marinus* were added to the reaction mixtures catalysed by the recombinant BEPIS. The phospholipids produced were extracted as described above and the organic phase was concentrated under a stream of nitrogen. The product obtained was purified by TLC and analysed by LC/MS using an inductively coupled plasma-DRC2 mass spectrometer in a negative ionization mode (Avanti Polar Lipids, Inc, Alabaster, USA).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Reversed-phase HPLC chromatogram of the reaction products synthesized by *Rhodothermus marinus* membranes using CDP-inositol. Peaks are labelled with m/z (Da). The minor components originated fragmentation patterns similar to that of the major product.

Fig. S2. Thin-layer chromatogram of total lipids extracted from *R. marinus* (lanes 1–3) and of the phospholipid product obtained by incubating the membrane fraction of *E. coli*-pBEPIS with radiolabelled CDP-inositol and the ether glycerolipid fraction obtained from acid methanolysis of *R. marinus* lipids (lane 4). Total lipids of *R. marinus* were obtained using the trichloroacetic acid Bligh–Dyer extraction method and applied to the TLC plate: 2.5 μl (lane 1), 5 μl (lane 2), 10 μl (lane 3). The membrane fraction of *E. coli*-pBEPIS (200 μg) was incubated at 65°C for 1 h with CDP-L-*myo*-[^{14}C]inositol plus *R. marinus* ether glycerolipids (lane 4). Other constituents of the reaction mixture were 50 mM Tris-HCl (pH 8.0); 20 mM $MgCl_2$; 5 mM β-mercaptoethanol, and 0.5% Triton X-100. After the enzymatic reaction, the lipid product was extracted and applied to a TLC plate. Lipids were separated using the solvent system chloroform/methanol/acetic acid/water (80:30:20:10 v/v). Lipids were visualized by spraying with molybdic acid reagent followed by charring at 120°C (left panel), or by autoradiography (right panel), s.f., solvent front. The results show that *R. marinus* cells contain the dialkylether glycerophosphoinositol.

Fig. S3. Thin-layer chromatogram of phospholipid products obtained from incubation of the membrane fraction of *R. marinus* (200 μg) with: radiolabelled CDP-inositol plus DAG (lane 1); radiolabelled CDP-inositol (lane 2); radiolabelled inositol-1-phosphate (lane 3). Other constituents of the reaction mixture were 50 mM Tris-HCl (pH 8.0); 20 mM $MgCl_2$; 5 mM β-mercaptoethanol, and 0.5% Triton X-100. After 1 h at 65°C, the lipid product was extracted and applied to the TLC plate. Lipids were separated using the solvent system chloroform/methanol/acetic acid/water (80:30:20:10 v/v). Autoradiography was used for spot visualization, s.f., solvent front.

Fig. S4. Initial rate of the reaction catalysed by IPCT as a function of the substrate concentration. (**A**) L-*myo*-inositol-1-phosphate (ino-1P); (**B**) CTP. The reaction mixtures, containing 8.8 mM CTP and 0 to 10 mM ino-1P or 10 mM ino-1P and 0 to 8.8 mM CTP, were incubated at 75°C. The reaction product, CDP-inositol, was quantified by ^{31}P-NMR. The kinetic parameters (K_m and V_{max}) were obtained by fitting the curves to a hyperbolic function using the ORIGIN 5.0 PROFESSIONAL software (Origin Labs). K_m for ino-1P, 2.6 mM; K_m for CTP, 1.8 mM; V_{max} = 75.7 pmol min^{-1} mg of protein^{-1}.

Table S1. Substrate specificity of recombinant IPCT[a].

Table S2. GI/GenBank NCBI accession numbers of the enzymes (AIS, PIS, PIPS, BEPIS and DIPPS) used to construct the phylogenetic tree.