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Stereochemistry of phosphatidylglycerols from thermotolerant bacteria isolated thermal springs

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ABSTRACT

Phosphatidylglycerol (1,2-diacyl-*sn*-glycero-3-phospho-glycerol) (PG) is one of the most abundant lipids in biological membranes. However, the chirality of the carbon atom in glycerol phosphate differs among the three kingdoms: bacteria, archaea, and eukaryotes. It is commonly assumed that archaea, as well as bacteria and eukaryotes, produce only one isomer of PG. Archaeal membranes consist of phospholipids with glycerol-1-phosphate in the *S* configuration, while the phospholipids of the other two kingdoms contain glycerol-3-phosphate with *(R)* stereochemistry. Another chiral atom is found in glycerol with non-esterified hydroxy groups. Considering the high temperatures that accompanied the origin of life on Earth, it becomes obvious that it is necessary to clarify the importance of membrane lipids in early evolutionary times. To reconstruct the effect of high temperatures on membrane lipids, it is ideal to use microorganisms originating from a thermophilic environment analogous to the early Earth, such as the thermal groundwater of the famous spa town of Karlovy Vary. Here, we prepared all four isomers of PG, i.e., *(R,S, R,R, S,R)*, and *(S,S)*, by organic synthesis and analyzed the representation of individual molecular species in seven bacteria isolated from the Karlovy Vary thermal springs using chiral chromatography - mass spectrometry. Our results provide evidence that five of these strains produce all four isomers of PG and that this production is highly dependent on the cultivation temperature. Subsequent analysis by chiral chromatography revealed that the ratio of isomers, enantiomers, and diastereoisomers depends on the cultivation temperature of individual strains.

1. Introduction

Phosphatidylglycerol (PG) is found in almost all types of bacteria and represents 20–25 % of phospholipids in most Gram-negative bacteria, which are present only in the inner membrane. *Escherichia coli*, a widely studied model organism, has up to 20 % PG in its membranes [1]. In Gram-positive bacteria, which have a single phospholipid bilayer coated with peptidoglycans, PG can constitute up to 60 % of phospholipids. In many bacteria, the diacyl form of the lipids predominates, but in others, the alkylacyl and alkenylacyl forms are more abundant [2,3]. The charge on the phosphate group means that it is an anionic lipid of neutral pH with a head group larger than expected due to hydration and an overall cylindrical shape. The molecule has two chiral centers with nonacylated glycerol usually attached to the phosphate through the *sn*-1′ position. Therefore, four isomers are theoretically possible, i.e. *(R,S, R, R, S,R),* and *(S,S)* (Fig. 1S).

The origin of chirality is still not fully understood, and speculation about where and how "enantiopure" life emerged from the primordial soup of abiotic mixtures of racemic molecules is very well reviewed [4–6]. One of the main conclusions is that all chiral molecules are formed in both enantiomers (racemates), suggesting that the symmetry imbalance between the two possible stereoisomers arose later (racemic/skacalemic/enantiomerically pure) [6,7]. For example, Fiore et al. [6] demonstrated that a racemic and scalemic mixture of *R*-1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (*R*-POPC) and *(S)*-POPC formed stable membranes similar to those made from homochiral *(R)*-POPC [7].

Based on the literature, only the *(R,S)* isomer of PG has been found in eukaryotic organisms, e.g., soybean or cabbage leaf [8], or algae (green algae *Enteromorpha* sp. and *Chlorella vulgaris*, raphidophyte alga *Heterosigma akashiwo*) [9]. One of the few exceptions, when the structure of

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glycerol 1-phosphate is also present in mammalian lipids, is the minorly represented phospholipid bis(monoacylglycero)phosphate, also known as lysobisphosphatidic acid [10]. On the other hand, two isomers have been identified in prokaryotic cells, specifically in *E. coli*, i.e., the isomer 1,2-diacyl-*sn*-glycero-3-phospho-1′-*sn*-glycerol (*R,S* configuration) and 1,2-diacyl-*sn*-glycero-3-phospho-3′-*sn*-glycerol (*R,R* configuration) [8]. The *(R,R)* isomer was also identified in 16 marine bacteria in amounts up to 6.5 % of total PG [11]. The composition of PG stereoisomers in bacteria can change depending on environmental conditions. In particular, with increasing temperature, the *(R,R)* isomer changes in *E. coli*, probably due to the adaptation of the cell to the temperature change [12].

In bacteria, PG is biosynthesized from phosphatidic acid by enzymatic reactions via an intermediate cytidine diphosphate diacylglycerol [13]. Other secondary biosynthetic pathways produce PG, e.g., the catabolism of cardiolipin or other phospholipids, catalyzed by phospholipase D, and eventually, cardiolipin synthase (ClsB) [14], which can partially change the stereochemistry of the second glycerol group (free glycerol). The other two isomers, i.e., *(S,R)*, and *(S,S)*, could also be present in organisms if PG biosynthesis starts with glycerol 1-phosphate ((2*S*)-2,3-dihydroxypropyl dihydrogen phosphate), which is the enantiomer of glycerol 3-phosphate. Both enantiomers are biosynthesized by reducing dihydroxyacetone phosphate by the respective enzyme, i.e., *sn*-glycerol-1-phosphate dehydrogenase (EC 1.1.1.261) or *sn*-glycerol-3-phosphate dehydrogenase (EC 1.1.1.8). So far, it has been assumed that in archaea, the starter unit is glycerol 1-phosphate while in bacteria and eukaryotic organisms it is its enantiomer glycerol 3-phosphate [15–17]. However, genetic analyses of archaea and bacteria showed that both enzymes are present in both domains. For glycerol-3-phosphate dehydrogenase, the bacterial vs archaeal ratio was found to be 477,032:2,634, whereas for glycerol-1-phosphate dehydrogenase it was 2,459:2,169, (sequences available in NCBI) [18]. It is clear from these values that, in some bacteria, the presence of all four PG isomers is not excluded. The analysis or determination of individual PG isomers can be carried out in several ways. Direct measurement of optical rotation was insufficiently sensitive to distinguish between diastereomeric PG [19]. Circular dichroism used by Batrakov et al [20]. makes it possible to determine the absolute configuration of chiral compounds. However, its use is not simple and practical in obtaining the composition of the mixture. Therefore, only chiral chromatography of derivatized phospholipids is used. One of the most suitable derivatizing agents is 3, 5-dinitrophenyl isocyanate, which reacts with free OH groups of PG to form 3,5-dinitrophenylurethane (DNPU). These derivatives of PG can be separated on a stationary column with a chiral phase (e.g., phase based on (*R*)- and (*S*)-1-(1-naphthyl)ethylamine), as has already been described many times [8,21–23].

Chiral chromatography can be used to separate isomers of phospholipids, i.e. compounds having two acyls and a substituted phosphoric acid on a glycerol backbone. A phenylcarbamate-based group, most often 3,5-dimethylphenylcarbamate, 3-chloro-4-methylphenylcarbamate or 4-chlorophenylcarbamate are chemically bound to oligo- or polysaccharides, e.g., cyclodextrins, amylose or cellulose [23,24]. Other immobilized selectors can also be used, such as a triazole quinidine derivative for the separation of dafachronic acid epimers or N^{α}-Boc amino acids [25,26].

For an exact determination of the structure of the isomer (diastereomers and/or enantiomers), standards must be used. These standards are not commercially available, so they must be synthesized. The synthesis of individual PG isomers has been described several times. Two types of synthesis are possible, either total organic synthesis, based mainly on (*R*)-and (*S*)-solketals, in which the respective isomers are obtained by multiple synthesis [27–29], or a reaction of phosphatidylcholine with (*R*)-and (*S*)-solketals in the presence of phospholipase D [12,30].

From the point of view of the origin of life, it is necessary to clarify the importance of membrane lipids in early evolutionary times. It is assumed that the original microorganisms lived in environments similar to hydrothermal vents and crust fissures or volcanoes. The ancient (supposedly 35,000 years old when reaching the surface) [31] underground waters of the famous spa town of Karlovy Vary (Carlsbad), Czech Republic, represent an ideal source of thermotolerant bacteria potentially relevant to the study of early evolutionary times. We studied bacterial isolates from these thermal springs and their physiological adaptation to the lowest and highest cultivation temperatures. Two different temperatures illustrate the climatic changes that occurred between the origin of life and the time when the Earth began to cool. In this study, we have reported for the first time the presence of four PG isomers (i.e. *R,S, R,R, S,R,* and *S,S*) identified by chiral chromatography in seven thermotolerant bacteria (Table 1S in Supplementary Information) at different concentrations. The assumption stated in many papers that archaea vs bacteria and eukaryotes produce only one isomer of PG was not confirmed.

2. Experimental section

2.1. Chemicals and Standards

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 3-palmitoyl-2-oleoyl-*sn*-glycero-1-phosphocholine (Ent-POPC), 15:0/15:0-PG (1,2-dipentadecanoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol)), (*R*)-(-)-2,2-dimethyl-1,3-dioxolane-4-methanol ((L)-(-)-solketal), (*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol (D)-(+)-solketal), and phospholipase D of *Streptomyces chromofuscus* were obtained from Sigma–Aldrich (now Merck, Czech Republic). All the other standards, chemicals, reagents, and solvents used in this study are fully described in the Supporting Information. Both enantiomers of solketals ((*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol and (*R*)-(−)-2,2-dimethyl-1,3-dioxolane-4-methanol) were sold with a purity of more than 95 molar percent; see, e.g., the paper Itabashi et al.[32] describing the chiral separation of both enantiomers purchased from Merck and Sigma.

2.2. Strains and cultivation

All strains examined in this study originated from Carlsbad thermal springs and were previously isolated and identified by methods described by Smrhova et al. [33] The strains were reinoculated from University of Chemistry and Technology collection glycerol stocks stored at -80°C on TSA plates (1.7 % Casein Peptone, 0.3 % Soy Peptone, 0.25 % Dextrose, 0.25 % Dipotassium Phosphate, 0.5 % NaCl solidified with 1.8 % agar). The plates were then incubated at 37°C in the dark until visible growth was observed. The identity of the grown strains was verified by MALDI-TOF mass spectrometry measurement [34]. Each strain was tested for notable growth in liquid TSB medium (TSA without agar) at the lowest and highest possible temperatures. Subsequently, the strains were cultivated under tested temperatures and 130 rpm in the dark. The cultures were sustained until stationary phase reaching the optical density in the range of 1.2–1.5 McFarland to harvest dried biomass. In the case of the lowest growth temperature, this took up to 4 days. Cell cultures were centrifuged at 5000 g for 5 min and the resulting pellets were lyophilized for 24 h.

2.3. Isolation of lipids

Total lipids were extracted using the method of Bligh and Dyer.[35] The extracts were evaporated to dryness under reduced pressure. For further analysis, total lipids were dissolved in the mobile phase (methanol/acetonitrile/aqueous 1 mM ammonium acetate (50:30:20, *v/v/v*)).

2.4. Semi-preparative HILIC-ESI-MS

Semipreparative HPLC equipment consisted of a 1090 Win system, a PV5 ternary pump (400 bar pressure (5801 psi)), an automatic injector

(HP 1090 series, Hewlett Packard, USA), and HILIC HPLC ZIC®-HILIC 250 × 10 mm, 5 μm, 200 Å column. An elution with a flow rate of 4.5 mL/min was performed, and a linear gradient was carried out from the mobile phase containing methanol/acetonitrile/aqueous 1 mM ammonium acetate (10:70:20, *v/v/v*) to methanol/acetonitrile/aqueous 1 mM ammonium acetate (50:30:20, *v/v/v*) for 60 min. The column temperature was 35°C, and the reequilibration period between runs was 30 min. The mixture of cardiolipin (CL), free fatty acid (FFA), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS) was used as an external standard. The eluent from the HPLC column was split so that 5 % of the flow was introduced into ESI–MS, and 95 % of the flow containing fractions of lipid classes was collected manually. The fractions were then evaporated and hydrolyzed.

The LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was equipped with a heated electrospray interface (HESI) in positive and negative ionization modes. The MS scan range was performed in the FT cell and recorded within a window between 150–2000 *m/z*. The mass resolution was set at 105,000, and the ion spray voltage was -2.5/+3.5 kV in the negative/positive ionization mode. The ionization mode used the following parameters: N_2 was used as a nebulizer gas and set at 18 arbitrary units (sheath gas), and 7 arbitrary units (auxiliary gas); ion source temperature, 250°C; capillary temperature, 230°C; capillary voltage, 50 V; and tube lens voltage, 170 V. Helium was used as a collision gas for collision-induced dissociation (CID) experiments. The CID normalization energy of 35 % was used to fragment the parent ions. The MS/MS product ions were detected in a high-resolution FT mode.

The MS spectrometer was calibrated using a Pierce LTQ Orbitrap positive and/or negative ion calibration solution (Thermo Fisher Scientific, San Jose, CA, USA). The internal lock mass was used in the acquisition of mass spectra, that is, 255.2330 m/z $[M-H]^-$ of palmitic acid in negative ESI. The mass accuracy was better than 1.0 ppm. The chemical structures of the compounds were confirmed with the help of the LIPID MAPS® Lipidomics Gateway spectral database (http://www.lipidmaps.org/). Tandem MS confirmed the structure of one of the most abundant peaks; see below.

2.5. Preparation of diastereomeric isopropylidene PGs [12]

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-1′,2′-*O*-isopropylidene-*sn*-glycerols (*R,R* and/or *R,S* configurations) were prepared from PC (16:0/18:1-PC) and (*S*)-(+)-solketal or (*R*)-(-)-solketal by transphosphatidylation catalyzed by phospholipase D (Fig. 1S). Similarly 3-palmitoyl-2-oleoyl-*sn*-glycero-1-phospho-1′,2′-*O*-isopropylidene-*sn*-glycerols (*S,R* and/or *S,S* configurations) were prepared from ent-16:0/18:1-PC and (*S*)-(+)-solketal or (*R*)-(-)-solketal by phospholipase D catalyzed transphosphatidylation (Fig. 1S). Briefly, a mixture containing 2.5 mg of PCs (16:0/18:1-PC or ent-16:0/18:1-PC), 1 mL of 0.4 M acetate buffer (pH 5.6), 0.5 mL of 0.2 M $CaCl_2$, 0.1 mL of *S*- or *R*-solketal/0.4 M acetate buffer (1:4, vol/vol), and 1 mL of water was mixed at 30°C for 10 min. Then the phospholipase D (0.4 M acetate buffer, pH 5.6) from *S. chromofuscus* (25 units) was added. The reaction was initiated by the addition of 2.5 mL of diethyl ether and performed at 30°C for 0.5 h with stirring. The products were extracted by 5 mL of chloroform/methanol (2:1, vol/vol), dried over anhydrous sodium sulfate, and evaporated under a stream of nitrogen.

Undesired hydrolysis to form PA was suppressed by size type and mixing conditions and supplemented with $CaCl_2$ [12,36]. In this work, almost no byproduct, i.e., PA, was detected by MS after the reaction.

Preparation of diastereomeric PG [12] (Fig. 1S). Isopropylidene PG isomers (diastereomers and enantiomers, i.e., *(S,R, S,S, R,S),* and *(R,R)* (see above) were dissolved in 1 mL of trimethyl borate, 50 mg of boric acid was added, and the mixture was heated at 100°C for 2.5 h. After the solvent was evaporated under a stream of nitrogen, the reaction mixture was dissolved in 6 mL of chloroform/methanol (2:1, vol/vol), then washed with 1 mL of distilled water, and the chloroform layer was dried over anhydrous sodium sulfate. The solvent was evaporated, and the crude PGs were analyzed by chiral HPLC.

2.6. Preparation of DNPU

The PGs were converted to bis(3,5-dinitrophenylurethanes), i.e., bis-DNPU derivatives, as described by Itabashi and Kuksis [23], with a minor modification. The products (~ 1 mg), all four isomers, were dissolved in 1 mL of dry toluene, 5 mg of 3,5-dinitrophenyl isocyanate, and 20 μL of dry pyridine was added, and the solution was stirred gently at 30°C. After 12 h, the solvent was evaporated to dryness, and LC-MS analyzed the resulting bis-DNPU derivatives on the chiral column; see below.

2.7. Analysis of PG

PG analysis using chiral LC/ESI-MS. The same LC system used in the HILIC mode was also used for separation in the chiral mode. PGs (~ 2.5 $mgmL^{-1}$ in mobile phase) were chromatographically separated on two Astec cyclobond™ I 2000 DMP (3,5-dimethyl-phenyl-carbamate modified β-cyclodextrin) chiral LC columns (5 μm, 25 cm × 4.6 mm) (Sigma-Aldrich, Ltd.), connected in series. The mobile phase was a gradient from 80 % A and 20 % B at 0 min to 40 % A and 60 % B over 30 min, and then isocratically maintained for 5 min, where A was hexane/2-propanol/trifluoroacetic acid (70:29.8:0.2 *v/v/v*) and B was hexane/2-propanol/trifluoroacetic acid (30:69.8:0.2 *v/v/v*). The flow rate, the column temperature, and the injection volume were 0.5 $mLmin^{-1}$, 24°C, and 10 μL, respectively. Other conditions were the same as those used for HILIC (see above).

2.8. Calibration

The synthesized standard DNPU-16:0/18:1-PG was analyzed separately. The ion at *m/z* 152.9958 (glycerol 3-phosphate ion with loss of H_2O) from the precursor ion scan (PIS) was used to generate a calibration curve using the peak area of the tandem MS corresponding to the different concentrations of the standard (1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100, 200, 500, 1000, 2000, 5000, 10000, 20000, and 50000 $fmol\mu L^{-1}$). The linear regression of the calibration curve and the signal/noise (S/N=3) ratio were calculated. The assays were linear throughout the concentration range, ranging from 5 to 20000 $fmol\mu L^{-1}$, with a mean coefficient of determination R^2 of 0.9977. The fifth point of the calibration curve (20.0 $fmol\mu L^{-1}$) corresponds to the limit of quantification (LOQ). The detection limit (LOD) for DNPU-16:0/18:1-PG was determined; This concentration was 5.0 $fmol\mu L^{-1}$, consistent with previously published data. Schoeny et al.[37] determined that PG LOD is 0.003 $\mu molL^{-1}$ (i.e. 3.0 $fmol\mu L^{-1}$), Huang et al. [38] determined that the LOD of 17:0/14:1-PG 0.80 $pmolmL^{-1}$ (i.e. 0.8 $fmol\mu L^{-1}$) and LOQ 2.64 $pmolmL^{-1}$ (2.64 $fmol\mu L^{-1}$) using the precursor ion at *m/z* 153 in negative ion mode. The partial difference between our LOD and the published detection limits may be moderately influenced by the different structures of the detected compounds, i.e., our measured DNPU-PG vs the published LOD of phosphatidylglycerols. For comparing two cultivations, a t-test for two independent samples was applied for each combination of ID and temperature separately. T-test was performed in each row separately for *R,S; R,R; S,R* and *S,S* isomers.

3. Result and discussion

3.1. Synthesis of Standards

The method described for the synthesis of PG diastereomers and enantiomers was used [12]. All four available isomers of PG, i.e. *R,R, R, S, S,R,* and *S,S,* were prepared using commercially available phospholipase D from *Streptomyces*; see also D'Arrigo et al.[30] and

commercially available enantiomers of both PC (16:0/18:1-PC, i.e., *R* enantiomer and Ent-16:0/18:1-PC, i.e., *S* enantiomer), as well as both enantiomers of solketals ((*R*)-(−)-2,2-dimethyl-1,3-dioxolane-4-methanol and/or (*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol), see Experimental section and Fig. 1S. In addition, these isomers were derivatized with bis(3,5- dinitrophenyl) isocyanate to form the corresponding urethanes, e.g., tandem MS of DNPU-16:0/18:1-PG. The LC-MS separation of the above isomers on a chiral column is shown in Fig. 2S.

3.2. Hydrophilic interaction liquid chromatography – HILIC

Total lipids were extracted from lyophilized bacterial cells after cultivation according to Bligh and Dyer [35]. Cultivation temperatures, biomass yields, total extracted lipids, and percentage of PG of total lipids are summarized in Table 1S. Production of biomass, total lipids, and PG follows previously published results for several strains of the genus *Bacillus* and related genera of the family Bacillaceae [39–41]. The profile of the lipid class of the bacteria was analyzed using HILIC, where Fig. 1 shows standard chromatograms and profiles of two strains (*Bacillus cereus* and *B. licheniformis*).

The phospholipid classes were divided at baseline and the separation time on a semi-preparative column with a diameter of 10 mm ranged from 10 to 45 min. The preliminary structure of the PGs was determined on the basis of retention time (tR) compared to the standards. The PG fraction was collected manually and further analyzed by ESI precursor ion scanning tandem mass spectrometry (PIS MS/MS) at *m/z* 152.9958, the predicted structures of this ion; see Fig. 3S [42]. Fig. 2 shows the shotgun analysis of a mixture of PG homologs from three thermotolerant bacteria isolated in our study. Table 1 shows the relative abundance of the molecular species of these bacteria. Analysis of strains cultured at different temperatures shows that the majority of molecular species of PG were 30:0-PG, more precisely 15:0/15:0-PG. As an example, the natural and standard negative tandem high-resolution mass spectrum of the major molecular species, i.e., 15:0/15:0-PG spectrum, is shown in Fig. 3 and Table 2S. Briefly, in tandem MS, abundant ions resulted from the loss of acyl chains *sn*-1 and *sn*-2 as a ketene (RCH=C=O) of $[M-H]^-$ at *m/z* 469.2574 and the neutral loss of the *sn*-1 and *sn*-2 RCOOH group of $[M-H]^-$ at *m/z* 451.2468. Other ions were also identified, i.e., precursor ion $[M-H]^-$ at *m/z* 693.4714 Da, *sn*-1 and *sn*-2 $RCOO^-$ (241.2174 Da), and glycerophosphoglycerol ion with loss of H_2O at *m/z* 227.0327, etc. (Table 2S and Fig. 3) [43]. Thus, tandem MS confirmed this structure.

PG was found to be the predominant phospholipid in bacteria of the genus *Bacillus* and its phylogenetic relatives [44,45]. Shah et al. [46] identified 15:0/15:0-PG with *m/z* 693.4712 in the thermotolerant *Geobacillus* sp. strain GWE1 as one of the main phospholipids. Taking into account the published data and the results obtained in this study, we confirm that PG is the main phospholipid in the seven strains of bacteria and therefore was selected for further analysis. In addition, other homologs (molecular species) were identified for all strains.

3.3. Resolution of diastereomeric and enantiomeric phosphatidylglycerols

Itabashi [22] describes in his paper that the separation of diastereomers, i.e. *(S,S, S,R, R,S)* and *(R,R)* on a conventional normal or reverse phase column was not possible due to the large distance between the two chiral centers. Unfortunately, although theoretically the diastereoisomers should be separable on a non-chiral column, we reached the same results as Itabashi. Therefore, chromatography on a chiral column, or on two columns connected in series, was used to separate standards and real samples.

Fig. 2S shows the resolution of bis-DNPU derivatives of synthetic 1-palmitoyl-2-oleoyl-rac-glycero-3-phospho-1-rac-glycerols derived from commercial phosphatidylcholines (see Experimental) and solketals by transphosphatidylation with *Streptomyces chromofuscus* phospholipase D in the two Astec cyclobond™ I 2000 DMP chiral LC columns (3,5-dimethyl-phenyl-carbamate modified β-cyclodextrin), connected in series. All PG derivatives were resolved into diastereomeric and enantiomeric peaks in an elution time of approximately 24 min.

A phospholipid with hydroxyl groups, such as PG, easily reacts with 3,5-dinitrophenyl isocyanate to form the corresponding urethane derivatives that are separable in chiral columns. This derivatization method was used to determine the configuration of PG prepared by PC

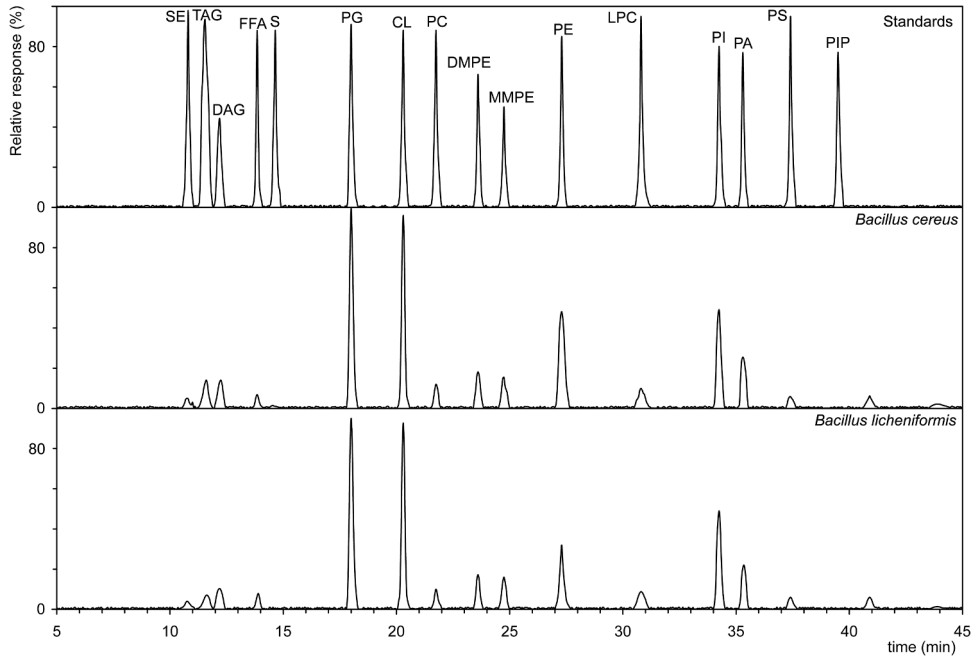

Fig. 1. Hydrophilic interaction liquid chromatography-HILIC/ESI-MS separation of the major lipid classes of the two different biological sources (*B. cereus, B. licheniformis*, and standards, i.e. SE - sterol esters, TAG - triacylglycerols, DAG - diacylglycerols, FFA - free fatty acids, S -sterols, PG -phosphatidylglycerols, CL - cardiolipins, PC - phosphatidylcholines, DMPE - dimethylphosphatidylethanolamines, MMPE - monomethylphosphatidylethanolamines, PE - phosphatidylethanolamines, LPC - lysophosphatidylcholines, PI - phosphatidylinositols, PA phosphatidic acids, PS - phosphatidylserine, and PIP - phosphatidylinositol phosphates). The TIC (total ion current) was filtered and exhibits ions in the interval from 200 to 1500 Da.

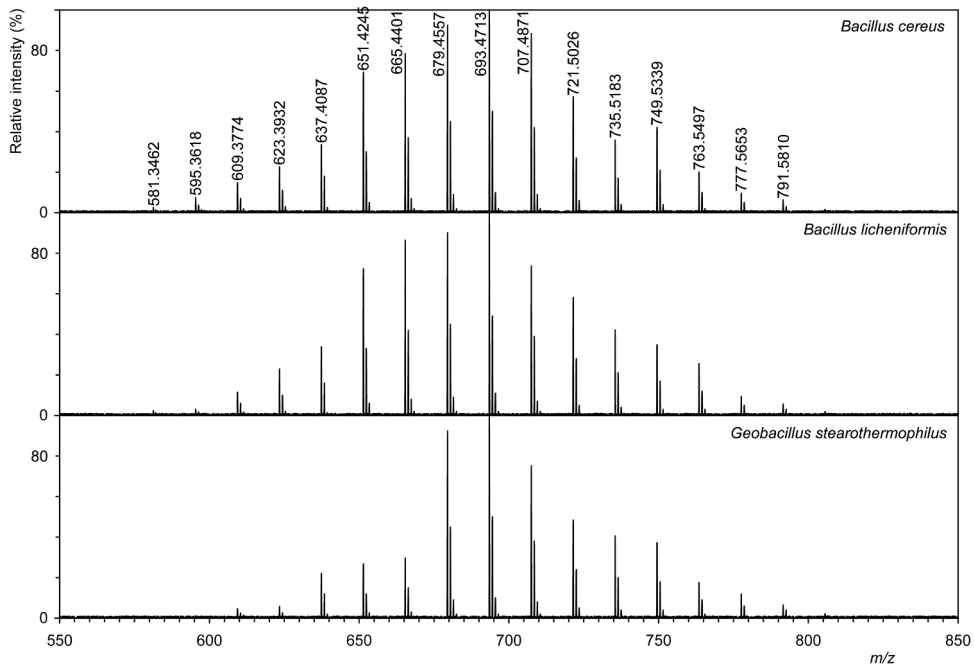

Fig. 2. High-resolution mass spectrum using the neutral loss scan method (neutral fragment at *m/z* 152.9928) by negative ESI.

Table 1
The relative abundance (% of total PG) of molecular species of *Bacillus cereus, B. licheniformis*, and *Geobacillus stearothermophilus.*

[M-H]$^-$	*Bacillus cereus*	*Bacillus licheniformis*	*Geobacillus stearothermophilus*	Sum of carbons in acyl chains
581.3461	0.4	0.4	0.0	22
595.3617	0.5	1.3	0.2	23
609.3773	1.7	2.2	0.9	24
623.3931	3.4	3.3	1.1	25
637.4086	5.0	4.9	4.2	26
651.4243	10.7	10.1	5.1	27
665.4399	12.8	11.5	5.7	28
679.4556	13.4	13.5	17.7	29
693.4712	14.8	14.6	19.1	30
707.4869	10.8	12.9	14.3	31
721.5025	8.6	8.4	9.2	32
735.5182	6.3	5.2	7.8	33
749.5338	5.2	6.2	7.1	34
763.5495	3.8	2.9	3.4	35
777.5651	1.4	1.4	2.3	36
791.5808	0.8	0.9	1.2	37
805.5964	0.3	0.2	0.4	38
819.6121	0.1	0.1	0.2	39
833.6277	0.0	0.0	0.1	40

phospholipase D and glycerol [47] or for the synthesis of pure stereoisomers of PG from PC enantiomeric and solketals [12,32]. The reaction in the presence of bacterial phospholipase D proceeded largely stereospecifically, in contrast to the phospholipase D of cabbage or peanut. The chiral phase HPLC also confirmed that some bacteria contain significant amounts of the PG diastereomer (*R,R* isomer), the proportion of which gradually increased with increasing growth temperature, suggesting adaptation to environmental change [12].

Depending on the source CID energy, fragmentation of the [M-DNPU]$^-$ ion (base peak) occurs. Applying a high voltage (source CID energy, 40 %) to the ion source, ion splitting occurs at *m/z* 1165.5327, i.e. [M-DNPU]$^-$. A series of ions is formed, the most important of which are [M-H-DNPU]$^-$ and [M+H-2 × DNPU]$^-$, at *m/z* 956.5254 and *m/z* 747.5182, respectively; see Fig. 4S and Table 3S. Other ions found in the tandem MS spectrum are identical to the ions of nonderivatized 16:0/18:1-PG (standard); see also [48] and Fig. 5S including Table 4S.

3.4. Diastereomeric and enantiomeric phosphatidylglycerols in thermotolerant bacteria

The *(R,R)* isomer in bacteria is supposed to increase in abundance with increasing culturing temperature [12]. To confirm this hypothesis, seven thermotolerant bacteria were cultured from Karlovy Vary springs at two different temperatures. As shown in Table 2 and Fig. 4, the abundance of other isomers of PG, i.e., *(R,R, S,R)*, and *(S,S)* increases with increasing cultivation temperature (see also Fig. 4, Fig. 6S, and Fig. 7S). In particular, the relative abundance of *(R,R)*-PG increased by more than 80 % in *Bacillus tequilensis* (isolate M10) when the cultivation temperature was changed from 15°C to 50°C. The representation of *(S,R)* and *(S,S)* isomers also increased; see Table 2 and Fig. 4 and the representation of the "natural" isomer *(R,S)* decreased. According to the values in both Table 2 and Fig. 4, the representation of the *(S,R)* isomer at 50 °C is more than double the representation of this isomer at 15 °C. They were published [49] thermodynamic parameters of the phase transitions in fully hydrated racemic (DL) and stereochemically pure (L) phosphatidylcholines, i.e., 16:0/16:0 PE, where phase transitions T for lamellar subgel-lamellar liquid crystal is 81.85 ± 0.50 (°C) for DL-16:0/16:0, while for L-16:0/16:0 it is only 66.36 ± 1.00. In the case of phosphatidylcholines (PC) [50], unfortunately, the phase transition temperatures for phosphatidylcholines, e.g., 1-16:0/2-16:0, 2-16:0/3-16: 0 and rac-16:0/16:0 almost identical 41.3 ± 1.8, 41.2 ± 0.5, 41.6 ± 0.8, respectively.

Phosphatidylglycerols (PG) are rarely found in significant amounts in eukaryotic cell membranes and for this reason, compared to PG and PE, papers describing their thermodynamic transitions have been published [51]. However, they are the main components, e.g., the chloroplast membranes of higher plants and the membranes of Gram-positive bacteria, so we analyzed bacteria of the genus *Bacillus* and related genera. From the above data, it is clear that the effect of temperature on PG can be, as it was shown above, one of the decisive factors in the ability of thermotolerant bacteria to live and mainly reproduce at different temperatures, e.g., *B. tequilensis* (15 vs 50°C). Salonen et al., [52] did compare two stereoisomers, i.e. 1,2-dimyristoyl-sn-glycero-3-phosphatidyl-sn-1′-glycerol and 1,2 -dimyristoyl-sn-glycero-

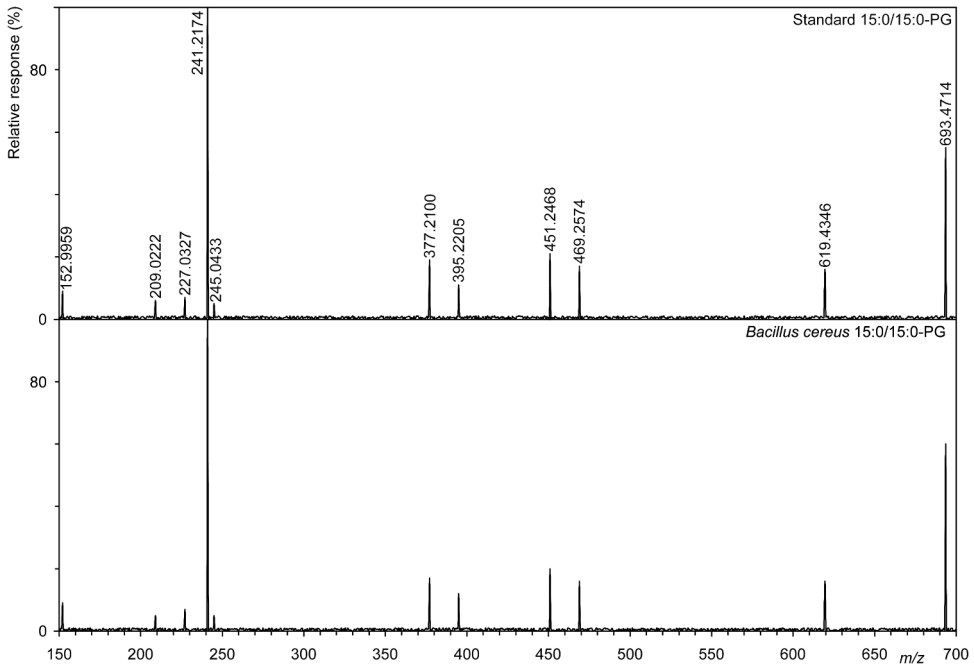

Fig. 3. Negative tandem high-resolution mass spectrum of the major molecular species, i.e., 15:0/15:0-PG (standard and PG from *Bacillus cereus*).

Table 2
The effect of the temperature on the representation of enantiomers and diastereoisomers (relative %) of PG in seven strains of bacteria cultivated at different temperatures.

ID	temp (°C)	*(R,S)*	*(R,R)*	*(S,R)*	*(S,S)*	glycerol-1-phosphate dehydrogenase	glycerol-3-phosphate dehydrogenase
Bacillus cereus	44	85.1 ± 0.9	7.7 ± 0.8	2.4 ± 0.2	4.8 ± 0.5	CUB15915.1	ETT84063.1
Bacillus cereus	12	91.5 ± 0.8	4.1 ± 0.5	1.6 ± 0.3	2.8 ± 0.4	CUB15915.1	ETT84063.1
Bacillus licheniformis	50	76.8 ± 1.0	8.6 ± 0.6	5.2 ± 0.1	9.4 ± 0.9	AOP16250.1	AOP15585.1
Bacillus licheniformis	20	88.0 ± 0.6	5.1 ± 0.4	2.8 ± 0.4	4.1 ± 0.3	AOP16250.1	AOP15585.1
Bacillus subtilis subsp. *inaquosorum*	50	77.2 ± 0.7	8.0 ± 0.8	5.6 ± 0.5	9.2 ± 0.5	AMA53404.1	WP_104010208.1
Bacillus subtilis subsp. *inaquosorum*	15	88.0 ± 0.5	4.8 ± 0.5	3.2 ± 0.2	4.0 ± 0.2	AMA53404.1	WP_104010208.1
Bacillus tequilensis	50	81.0 ± 0.6	6.7 ± 0.2	4.5 ± 0.4	7.8 ± 0.6	WP_174226813.1	WP_167873825.1
Bacillus tequilensis	15	87.6 ± 0.3	4.0 ± 0.9	2.0 ± 0.2	6.4 ± 0.7	WP_174226813.1	WP_167873825.1
Geobacillus stearothermophilus	60	79.1 ± 0.8	9.6 ± 0.7	3.8 ± 0.7	7.5 ± 0.5	KZM53192.1	KYD34837.1
Geobacillus stearothermophilus	46	80.7 ± 0.7	8.7 ± 0.8	3.4 ± 0.3	7.2 ± 0.3	KZM53192.1	KYD34837.1
Paenibacillus favisporus	46	87.8 ± 0.5	5.9 ± 0.5	2.3 ± 0.5	4.0 ± 0.4	n.d.[b]	WP_145141714.1
Paenibacillus favisporus	17	98.7 ± 0.4	1.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	n.d.	WP_145141714.1
Priestia aryabhattai	46	99.1 ± 0.9	0.9 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	n.d.	KMO00992.1
Priestia aryabhattai	14	98.9 ± 0.7	1.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	n.d.	KMO00992.1

[a] Data of technical replicates (six chromatographic analyses) ± standard deviations (S.D.).
[b] Presence was not described.

3-phosphatidyl-sn-3′-glycerol, but only the effect of NaCl on their thermotropic behavior.

For two bacterial species used, *Priestia aryabhattai* (isolate M7) and *Paenibacillus favisporus* (isolate P22), there are no records of the presence of *sn*-glycerol-1-phosphate dehydrogenase (EC 1.1.1.261) in GenBank, which explains the absence of *(S,R)* and *(S,S)* isomers (Table 2). Similarly, the very low representation of the *(R,R)* isomer in *P. aryabhattai* cultured at 46°C, up to one order of magnitude lower than, for example, *Bacillus licheniformis* (isolate V2) cultured at a similar temperature (50°C), can be explained by biosynthesis of this isomer differently than by biosynthesis through the cytidine derivatives of diphosphate diacylglycerol. As published by Yokobori et al., [53] both dehydrogenases (*sn*-glycerol-1-phosphate dehydrogenase (EC 1.1.1.261) or *sn*-glycerol-3-phosphate dehydrogenase (EC 1.1.1.8)) were identified in the

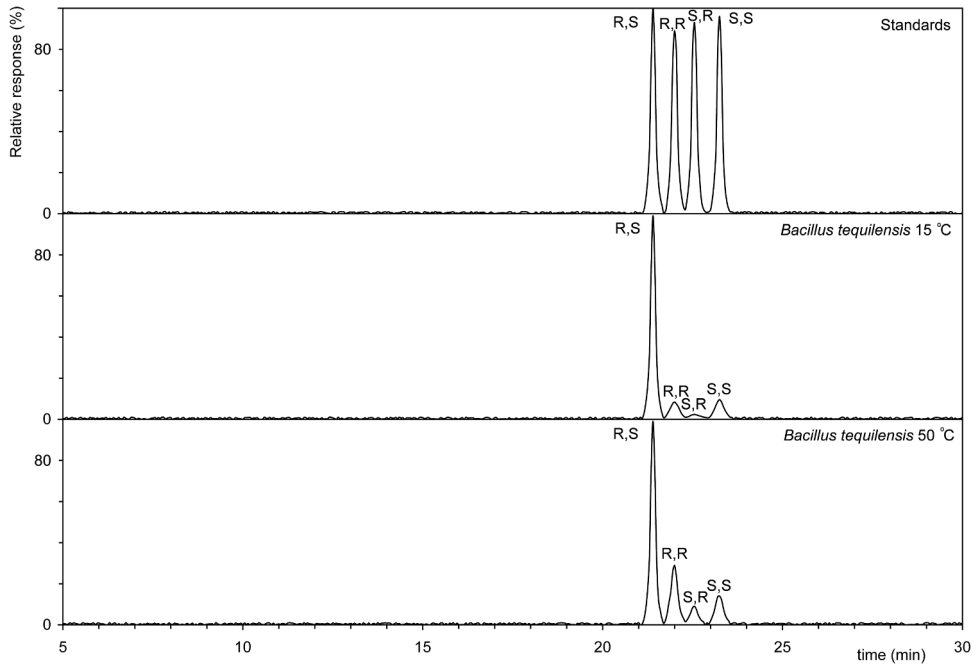

Fig. 4. Separation of four standards, prepared synthetically and four isomers from *Bacillus tequilensis* cultivated at 15 °C and 50 °C, respectively.

phylum Bacillota (formerly Firmicutes) which includes our studied isolates (*B. cereus* P23, *B. licheniformis* V2, *B. subtilis* subsp. inaquosorum S8, *Geobacillus stearothermophilus* P1, and *Paenibacillus favisporus* P22). Homochiral compounds are a basic feature of living matter and include, for example, L-amino acids and D-saccharides [54]. However, in the case of phospholipids, a case of dual homochirality is known with archaeal membranes being based on *sn*-glycerol 1-phosphate (G1P), i.e., enantiomers with stereochemistry *S*, while bacteria and eukaryotes on *sn*-glycerol-3-phosphate (G3P), i.e., enantiomers with stereochemistry *(R)* [55–57]. This phenomenon of different homochirality of phospholipids that exist in nature is known as the lipid divide. Archaea and bacteria, the two fundamental domains of life, share a common ancestor, called the last universal common ancestor (LUCA) [58]. The reason why the split occurred is still unknown, and several theories have addressed this issue, including that the LUCA membrane was heterochiral or racemic and that instability between the two phospholipid enantiomers eventually led to lipid partitioning [59]. However, the robustness of cells with a hybrid heterochiral lipid membrane has been reported to be higher than that of cells with a homochiral lipid membrane [60]. The LUCA membrane could be homochiral, see articles Yokobori et al., [53] or it could also be achiral. Phospholipids may have disappeared during evolution with the help of homochiral enzymes that synthesize membrane lipids.

4. Conclusion

There are several possible pathways for the biosynthesis of PG isomers in biological systems, but it is not yet clear which of them are currently in use. In this study, we analyzed seven strains of thermotolerant bacteria by chiral HPLC and, for the first time, detected all four PG isomers in the five of the bacteria examined. Although there are many studies on the molecular species composition and physiological functions of PGs from microorganisms, only a few studies distinguish between stereoisomers. Details on the distribution of PG isomers in microorganisms and differences in metabolic and physiological functions between stereoisomers can be expected to be elucidated in the future. Based on the results of the analysis presented in this paper and the articles mentioned above, it can be assumed that with the development of instrumental analytical techniques and the progress of whole genomes, especially for bacteria isolated from extreme environments, it will gradually be clarified.

Theoretical predictions and experimental evidence confirm that phospholipids are formed abiotically as racemic mixtures. The synthesis of either chiral products or selective catalytic reactions should initiate the appearance of enantiomeric phospholipids. In this study, we propose a scenario describing the evolution of polar lipid chirality in cell membranes. Our results confirm the heterochirality of polar lipids as described in the publication of Shimada and Yamagishi. [61] Their study showed that a heterochiral polar lipid membrane is not less stable than a homochiral polar lipid membrane.

Finally, our results lead to the conclusion that lipid partitioning in terms of stereochemistry is not as strict as originally suggested, including the presence of both enzymes (EC 1.1.1.261 or EC 1.1.1.8) related to the formation of G1P and G3P in bacterial [62,63] and archaeal [64] genomes, as well as the detection of G1P and G3P-based lipids in engineered *E. coli* [60] and several gram-positive bacteria [17].

CRediT authorship contribution statement

Andrea Palyzová: Writing – original draft, Visualization, Investigation, Funding acquisition. **Tereza Šmrhová:** Writing – original draft, Visualization, Methodology. **Gabriela Kapinusová:** Methodology, Formal analysis, Data curation. **Zdena Škrob:** Visualization, Formal analysis. **Ondřej Uhlík:** Writing – original draft, Project administration, Investigation, Funding acquisition. **Tomáš Řezanka:** Writing – original draft, Project administration, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

cardiolipin (CL); heated electrospray interface (HESI); HILIC-ESI-MS hydrophilic interaction chromatography-electrospray ionization-mass spectrometry; 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC); 3-palmitoyl-2-oleoyl-*sn*-glycero-1-phosphocholine (Ent-POPC); free fatty acid (FFA); phosphatidic acid (PA); phosphatidylcholine (PC); phosphatidylethanolamine (PE); phosphatidylglycerol (PG); phosphatidylinositol (PI); and phosphatidylserine (PS).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.465517.

Data availability

Data will be made available on request.

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