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ELECTROPHORESIS

RESEARCH ARTICLE

Stereochemistry of aminoacylated cardiolipins and phosphatidylglycerols from bacteria

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Abstract

Hydrophilic interaction liquid chromatography (HILIC) connected with electrospray high-resolution tandem mass spectrometry (MS) was used for the analysis of unusual amino acid (AA) substituted phosphatidylglycerols (PG) and cardiolipins (CL) in mesophilic and thermophilic bacteria. Individual peaks from the lipid class separation by HILIC were isolated and hydrolyzed to determine the absolute configuration of the aminoacyl side chain. The configuration of the aminoacyl side chain was assigned by indirect liquid chromatography (LC) enantiomer separation after the hydrolysis of the aminoacylated (aminoacyl) lipids using *N*-(4-nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester as chiral derivatizing agent and reversed phase LC–MS for analysis. When two chromatographic methods were combined, less common AAs, such as D-allo-Ile and D-allo-Thr, were identified. The taxonomic classification of bacteria showed that bacteria of the family Bacillaceae (*Bacillus* and *Geobacillus*) produce branched-chain AAs, that is, D-allo-Ile, D-Ile, and D-Leu. These AAs were present only in the genera *Bacillus* and *Geobacillus* and not in *Alicyclobacillus acidoterrestris* (family Alicyclobacillaceae). On the contrary, hydroxy AAs, that is, L- and D-Thr, and L- and D-allo-Thr, were identified as aminoacyl-PG and aminoacyl-CL in *A. acidoterrestris* and were not present in the genera *Bacillus* and *Geobacillus*. Therefore, the complete analysis made it possible to identify the stereochemistry of AAs in aminoacyl PGs and CLs and use this fact for chemotaxonomy.

KEYWORDS

achiral chromatography, amino acids, aminoacylated cardiolipins, aminoacylated phosphatidylglycerols, HILIC

1 | INTRODUCTION

Phosphatidylglycerol (PG) is one of the main components of some bacterial membranes, where it occurs as an anionic lipid in a cylindrical shape. The molecule has two chiral centers and a glycerol attached to a phosphate via the *sn*-3′ position. PG represents 20%–25% of phospholipids in most G^- bacteria. In G^+ bacteria that have a single-phospholipid bilayer, PG can make up to 60% of total phospholipids. It is an important component of membranes and is also a key intermediate in the biosynthesis of several other lipids, especially cardiolipin (CL), acylphosphatidylglycerol, and complex

Abbreviations: (S)-NIFE, *N*-(4-nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester; CL, cardiolipin; Lys, lysine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; tR, retention time.

lipoamino acids, which may have essential functions in bacteria.

Lysyl-PG is the main membrane lipid (20%–40%) in *Staphylococcus aureus* [1], whereas ornithyl-PG was found in the genus *Mycobacterium* [2] and alanyl-PG in *Clostridium perfringens* (formerly *C. welchii*) [3]. In *Bacillus subtilis*, an *N*-succinylated L-lysyl-PG has been detected that interestingly reverses the net positive charge of lysyl-PG [4]. *Enterococcus faecalis* has been reported to contain alanyl-PG, 2′-lysyl-PG, 3′-lysyl-PG, 2′,3′-dilysyl-PG and arginyl-PG (together with a diglucosyl derivative of PG) [5].

In some bacterial species, there are complex related lipoamino acids derived from CL. Structural analogs of CL, such as phosphatidylglycerophosphoglycerol, D-glucopyranosyl-CL, D-alanyl-CL, and L-lysyl-CL, have been found in both gram positive and gram negative bacteria [6–9].

Compared to the precursors PG and CL, which have negatively charged head groups, aminoacylated products are cationic or zwitterionic, causing a reduction in negative charge and protecting them from bacteriocins, which facilitates the interaction of pathogenic bacteria with their host [10]. Furthermore, these modified phospholipids protect cells from environmental stresses, for example, high osmotic pressure or low pH [11], because membranes containing these lipids are much less permeable than membranes containing only PG and/or CL.

Aminoacylphosphatidylglycerol synthases (aaPGS) are enzymes that transfer amino acids (AA) from aminoacyl-tRNA to PG. The enzyme "multiple peptide resistance factor" is an aminoacyl PG synthase identified in bacteria that can transfer lysine (Lys) or alanine from the respective aminoacyl-tRNAs to the hydroxyl group of PG or CL. Related enzymes are present in many other bacterial species, some of which can utilize only one lipoamino acid, whereas others can produce several products [12].

The genomic sequences available encoding two or more aaPGS paralogs were identified in 213 different species (96 different genera of microorganisms) [13]. Several years later, 174 bacterial species, each containing 1 or more aaPGS homologs, have been identified [14].

The occurrence of less common AAs in bacteria is not surprising because, for example, bacteria from the order *Bacillales* produce linear or cyclic lipopeptides that contain, very often, less common AAs, for example, D-allo-Thr and/or D-allo-Ile. Examples include fengycins and plipastatins (D-allo-Thr), fusaricidin/LI-F family (D-allo-Thr and D-allo-Ile) [15], cerexin A and cerexin B (D-allo-Thr and D-allo-Ile) [16, 17], and plusbacins D-allo-Thr [18]. *Paenibacillus polymyxa* produces tridecaptin analogs containing D-allo-Ile [19]. Lipopeptide similar to fengycin and containing D-allo-Thr was isolated from *Bacillus thuringiensis* [20].

There are several methods for the analysis of AA by high-performance liquid chromatography (HPLC), which can generally be divided into direct or indirect analysis (non-derivatized vs. derivatized AA). Unfortunately, racemization can occur during acid hydrolysis, so to exclude racemization, deuterated DCl is used in the D_2O or CD_3COOD mixture [21]. In the case of racemization, each racemized AA is marked with a D atom on the α-carbon, and thus, the molecular weight increases by 1 Da during subsequent analysis by mass spectrometry (MS). In the direct analysis of AA, a column with a chiral phase is used, in the case of indirect analysis, after derivatization with a suitable reagent, for example, 1-(9-fluorenyl)ethyl chloroformate, *N*-(4-nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester ((S)-NIFE), 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide [21–23], the mixture of diastereomers is also more often separated on a column with a reversed phase.

Based on these articles and our previous articles [24–27] that describe the identification of regioisomers and enantiomers of different lipids, the derivatives of PG and CL from three different bacteria were analyzed. Using several analytical techniques, that is, hydrophilic interaction liquid chromatography (HILIC), the hydrolysis of AA PGs and CLs, reversed phase-HPLC/tandem electrospray ionization high-resolution MS (RP–HPLC/ESI–HR–MS) in positive ionization mode proved the presence of less common AAs, such as D-allo-Thr or D-allo-Ile in *O*-amino-acid esters of PG or CL.

2 | MATERIALS AND METHODS

2.1 | Chemicals and standards

AA standards L and D were obtained from Sigma-Aldrich (now Merck). All the other standards, chemicals, reagents, and solvents used in this study are fully described in the Supporting Information section. (S)-NIFE was purchased from Cayman Europe OÜ.

2.2 | Strain and cultivation

The genera *Bacillus* and *Geobacillus* belong to the order *Bacillales*, family Bacillaceae, whereas the genus *Alicyclobacillus* also belongs to the order *Bacillales* but to the family Alicyclobacillaceae. The three strains were cultured in 500 mL Erlenmeyer flasks filled with 200 mL of medium. *Alicyclobacillus acidoterrestris* CCM 4659 and *Geobacillus stearothermophilus* CCM 2062 were grown under aerobic conditions at 45°C, respectively, for 24 h at

55°C [28]. *Bacillus licheniformis* CCM 2145 was grown at pH 6.5 and 50°C; see Supporting Information.

2.3 | Isolation of lipids

Total lipids were extracted using the method of Bligh and Dyer [29], and the aqueous phase was again extracted according to Markham and Jaworski [30] using a mixture of the lower phase of isopropanol/hexane/water (55:20:25 v/v/v). The extracts were evaporated to dryness under reduced pressure. For further analysis, total lipids were dissolved in the mobile phase (methanol/acetonitrile [ACN]/aqueous 1 mM ammonium acetate [50:30:20, *v/v/v*]).

2.4 | Semipreparative 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), 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/ACN/aqueous 1 mM ammonium acetate (10:70:20, *v/v/v*) to methanol/ACN/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 CL, free fatty acid, phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), 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 to ESI–MS, and 95% of the flow containing fractions of lipid classes was collected manually. The fractions were further evaporated and hydrolyzed.

The LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) was equipped with a heated electrospray interface in positive and negative ionization modes. The MS scan range was performed in the FT cell and recorded within a window between 100 and 2000 m/z. The mass resolution was set to 105 000, and the ion spray voltage was set at −2.5/+3.5 kV in the positive/negative 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 for the fragmentation of the parent ions. The MS/MS product ions were detected in a high-resolution FT mode.

The MS spectrometer calibration was conducted using a Pierce LTQ Orbitrap positive and/or negative ion calibration solution (Thermo Fisher Scientific). The internal lock mass was used in the acquisition of mass spectra, that is, 255.2330 m/z $[M–H]^-$ palmitic acid in the 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/). The structure of one of the most abundant peaks was confirmed by tandem MS; see below.

2.5 | Identification of amino acids

The fractions collected from the separation of HILIC separation (~0.1 mg) were hydrolyzed for 1 h at 50°C in DCl/D_2O (20% solution); water of 400 µL was added, and the sample was lyophilized. The residue was redissolved in 100 µL of water, followed by the addition of 50 µL of 0.2 M sodium tetraborate and 100 µL of a 3 mg/mL solution of (S)-NIFE in ACN. This mixture was incubated at room temperature for 10 min. The reaction was terminated by adding 30 µL of 4 M HCl and 1 mL of water. The reaction solutions were then filtered and diluted with water. A set of standard solutions containing L- and D-AA derivatives was processed in parallel.

The liquid chromatography equipment consisted of a 1090 Win system, a PV5 ternary pump, and an automatic injector (HP 1090 series, Agilent), and three Luna Omega, C18, columns 150 × 2.1 mm^2 × 1.6 µm connected in series. A flow rate of 0.97 mL/min, an injection volume of 10 µL, and a column temperature of 45°C were used. (S)-NIFE derivatives were separated using a solvent gradient program with water containing 0.1% trifluoroacetic acid (TFA) (phase **A**) and ACN containing 0.1% TFA (phase **B**) as follows: initial A/B (95:5, v/v), a linear change to 15:85 A/B (v/v) from 0 to 45 min, and hold for 5 min. The composition was returned to the initial conditions over 5 min. The 100% HPLC flow was introduced into the ESI source. The backpressure of the system reached 1000 bar during the gradient analysis. Although the manufacturer's efficiency was not achieved, that is, 350 000 theoretical plates/meter (see manufacturer's literature), the efficiency achieved in our case was ~217 000 theoretical plates/meter. *N*,*N*-bis(3-phenylpropionic acid methoxyethyl ester 2-yl)urea (by product) with a tR of 48.71 min was used as an internal standard. The LTQ Orbitrap Velos (Thermo Fisher

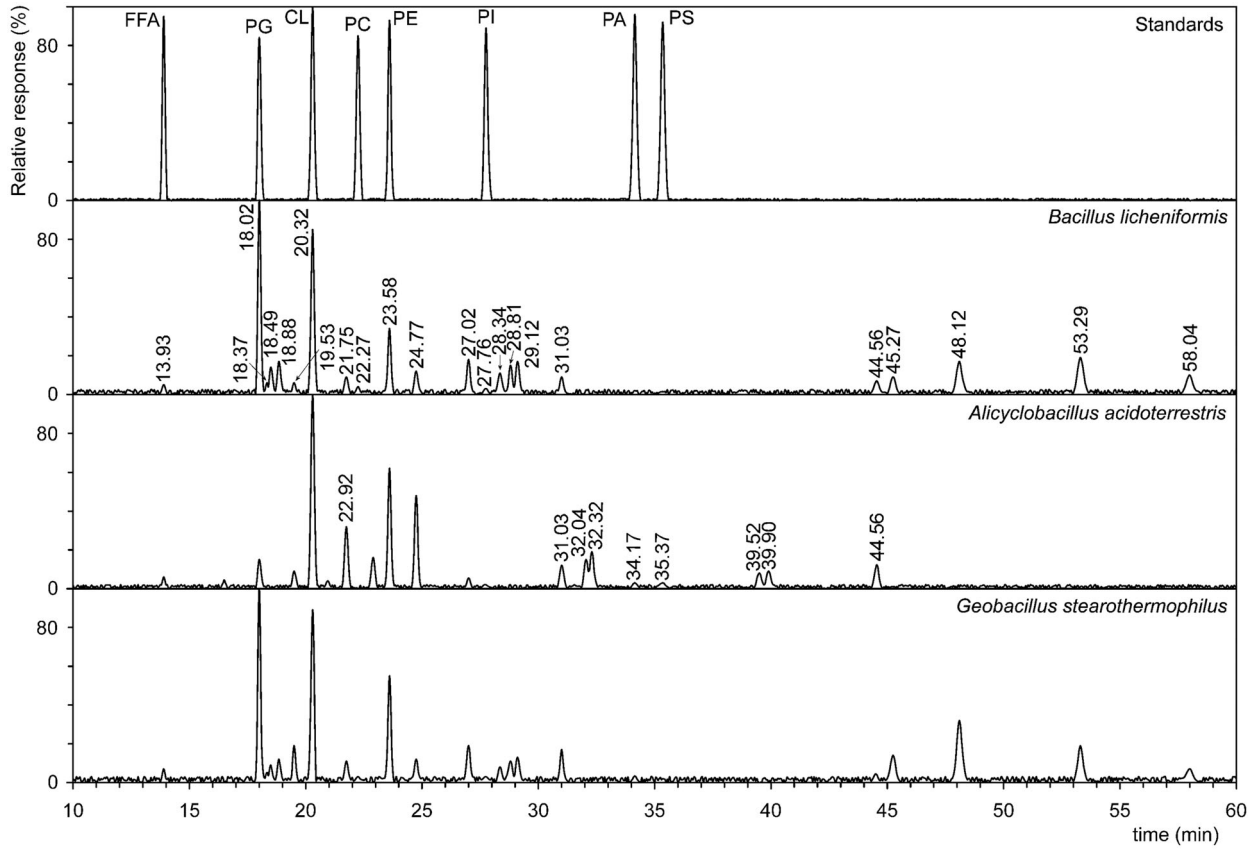

FIGURE 1 Hydrophilic interaction liquid chromatography–HILIC/ESI–mass spectrometry (MS) separation of the major lipid classes of the three bacteria (*Alicyclobacillus acidoterrestris* CCM 4659, *Bacillus licheniformis* CCM 2145, and *Geobacillus stearothermophilus* CCM 2062). The total ion current (TIC) was filtered and exhibits ions in the interval from 200 to 1500 Da.

Scientific) high-resolution hybrid mass spectrometer was used for the ESI–MS analysis, under conditions as described above.

3 | RESULTS AND DISCUSSION

3.1 | Hydrophilic interaction liquid chromatography—HILIC

Cells from three strains of the order *Bacillales* (*A. acidoterrestris* CCM 4659, *B. licheniformis* CCM 2145, and *G. stearothermophilus* CCM 2062) were cultured as described in Section S2 and harvested in the log phase when the optical density of the culture was maximal. After cultivation, the biomass was concentrated by centrifugation and lyophilized. However, due to the different relative hydrophobicities of lipid molecules, it is impossible to extract all lipid classes from biomass [31]. The chloroform/methanol mixture (extraction according to Bligh and Dyer) has already been published several times [30, 32, 33], and it is not suitable for very polar lipids. Therefore, after extraction, according to Bligh and Dyer, a mixture of lower phase of isopropanol/hexane/water (55:20:25 v/v/v) was used for the further extraction of polar lipids [30, 33, 34]. The total lipids of three bacteria were separated by HILIC (Figure 1 and Table S1). The fractions, see Table S1 (marked in bold), were collected manually. Separation of the diacyl standards of PC, PE, PS, PI, PA, and CL was not a problem, and these classes of phospholipids were separated at a baseline.

The preliminary structure of individual classes of phospholipids was determined based on retention times (tR) compared to standards and high-resolution tandem mass spectra of lipoamino acids (aminoacylated PGs (aminoacyl-PGs) and aminoacyl CLs (aminoacyl-CL) [35, 36]. Shotgun analysis of a mixture of three AA–PGs (allo-Thr-PG, Lys-PG, and Ala-PG) from thermophilic bacteria isolated from hot springs is shown in Figure S1. Shotgun analyses (MS1) of a mixture of AA-CLs are given in Table S2. Tandem MS of allo-Ile-PG shows a series of fragments, mainly fatty acyl anions ($RCOO^-$). Other ions, as described by Atila and Luo [37], are aminoacyl ions, that is, ions of type $[AA\text{-}H]^-$, for example, for the AA Lys, it is the ion at m/z 145.0984, which was previously described [38]. Thus, using tandem MS, aminoacyl-PG

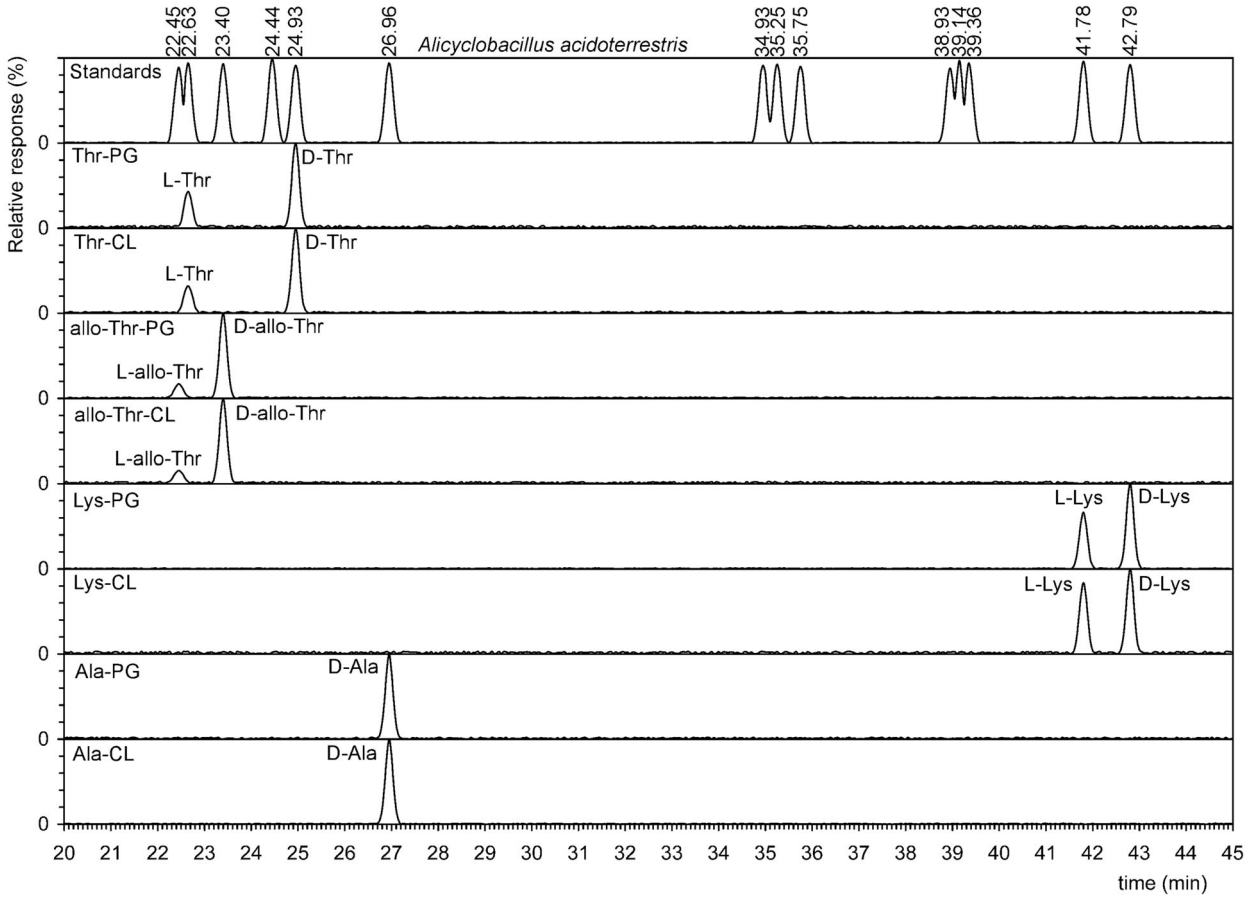

FIGURE 2 Reversed phase liquid chromatography/electrospray ionization–mass spectrometry (RP–LC/ESI–MS) product ion mass spectra of $[M+H]^+$ ions providing m/z 120.0809 *N*-(4-nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester ((S)-NIFE derivatives) as dominating molecular species from *Alicyclobacillus acidoterrestris*. Probable structure of the ion at m/z 120.0809; see Figure S4. Table S4 lists the names of the standards used; see also Section 2.4 for conditions.

and aminoacyl-CL were identified. As an example of a yet-unpublished compound, the tandem mass spectrum of aminoacylated PG (one of three possible AAs, i.e., allo-Ile-, Ile-, or Leu-PG) is presented in Figure S2, and data are provided in Table S3. From this spectrum, it is clear that the ion at m/z 130.0877 is present, that is, the ion of type $[\text{allo-Ile-H}]^-$. The description of the fragmentation of precursor ions of ions of the type $[\text{PG–AA}]^-$ and $[\text{CL–AA}]^-$, that is, ions not containing AA, was previously described in detail [38, 39]. In total, seven aminoacyl-PGs and eight aminoacyl-CLs were identified. Atila and Luo [37] identified a total of 17 types in *B. subtilis*.

As an example of cleavage in tandem MS, the molecular species at m/z 1522.1069, that is, Lys–CL, is given; see Figure S3, describing the fragmentation pathways. As the structures and cleavage in tandem MS have already been described several times, the results are presented only briefly. The $[M–H]^-$ ion found at m/z 1522.1069 loses an AA (Lys) to form an ion at m/z 1394.0121. The characterization of Lys–CL consisting of two different diacylglycerol moieties is illustrated by the product ion spectrum at m/z 1522.1069. The spectrum contains sets of ions at m/z 605.4190 (**a** ion), at m/z 661.4452 (**a** + 56), at m/z 741.4115 (**a** + 136) and at m/z 731.5598 (**b**), at m/z 661.4452 (**b** + 56), at m/z 867.5524 (**b** + 136), nomenclature according to Hsu et al. [27]. In the spectrum, there is an ion of m/z 731.5598, for which the structure was determined to be 19:0/19:0-PA. This ion is formed by the loss of a neutral particle of mass 135.9925, structure, see Figure S3. In addition to the ion at m/z 605.4190, the spectrum is also dominated by the ion at m/z 363.1943, resulting from the loss of the 15:0 acid in *sn*-2, and the less prominent ion at m/z 377.2099, resulting from the loss of 14:0 acids in sn − 1, showing the 14:0/15:0-PA structure. Similarly, the "right" part of CL was identified, where the dominant ions (see Figure S3) indicate the presence of **b**-type ions. Based on the facts mentioned above, it can be concluded that the ion at m/z 1522.1069 probably represents the substituted as the majority component: Lys-14:0/15:0–19:0/19:0–CL.

Both the CL content in bacteria and their identification are described quite often [29–31], so in the next section, the main attention was paid to the AA parts of PG and

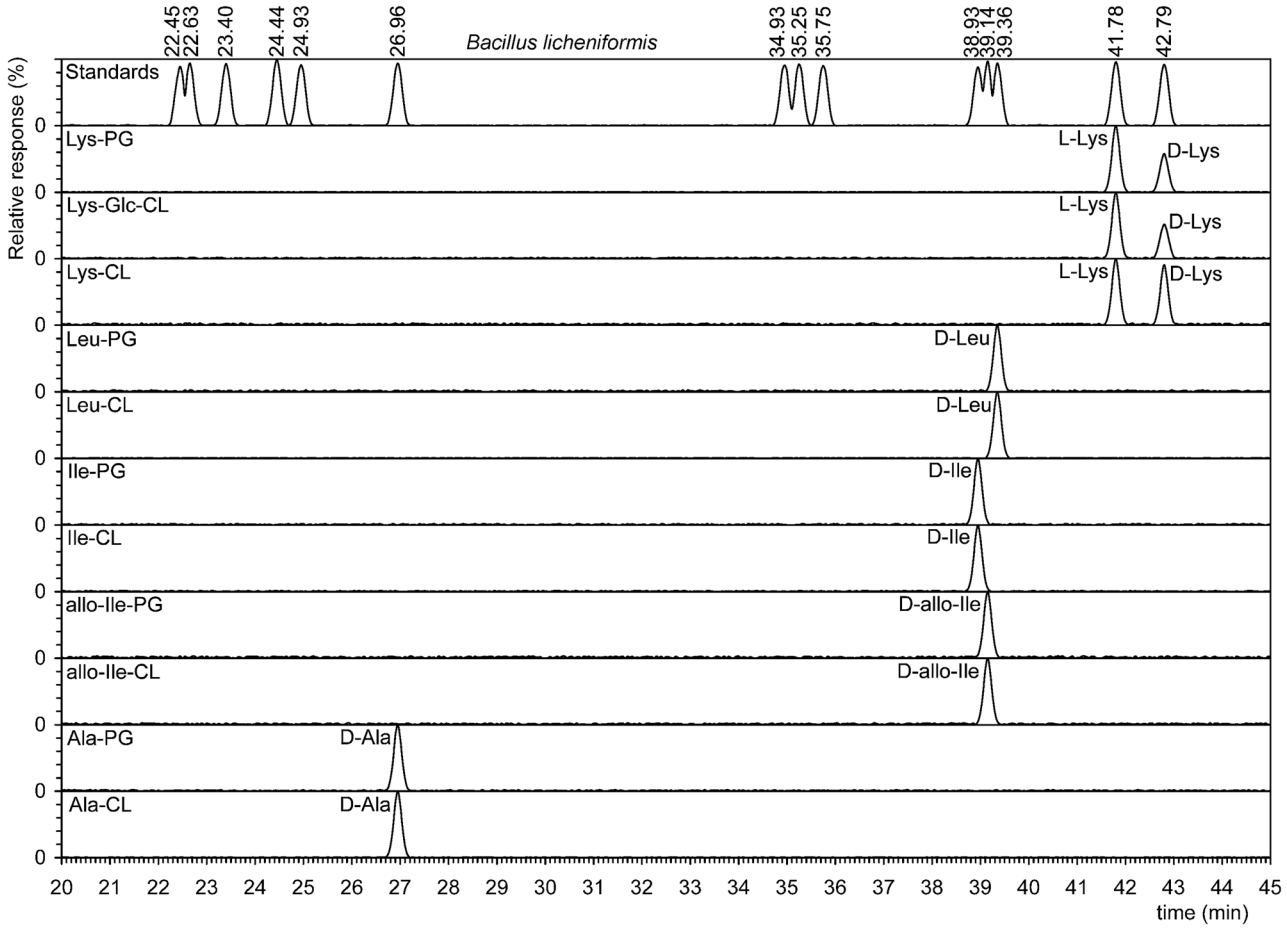

FIGURE 3 Reversed phase liquid chromatography/electrospray positive ionization–mass spectrometry (RP–LC/ESI–MS) chromatogram of product ion at *m/z* 120.0809 *N*-(4-nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester ((S)-NIFE derivatives) from *Bacillus licheniformis*; see also notes on Figure 2.

CL. First, the mixture of AA-PG and AA-CL obtained from HILIC was hydrolyzed, and the resulting AAs were further identified.

3.2 | Reversed phase separation of amino acid diastereomers

The 15 fractions, see Table S1 (marked in bold), were collected manually, and after hydrolysis, the free AAs were derivatized by (S)-NIFE, see above. To determine the degree of AA racemization during hydrolysis, a solution of DCl in D_2O (20% solution) was used. Ester bonds were hydrolyzed under much milder conditions (1 h at 50°C) than used in the hydrolysis of peptides. When a drastic hydrolysis condition of peptides was used (110°C for 20 h), the racemization of AA occurred to form enantiomers. Danielsen et al. (2020) described that the content of D-His increased by two orders of magnitude under the conditions of peptide hydrolysis [40]. To clarify the degree of racemization in our case, L-Ile was subjected to the same reaction as phospholipids after HILIC separation. The L-Ile standard was found to contain 0.05% of the enantiomer, that is, D-Ile, prior to "hydrolysis." After the "hydrolysis," the D-Ile content increased to only 0.08%, based on the basis of L-Ile. The ratio of both enantiomers as (S)-NIFE derivatives was determined using precursor ion values, that is, the ion at *m/z* 381.2022 versus the ion at *m/z* 382.2084. (S)-NIFE reacts with all AAs, and (S)-NIFE diastereomeric derivatives are easily formed in 15–20 min. In addition to 4-nitrophenol, phenylalanine methoxyethyl ester and *N*,*N*-bis(methoxyethyl 3-phenylpropionat-2-yl)urea, are formed as side products; the derivative of urea was used as internal standard at *m/z* 473.2283 $[M+H]^+$ with a tR of 48.71 min.

Individual diastereomers of (S)-NIFE were separated and identified by RP–HPLC/ESI–MS. The separation of synthetically prepared standards from commercially available L- and D-AAs was successful. Figures 2–4 show the separation of (S)-NIFE diastereomers obtained by the hydrolysis of AA–PG and AA–CL, that is, natural classes of lipids from three bacteria. After derivatization using

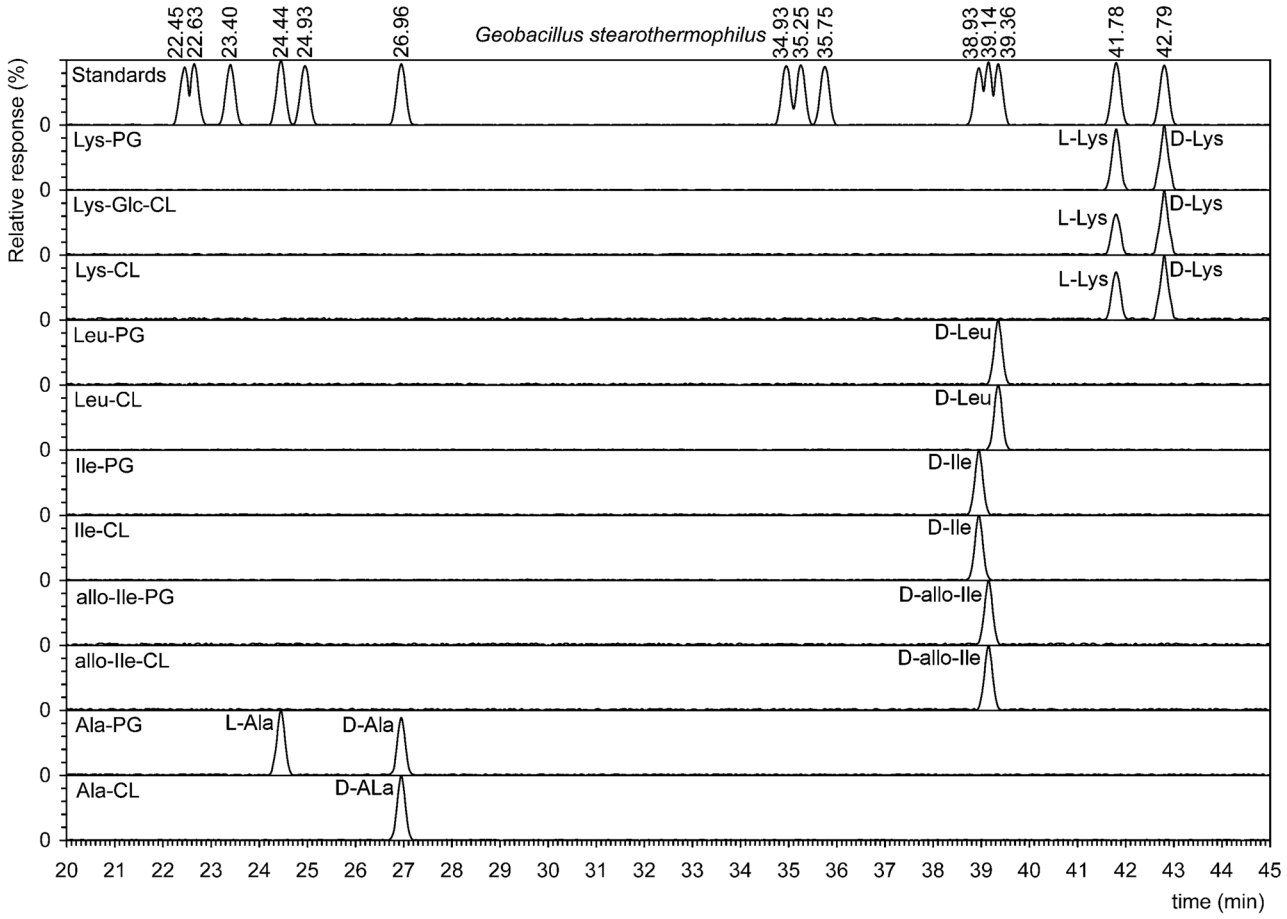

FIGURE 4 Reversed phase liquid chromatography/electrospray positive ionization-mass spectrometry (RP–LC/ESI–MS) chromatogram of product ion at *m/z* 120.0809 *N*-(4-nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester ((S)-NIFE derivatives) from *Geobacillus stearothermophilus*, see also notes on Figure 2.

(S)-NIFE, it was possible to separate Thr and Ile isomers, such as L-, D-, L-allo and D-allo AA derivatives based on the similarity of tR of natural AA derivatives with synthetically prepared standards. Identification of all (S)-NIFE AA derivatives was performed using precursor and product ions. Based on analyses already published [41–43], the ion at *m/z* 120.0809 was chosen as the product ion. A probable structure is shown in Figure S4, see also the already published paper [43]. For all (S)-NIFEs, the D-enantiomers were eluted without exception after the L-enantiomers. The tRs and the separation factors (α) of the (S)-NIFE derivatives are listed in Table S4.

4 | CONCLUDING REMARKS

The presence of aminoacyl-PG and aminoacyl-CL in all three bacteria is not surprising, as several aminoacyl-tRNA synthetases have been identified in all three bacteria; see examples in Table S5. It is generally assumed that aminoacyl-PG and aminoacyl-CL biosyntheses occur from PG and/or CL using the respective aminoacyl-tRNA synthetases (aminoacyl-tRNA ligases) [44]. Using two HPLCs on different phases, that is, HILIC and achiral chromatography, it was proven that all three bacteria biosynthesize different aminoacyl-PG and aminoacyl-CL. On the basis of the analyses, less common AAs, such as allo-Ile and allo-Thr, were identified, and, with exceptions, always the D-enantiomer. Only in *G. stearothermophilus* was L-Ala-PG identified, and in *B. licheniformis*, both allo-Thr enantiomers and both Thr enantiomers. Furthermore, the taxonomic classification of the bacteria showed that the bacteria of the family Bacillaceae (*Bacillus* and *Geobacillus*) produce a different type of aminoacyl-PG and aminoacyl-CL than the bacterium *A. acidoterrestris* (family Alicyclobacillaceae). These are mainly branched chain AA, that is, D-allo-Ile, D-Ile, and D-Leu, which are present only in the genera *Bacillus* and *Geobacillus* and not in *A. acidoterrestris*. In contrast, both L- and D-Thr and L- and D-allo-Thr were identified as aminoacyl-PG and aminoacyl-CL in *A. acidoterrestris*, which were not present in the genera *Bacillus* and *Geobacillus*.

It has been reported [45, 46] that the aminoacylation of phospholipids generally results in the incorporation of a positively charged or zwitterionic molecule into the otherwise negatively charged bacterial cytoplasmic membrane. The consequence is the neutralization of the membrane surface, a reduction of electrostatic affinity to cations, and the stiffness, fluidity, and permeability of the membrane are affected. The incorporation of aminoacylated phospholipids into artificial lipid vesicles stabilizes the lipid bilayer and changes the binding behavior of peptides [47, 48]. On the basis of the previous published work, we believe that mesophilic and even more thermophilic bacteria change their membrane structure in this way to have optimal stiffness (fluidity) at the optimal culture temperature.

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available in the Supporting Information section for the article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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