methods

Bacterial phospholipid molecular species analysis by ion-pair reversed-phase HPLC/ESI/MS

Nicolas Mazzella,* Josiane Molinet,* Agung Dhamar Syakti,* Alain Dodi,† Pierre Doumenq,[1,*] Jacques Artaud,* and Jean-Claude Bertrand[§]

Laboratoire de Chimie Analytique de l'Environnement* Unité Mixte de Recherche 6171, Institut Fédératif de Recherche Pôle Méditerannéen des Sciences de l'Environement 112, Europôle de l'Arbois, BP 80, 13545 Aix-en-Provence Cedex 4, France; Laboratoire d'Analyses Radiochimiques et Chimiques,† Commissariat à l'Énergie Atomique, Centre d'études de Cadarache, 13108 St Paul lez Durance, France; and Laboratoire de Microbiologie, Géochimie et Ecologie Marine,§ UMR 6117, case 901, Université de la Méditerranée, 163 avenue de Luminy, 13228 Marseille Cedex 09, France

Abstract This work set out to optimize the detection and separation of several phospholipid molecular species on a reversed-phase column with the use of an electrospray ionization/mass spectrometry-compatible counter-ion. An application of this technique concerned a qualitative and quantitative analysis of bacterial membrane phospholipids extracted from *Corynebacterium* species strain 8. The phospholipid classes of strain 8 were identified as phosphatidylglycerol, phosphatidylinositol, diphosphatidylglycerol, and a peculiar lipid compound, acyl phosphatidylglycerol. Most of the molecular species structures were elucidated, and regarding phosphatidylglycerol, the fatty acid positions were clearly determined with the calculation of the *sn-2/sn-1* intensity ratio of the fatty acyl chain fragments.—Mazzella, N., J. Molinet, A. D. Syakti, A. Dodi, P. Doumenq, J. Artaud, and J-C. Bertrand. **Bacterial phospholipid molecular species analysis by ion-pair reversed-phase HPLC/ESI/MS. *J. Lipid Res.* 2004.** 45: **1355–1363.**

Supplementary key words reversed-phase liquid chromatography/electrospray ionization/mass spectrometry • negative ionization • counter-ion • *sn-1* and *sn-2* fatty acid positions • *Corynebacterium* species

Phospholipids are the major components of bacterial cell membranes. These lipid compounds possess closely related and complex structures classed according to the polar head group linked to the phosphate moiety. The diversity of the polar head groups is important, and as far as prokaryotic organisms are concerned, the main classes of phospholipids are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and their respective derivatives: mono-, bi-, or trimethylated forms of PE and a dimeric form of PG [diphosphatidylglycerol (DPG)]. The other phospholipid classes, such as phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylserine (PS), are considered to be less characteristic of bacterial cell membranes (1, 2). In addition, phospholipid classes are distinguished according to their anionic (PG, DPG, PS, PI) or zwitterionic (PE, PC) properties. At neutral pH, the former are negatively charged and the latter are globally neutral. Each phospholipid class is made up of several molecular species. The diversity of these molecular species depends on the two fatty acyl chains linked to the glycerol backbone (mainly saturated or monounsaturated and from 16 to 18 carbon atom chain length). Moreover, the study of the fatty acid distribution (*sn-1* or *sn-2* position) is of interest to determine the stereochemistry of the molecular species.

Numerous studies of phospholipid classes have dealt with applications of HPLC exclusively combined with an evaporative light-scattering detector (ELSD) (3–8). Some of these methods have involved complex binary or ternary solvent gradients (4, 5, 7, 8), whereas chromatographic separations have been systematically carried out on normal-phase columns. However, the main drawback of such an analysis is that the information provided is limited to the phospholipid classes. In addition, the use of an ELSD requires that the additives and buffers have to be volatiles

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Abbreviations: APG, acyl phosphatidylglycerol; DMDS, dimethyl disulfide; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol; DPG, diphosphatidylglycerol; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; ELSD, evaporative light-scattering detector; ESI, electrospray ionization; FAME, fatty acid methyl ester; IPMS, intact phospholipid molecular species; MSM, mineral salt medium; PC, phosphatidylcholine, PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLFA, phospholipid ester-linked fatty acid; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-glycerol; PS, phosphatidylserine; TBAAc, tetrabutyl ammonium acetate; TBABr, tetrabutyl ammonium bromide; UV, ultraviolet light.

[1] To whom correspondence should be addressed.
e-mail: pierre.doumenq@univ.u-3mrs.fr

and that the amount of water, although it is essential for attaining the correct elution, ought to be low.

Other works have included analyses carried out on reversed-phase columns with either an ultraviolet light (UV) detector (9) or both an ELSD and a UV detector (10). These separations provided information on molecular species, but the choice of buffers, acids, counter-ions, and solvents was restricted (volatile for ELSD and low absorbance for UV detector). Furthermore, a preliminary isolation of each phospholipid class was necessary. A time-consuming but efficient method consists of phospholipid derivatization (alkylation or aminoalkylation) to improve their separation and their UV detection (10).

Finally, the most recent and convenient approach is related to electrospray ionization (ESI) and mass spectrometry detection. Most authors have used either normal-phase columns (11–15) or, to a lesser extent, reversed-phase columns (16, 17) coupled with an electrospray interface equipped with an ion trap or a triple quadrupole analyzer (tandem mass spectrometry). These techniques are the most efficient ones for achieving qualitative and quantitative analyses of both classes and molecular species. On the other hand, fewer works have used a single quadrupole. In this case, the authors were required to perform the analyses on reversed-phase columns to exhaustively separate and analyze the phospholipid molecular species (2, 18–20).

As far as our work is concerned, we developed an optimized phospholipid detection method involving a methanol-dichloromethane-water gradient that was derived from the mixture usually used for lipid extraction (21). Furthermore, we suggested a mechanistic study concerning the formation of the carboxylate anion fragments that are generally used to determine the fatty acyl chain *sn* position. In addition, we developed an original reversed-phase ion-pair chromatography technique to separate and quantify both phospholipid classes and molecular species. In this way, we chose a tetraalkyl ammonium homolog [tetrabutyl ammonium bromide (TBABr)] as ion-pairing reagent. To our knowledge, such a counter-ion was not previously used for HPLC/ESI/MS phospholipid analysis. Then we were able to separate and identify most of the phospholipid molecular species of a Gram-positive bacterium (*Corynebacterium* species strain 8) cultured in vitro. Finally, we analyzed the ester-linked phospholipid fatty acids as fatty acid methyl esters (FAMEs) by GC-MS to obtain some complementary information on monounsaturated and methyl branched fatty acyl chains.

MATERIALS AND METHODS

Chemicals and materials

Acetone, dichloromethane, methanol, heptane, water (CHROMASOLV grade), diethyl ether (purity >99.9%), and TBABr or acetate (ion-pair reagent; purity, ⩾99%) were purchased from Fluka. TLC Si 60 F254 and Silica gel 60 (0.063–0.200 mm) were purchased from Merck. GF/F filters (47 mm Ø) and Silica gel plus Sep-Pak cartridges were purchased from Whatman and Waters, respectively. Boron trifluoride (10% in methanol, m/v), dimethyl disulfure (99%), iodine (99.99%), and pyrrolidine (99%) were purchased from Aldrich.

1,2-Dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol (DMPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-glycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1,2-diacyl-*sn*-glycero-3-phospho-*rac*-glycerol from egg yolk lecithin (PG), DPG sodium salt from bovine heart, and L-α-PI sodium salt from bovine liver were purchased from Sigma.

Bacterial culture

The Gram-positive marine hydrocarbon-degrading bacterium *Corynebacterium* species strain 8, previously named *Pseudomonas* species strain P8 (22), was cultured in darkness at 20°C, under aerobic conditions, in 500 ml inverted T-shaped flasks containing 200 ml of synthetic mineral salt medium (MSM) supplemented with 3 g/l ammonium acetate. The synthetic MSM was composed of 23 g/l NaCl, 0.75 g/l KCl, 5 g/l Tris(hydroxymethyl)aminomethane, 1 g/l NH_4Cl, 3.9 g/l $MgSO_4$, 5 g/l $MgCl_2$, 1.5 g/l $CaCl_2$, 0.12 g/l K_2HPO_4, 0.002 g/l $FeSO_4$, and $7H_2O$. Aeration was provided by agitation on a reciprocal shaker (1.5 Hz). After 72 h, the bacterial cells were centrifuged (3,500 *g*). The cell pellet was washed twice with MSM and frozen.

Lipid extraction and fractionation

Bacterial cells were extracted as a wet pellet using a modified Bligh and Dyer method (21). Cell pellet (0.5–1 g) was extracted at 4°C for 24 h by stirring with a mixture of chloroform-methanol-water (10:20:8, v/v). After centrifugation at 34,300 m/s^{-2} for 5 min, the mixture was filtered and divided into two phases by adding 16 ml of chloroform and 20 ml of water. After settling (24 h), the lower phase was collected and the aqueous phase was reextracted with 26 ml of chloroform. The two organic phases were added and roto-evaporated under reduced pressure.

The lipid extract (5–10 mg) was fractionated on a silica gel Sep-Pack cartridge (1 g). The sample was loaded on the top of the cartridge and eluted successively with chloroform (10 ml), acetone (10 ml), acetone-methanol (10 ml; 95:5, v/v), and methanol (30 ml). The last fraction, which contained purified phospholipids, was dried under nitrogen and stored at −18°C until HPLC/ESI/MS analysis or derivatization prior to GC-MS analysis was performed.

Analysis of FAMEs and derivatives by GC-MS

Phospholipid ester-linked fatty acids (PLFAs) were transmethylated (BF_3/methanol) to produce FAMEs (23, 24). The double bond position and the geometry of monounsaturated fatty acids were determined by forming dimethyl disulfide (DMDS) adducts (25). Elucidation of the methyl branching position was achieved according to the Andersson and Holman method (26). The *N*-acylpyrrolidine derivatives were prepared by FAME treatment and purified by thin-layer chromatography (heptane-diethyl ether, 80:20, v/v) before GC-MS analysis. FAMEs and their derivatives were separated and identified using a Hewlett-Packard 5890 series II gas chromatograph (Geneva, Switzerland) equipped with an AT-5 mass spectrometer (Alltech) and a 60 m × 0.25 mm (0.25 μm) capillary column and coupled to a Hewlett-Packard 5898A MS Engine mass spectrometer. The transfer line was held at 298°C and the source at 240°C. Electron impact mass spectra were acquired at 70 eV. For the complete compounds, total ion currents (full scan) were acquired from 40 to 600 Da.

A volume of 1 μl was injected into a splitless injector. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. For

FAMEs and DMDS derivative analyses, the oven program was 30°C for 1 min, then 50°C/min up to 70°C, 10°C/min up to 120°C, 2°C/min up to 290°C, and finally held for 10 min. For pyrrolidide derivative analyses, the column was held at 30°C for 1 min, ramped to 100°C at 50°C/min, ramped to 200°C at 20°C/min, ramped to 290°C at 2°C/min, and finally held for 20 min.

Analysis of phospholipids by HPLC/ESI/MS

An HP 1100 series LC/MSD system (Agilent Technologies) was used. The phospholipids were separated on a Microsorb $5C_8$, 150 × 4 mm (5 μm) column with a Microsorb $5C_8$ (5 μm) precolumn (Varian). The HPLC system was coupled with a single quadrupole mass spectrometer equipped with an ESI source. The ionization mode was negative, the nebulizing gas (N_2) pressure was 345 kPa, and the drying gas (N_2) flow and temperature were 9 l/min and 300°C, respectively. The electrospray needle was at ground potential, whereas the capillary tension was held at 4,000 V. The cone voltage was kept at 250 V. The mass resolution was 0.13 Da, and the peak width was set to 0.12 min. For a qualitative analysis, total ion currents (full scan) were acquired from 200 to 1,600 Da. A ternary solvent system was used for the phospholipid elution: dichloromethane (phase A), methanol (phase B), and a 30 mM TBABr aqueous solution (phase C). The proportion of phase C was maintained at 15% during all experiments, resulting in a constant concentration of 4.5 mM TBABr. The gradient profile is given in **Table 1**. The flow rate was 0.5 ml/min, and the separations were carried out at room temperature. All samples and standards were dissolved in dichloromethane-methanol-water (25:60:15, v/v) before chromatography. The injection volume was set to 10 μl. DMPG, DPG, and PI were used as internal standards for each corresponding phospholipid class. The concentrations of internal standards were 10 μg/ml for PI and DMPG and 50 μg/ml for DPG. Quantitative analysis was carried out in single ion monitoring mode.

FAMEs and phospholipid nomenclatures

Fatty acids are designated according to the convention C:n, where C corresponds to the total number of carbon atoms and n refers to the total number of double bonds (e.g., octadecanoic acid is 18:0). The position of the double bond is indicated by the Δ number closest to the carboxyl carbon. The Z or E configuration is given after the Δ position of the double bond. Methyl branching is mentioned as the Δ position of an additional methyl from the carboxylic end of the molecule.

Phospholipids are constituted of a glycerol backbone esterified by two fatty acids on the *sn-1* and *sn-2* positions. The moiety esterified on the *sn-3* position refers to the polar head group (e.g., inositol for the PI or a glycerol for the PG). Each polar head group defines a phospholipid class, and each class can be divided into several molecular species according to the fatty acid composition and distribution.

Phospholipids are abbreviated as follows: PL (ΣC:Σn), where PL corresponds to the polar head group, ΣC refers to the sum of the total number of carbon atoms, and Σn is related to the total number of double bonds of the whole fatty acid. If the distribution and the number of carbons or double bonds of each fatty acyl chain are unknown, then the phospholipid is designated PL (ΣC:Σn) [e.g., PG (32:1), where 32:1 should correspond to either 16:0 and 16:1 or 18:1 and 14:0]. If the fatty acyl chain structures are resolved but the *sn-1* and *sn-2* positions remain unclear, then the phospholipids are designated PL (C:n*C:n). Finally, when the acyl chain composition and distribution are known, then the phospholipids are noted as PL ($C:n_1/C:n_2$), where $C:n_1$ and $C:n_2$ correspond to the fatty acids linked to *sn-1* and *sn-2* positions, respectively [e.g., PG (16:0/18:1) for 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol].

TABLE 1. Linear gradient composition for the separation of phospholipids on a $5C_8$ reversed-phase column coupled to an electrospray ionization/mass spectrometry source

Time	Percent A	Percent B	Percent C	Flow Rate
min				*ml/min*
0	25	60	15	0.5
15	25	60	15	0.5
28	51	34	15	0.5
33	51	34	15	0.5
35	25	60	15	0.5
38	25	60	15	0.5

A, CH_2Cl_2; B, methanol; C, 30 mM tetrabutyl ammonium bromide solution.

RESULTS AND DISCUSSION

ESI/MS fragmentation of phospholipid standards

Phospholipid response optimization and effect of an ion-pairing reagent on sensitivity. Among the various parameters considered (i.e., drying gas flow and temperature, mobile phase flow rate, capillary and cone voltages), only the latter two appeared influential. The others factors were fixed (see Materials and Methods). A multicriteria optimization using a desirability function (27) was then performed on NEMROD-W™ software (LPRAI SARL) to optimize the $[M - H]^-$ ion response of different standard phospholipids (PE, PG, DPG, and PI). **Figure 1** shows the area (in gray) where all of the responses of the multicriteria optimization are satisfied simultaneously, with capillary voltage ranging from 3,500 to 4,120 V and cone voltage ranging from 225 to 270 V.

On the other hand, a methanol-dichloromethane-water gradient was considered at first. We observed a poor separation of the phospholipid classes, and we were not able to distinguish the different molecular species. Abidi and Mounts (9) studied different tetraalkyl ammonium ho-

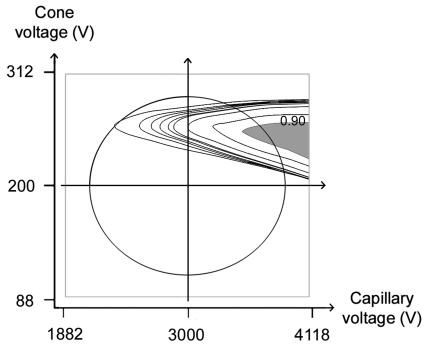
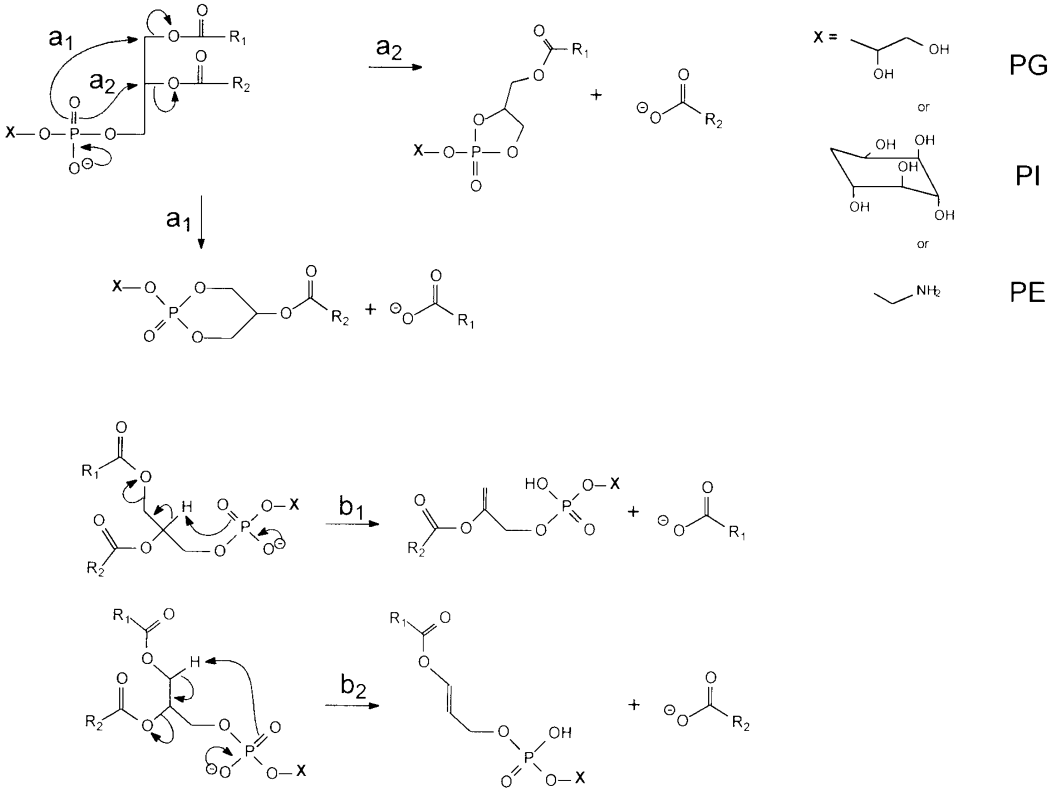


Fig. 1. Multicriteria optimization of the response of a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-glycerol (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), phosphatidylinositol (PI), and diphosphatidylglycerol (DPG) standard phospholipid mixture. The gray area corresponds to the optimal response for the two parameters considered (cone and capillary voltages).

mologs in reversed-phase ion-pair liquid chromatography with UV detection because such counter-ions are commonly used to separate acidic analytes (28, 29). They showed that both the nature of the alkyl chains and the concentration of the ion-pairing reagent could increase the retention and the separation of several molecular species of PE or PC. Thus, since tetraalkyl ammonium homologs were never used for HPLC/ESI/MS analysis of phospholipids as far as we know, we investigated an efficient counter-ion. We chose first the TBABr that was previously used for HPLC/ESI/MS analysis of cyclic nucleotides (29) and studied the effects of this ion-pairing reagent on phospholipid detection. Subsequently, we considered a dichloromethane-methanol-water (25:60:15, v/v) mobile phase supplemented with TBABr. The results were that TBABr did not significantly degrade the phospholipid standard responses until the counter-ion concentration, into phase C, was less than 40 mM. Beyond this limit, as previously reported by Witters et al. (29), both a pollution of the electrospray source and a progressive loss of sensitivity after several runs were observed. Furthermore, instead of using TBABr, tetrabutyl ammonium acetate (TBAAc) was used. This counter-ion, with equivalent concentration and separation improvement compared with TBABr, quickly decreased the detector sensitivity. In fact, it appeared that too high a concentration of such tetraalkyl ammonium homologs would perturb the ESI processes, because the mobile phase evaporation could be reduced. In addition, the analyte ions could be statistically outnumbered near the droplet surface, resulting in a decrease of the analyte signal (29). Finally, TBAAc was no longer used, and the TBABr concentration was kept constant during the entire study (30 mM into phase C).

Formation of $[R_1CO_2]^-$ and $[R_2CO_2]^-$ anions. All of the analyses were achieved in negative ionization mode for the purpose of studying the fatty acyl chain compositions and positions. Fragmentation mechanisms leading to the formation of carboxylate anions are proposed in **Scheme 1**. Two competitive processes could occur: the first one could involve a charge-driven fragmentation (a_1 and a_2), whereas the second one could be related to a charge-remote fragmentation (b_1 and b_2) (30). Both a_1 and b_1 produce the formation of $[R_1CO_2]^-$ anions, and in the same way, a_2 and b_2 produce $[R_2CO_2]^-$ fragments. In addition, some workers have studied the variation of the *sn-1/sn-2* abundance ratio according to the carbon chain length and the number of double bonds (12, 31). Their results, with regard to PE, PS, and PC, have showed that the *sn-2/sn-1* ratio is typically greater than 1 when the carbon chain length of each fatty acids is less than 20 and the number of double bonds is less than 4. Thus, because the aim of this work concerns bacterial phospholipid analysis (mainly characterized by 14:0, 16:0, 16:1, 18:0, and 18:1 fatty acids) (1, 32), we studied the *sn-2/sn-1* ratios of two standards (POPG and POPE) that we considered to be representative of the prokaryotic phospholipids. Concern-



Scheme 1. Proposed collision-induced dissociation pathways of phosphatidylglycerol (PG), phosphatidylinositol (PI), or phosphatidylethanolamine (PE) after an electrospray ionization showing the formation of $[R_1CO_2]^-$ and $[R_2CO_2]^-$ ions.

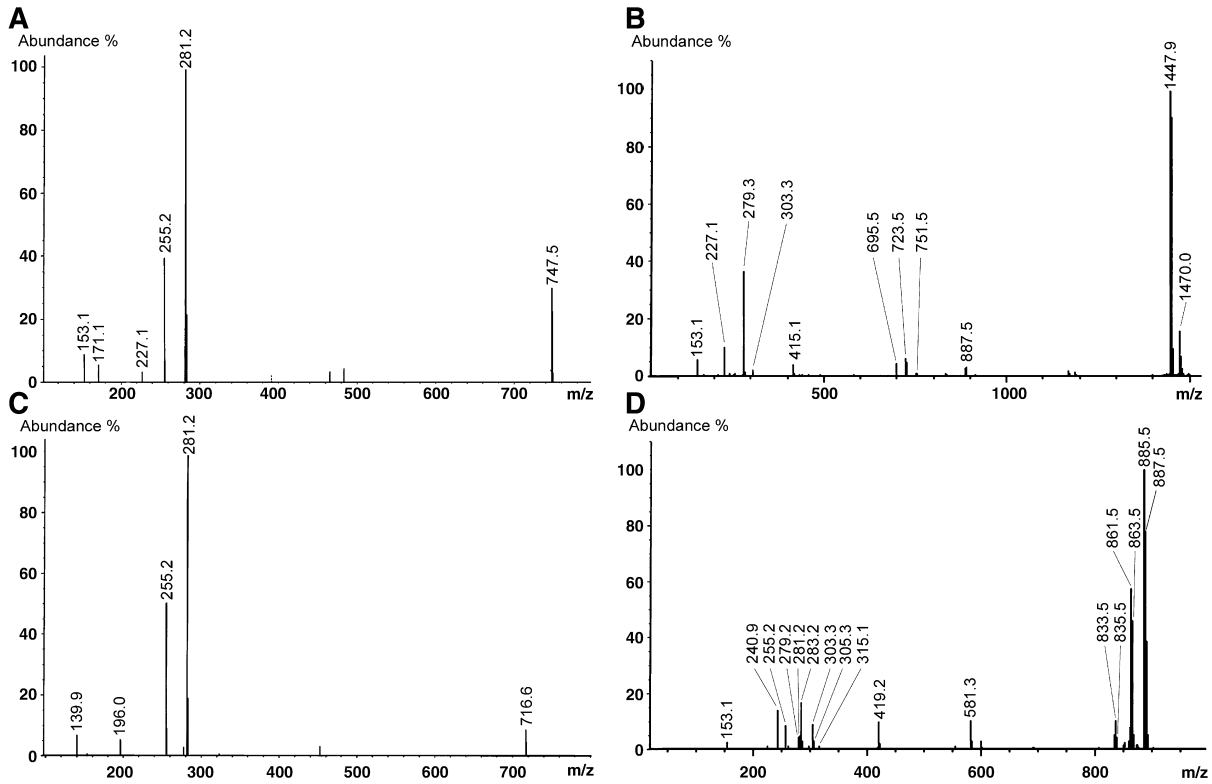


Fig. 2. Negative ionization mass spectra of phospholipid standards: POPG (A), DPG (B), POPE (C), and PI (D).

ing POPG and POPE (**Fig. 2A**, C), we found that the intensity of the carboxylate anion arising from *sn-2* (i.e., $[C_{18}H_{33}O_2]^-$, m/z = 281.2) was more abundant than the carboxylate anion arising from the *sn-1* position (i.e., $[C_{16}H_{31}O_2]^-$, m/z = 255.2). The *sn-2*/*sn-1* ratio, under our experimental conditions, was equal to or slightly higher than 2 for both standards. Such a result was in accordance with previous observations (30, 31, 33). However, unlike both POPG and POPE, we found for PI equivalent intensities of *sn-1* and *sn-2* carboxylate anions after LC separation of the different molecular species included in the standard (data not shown) and at different cone voltages (from 50 to 300 V with a 50 V step size).

PG and PI fragmentation pathways. Negative ESI/MS spectra of POPG (Fig. 2A) showed the $[M - H]^-$ ion with m/z at ~747.5 and the carboxylate anion fragments (m/z = 255.2 and 281.2). Concerning PI, the standard contained six different molecular species (Fig. 2D). The pseudomolecular anions were m/z = 833.5, 835.5, 861.5, 863.5, 885.5, and 887.5. The whole carboxylate anion fragments were characterized by m/z at 255.2, 279.2, 281.2, 283.3, 303.3, and 305.3. For both phospholipid classes, the low mass fragments are reported in **Table 2**. After the neutral loss of a fatty acid (R_1COOH or R_2COOH) followed by the neutral loss of a fatty acyl group as a ketone ($R_1CH{=}C{=}O$ or $R_2CH{=}C{=}O$), we observed the resulting m/z = 227.1 and m/z = 315.1 anions ($[M - R_xCOOH - R_yCH{=}C{=}O]^-$), which were characteristic of PG and PI polar head groups, respectively (33, 34). The neutral loss of the glycerol or the inositol moiety (X-H) was probably attributable to a proton transfer from the alcohol function closest to the phosphate and belonging to the polar head group. This led to the formation of the m/z = 153.1 anion ($[M - R_xCOOH - R_yCH{=}C{=}O - (X\text{-}H)]^-$) for both PI and PG. Finally, the m/z = 240.9 anion (Fig. 2D) is diagnostic of PI in that it corresponds to the inositol phosphate fragment after a loss of H_2O (Table 2) (34).

PE fragmentation pathways. The mass spectra of POPE (Fig. 2C) exhibited a pseudomolecular ion (m/z = 716.6) and two intense fragments (m/z = 255.2 and 281.2) corresponding to the 16:0 and 18:0 fatty acids, respectively. The product anions $[M - R_xCOOH - R_yCH{=}C{=}O]^-$ with a common mass of 196.0 Da (Table 2) were characteristic of PE (19). Unlike PG and PI, there was no neutral loss of the polar head group, because the ethanolamine polar head group did not contain any alcohol function. The only daughter ion (m/z = 139.9) of the m/z = 196.0 frag-

TABLE 2. Characteristic negative ions of PG, PI, and PE

Fragment Ion	PG	PI	PE
		m/z	
$[M - R_xCOOH - R_yCH{=}C{=}O]^-$	227	315	196
$[M - R_xCOOH - R_yCH{=}C{=}O - (X\text{-}H)]^-$	153[a]	153[a]	
$[HPO_4CH_2CH_2NH_2]^-$			140
$[HPO_4(C_6H_{11}O_5) - H_2O]^-$		241	

PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

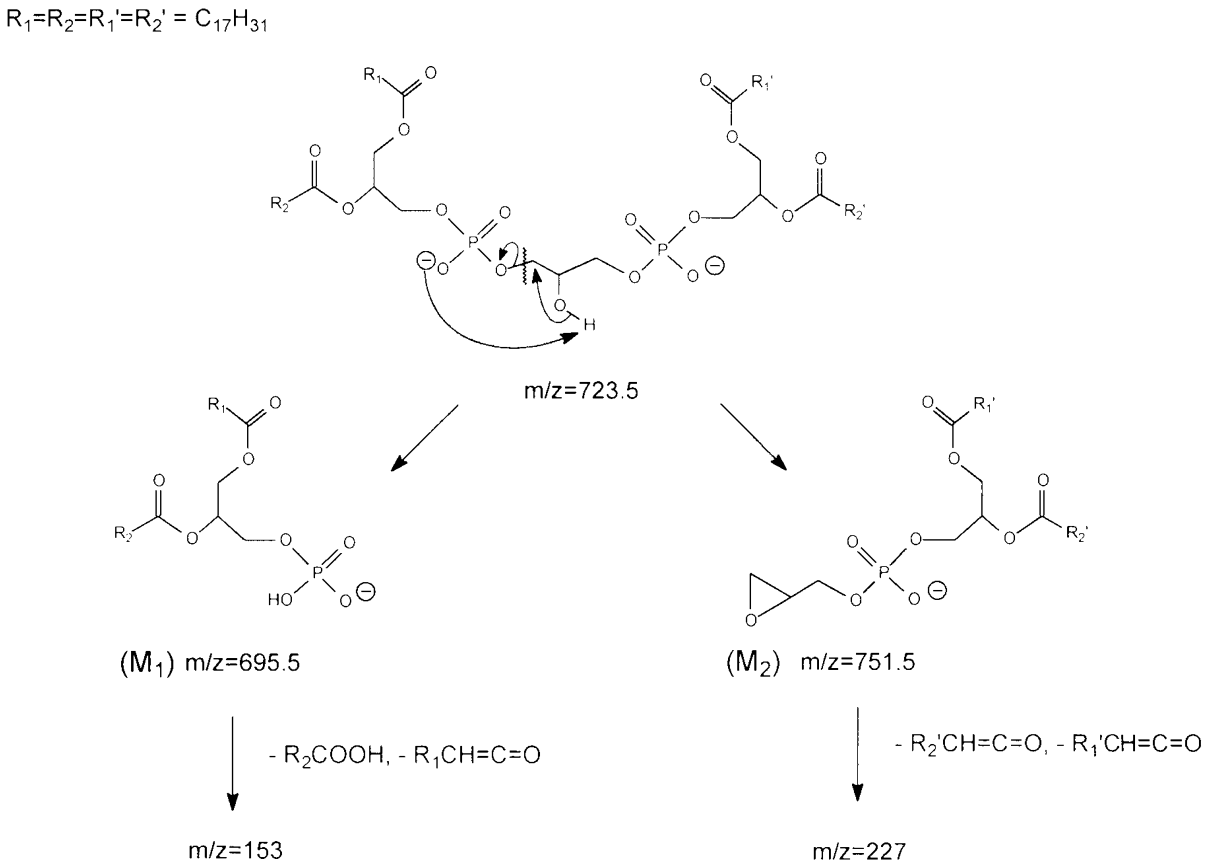
[a] X corresponds to either $CH_2CHOHCH_2OH$ for PG or $C_6H_{11}O_5$ for PI.

ment probably resulted from a neutral loss of a ketone (CH_2=CH-CH=O) (30).

DPG fragmentation pathways. Figure 2B represents the mass spectra of two molecular species of DPG (*m/z* = 1,447.9 and 1,470.0) and the corresponding fatty acids (*m/z* = 279.3 and 303.3). DPG is a unique phospholipid with dimeric structure, carrying four fatty acyl chains and one or two negative charges. DPG is composed of two phosphatidyl moieties linked by a central glycerol group. This phospholipid also contains two phosphate groups that have two different acidities: $pK_1 = 2.8$ and $pK_2 > 7.5$ (35). Generally, the quasi-molecular ion of DPG is $[M - 2H]^{2-}$ (31), but we observed mainly the $[M - H]^-$ anion when an increased cone voltage was applied ($V_{exit\ cap} > 200$ V). **Scheme 2** proposes a fragmentation of a DPG molecular species [DPG (4× 18:2)]. The $[M - H]^-$ anion had a mass of ~1,448 Da, and the four fatty acyl chains are characterized by *m/z* = 279.3 fragments. Under our conditions, the doubly charged form (*m/z* = 723.5) possessed a very low intensity compared with the single charged anion. The dimeric structure of DPG could generate two daughter ions. The first one should be a phosphatidic acid analog (*m/z* = 695.5, named M_1) and probably produced the *m/z* = 153.1 fragment ($[M_1 - R_2COOH - R_1CH=C=O]^-$), like PI and PG. The second one should be a PG analog (*m/z* = 751.5, called M_2) and probably led to the *m/z* = 227.1 anion formation ($[M_2 - R_2'CH=C=O - R_1'CH=C=O]^-$). Consequently, the polar head group fragments that derived from DPG were similar to those obtained from PG.

HPLC/ESI/MS analysis of a phospholipid standard mixture

A chromatographic separation of several molecular species of four phospholipid classes (PG, PI, PE, and DPG) is represented in **Fig. 3**. The DPPE, DPG, PG, and PI standards were constituted of one, two, four, and six molecular species, respectively. First, we noticed that there is some separation among individual molecular species of a particular phospholipid head group class [e.g., PG (18:0*18:2) and PG (18:0/18:1); Fig. 3]. Although the separation between some compounds was barely discernible [e.g., PG (16:0*18:2) and PG (16:0/18:1); Fig. 3], our operating conditions allowed us to achieve unambiguous mass spectra interpretation of the major molecular species. Moreover, it appeared that the separation (or selectivity α) increases with the total fatty acyl chain length [e.g., α ≈ 1.2 between PG (16:0/18:1) and PG (18:0/18:1); Fig. 3] and, conversely, decreases with the double bond incorporation [e.g., α ≈ 1.1 between PI (16:0*18:2) and PI (16:0*18:1); Fig. 3]. Nevertheless, most of the PI



Scheme 2. Proposed collision-induced dissociation pathways of diphosphatidylglycerol after electrospray ionization showing the formation of $[M_1 - R_2COOH - R_1CH=C=O]^-$ or $[M_2 - R_2CH=C=O - R_1CH=C=O]^-$ ions.

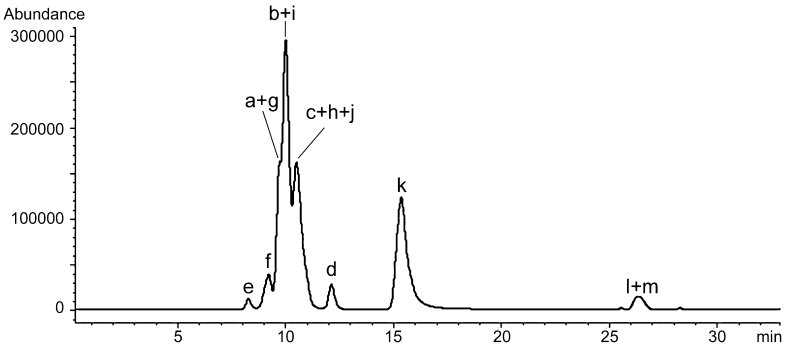


Fig. 3. Single ion monitoring of a standard phospholipid mixture: a, phosphatidylglycerol (PG; 16:0*18:2); b, PG (16:0/18:1); c, PG (18:0*18:2); d, PG (18:0/18:1); e, PI (16:0*18:2); f, PI (16:0*18:1); g, PI (18:0*18:2); h, PI (18:0*18:1); i, PI (18:0*20:4); j, PI (18:0*20:3); k, phosphatidylethanolamine (2× 16:0); l, DPG (4× 18:2); m, DPG (3× 18:2*20:4). Electrospray ionization/mass spectrometry (HPLC/ESI/MS) conditions are described in Materials and Methods.

and PG molecular species possess similar retention times. This could be attributable to a very close polarity of inositol and glycerol polar head groups. Further modifications of the gradient composition, as well as a concentration increase, did not significantly improve the separation of PG and PI. In addition, the retentions and the chromatographic patterns of DPG and acyl phosphatidylglycerol (APG) [not shown in Fig. 3 but present in the bacterial extract (**Fig. 4**)] were dramatically affected. Ultimately, a loss of sensitivity was observed when ion-pairing reagent concentration became important, as mentioned previously.

According to the results of Kim, Wang, and Ma (18), the phospholipid responses appeared largely more dependent on the polar head group than on the fatty acyl chain composition. Furthermore, the response of the PC molecular ion was investigated by Koivusalo et al. (36). They observed a 10% decrease in response when four atoms of carbon were added to the total acyl chain length. In the same way, they noticed that the incorporation of one double bond could improve sensitivity. However, they showed a large diminution of both previous effects at low concentration. With regard to phospholipid classes, the linear dynamic range was from 1 to 200 μg/ml for either PG or PI. The sensitivity of DPG was ~10-fold less important compared with that of PG and PI (linear dynamic range, 20–500 μg/ml). Thus, in accordance with the observations of Koivusalo et al. (36), we diluted our samples to ensure the correct reproducibility during quantitative analyses (molecular species areas of each class were comparable to the areas of the corresponding internal standards). A triplicate HPLC/ESI/MS quantitative analysis of the same sample was performed, and a variation of ⩽12% was obtained.

Qualitative and quantitative analyses of a bacterial extract

PLFA analysis. A preliminary study of PLFAs was carried out because a HPLC/ESI/MS analysis provided no information about the methyl branched fatty acyl chains or the double bond positions and geometry. Also, such information is largely used for microbial characterization (1, 20, 23). The phospholipid methanolysis of strain 8 cultured on ammonium acetate produced the following fatty acids: 14:0 (1.3%), 16:1Δ^{7Z} + 16:1Δ^{7E} (3.2%), 16:0 (37.7%), 18:1Δ^{9Z} (40.0%), 18:0 + 9-Me-18:1Δ^{10E} (11.1%), 10-Me-18:0 (3.6%), and two unidentified isomers x-Me-19:1 (both 3.0%). These results are in accordance with several previous works showing that bacterial PLFAs are characterized by the predominance of 14:0, 16:1, 16:0, 18:1, and 18:0 (1, 30). However, some peculiar PLFAs were found in strain 8 lipid composition: 10-Me-18:0 and a rare compound, 9-Me-18:1Δ^{10E} (coeluted with 18:0), previously observed among several *Corynebacteria* species (37).

Intact phospholipid class analysis. The *Corynebacterium* genus is characterized by the presence of the common bacterial phospholipids PG, DPG, and PI. Like most Gram-positive bacteria, their cell membranes are made up of small amounts of PE and its methylated derivatives (<10%) (1). Furthermore, the *Corynebacteria* species contain an unusual phospholipid, APG, which is a derivative of PG esterified by a fatty acyl chain on the *sn-1′* position of the glycerol polar head group (37, 38). Consequently, this lipid liberates three fatty acids after methanolysis or saponification (38). Additionally, some species, such as *Corynebacterium diphteriae*, *Corynebacterium xerosis*, *Corynebacterium equi*, and *Corynebacterium bovis*, contain some predominant lipids such as mannophosphoinositides (mannoside deriva-

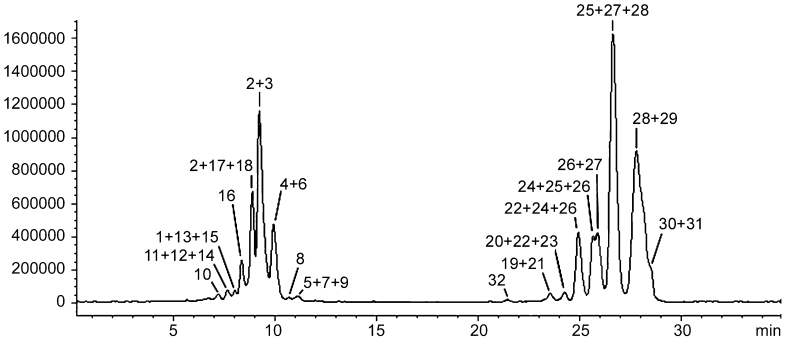


Fig. 4. Single ion monitoring of the molecular ions of the phospholipid extracted from *Corynebacterium* species strain 8 and separated on a $5C_8$ reversed-phase column. HPLC/ESI/MS conditions are described in Materials and Methods. See Table 4 for compound identifications.

TABLE 3. Intact phospholipid class composition of *Corynebacterium* species strain 8

Phospholipid Class	Amount
	relative %
PI	9[a]
PG	91[a]
Acyl phosphatidylglycerol	ND
Diphosphatidylglycerol	<1[a,b]

ND, not determined.

[a] Indicated values are means of three different analyses. The standard deviation did not exceed 12% of the mean.

[b] Diphosphatidylglycerol is present in a very low amount.

tives of PI) (39). As far as our study is concerned, we observed the PG, DPG, APG, and PI classes (Fig. 4). However, like Niepel et al. (37), we found no traces of PE and mannoside derivatives of PI. A quantification of the PG, DPG, and PI classes was performed (**Table 3**), but the amount of APG was not determined because no commercial standard was available to our knowledge. After the quantitation, DPG appeared as a very minor compound (<1%), whereas PG (except for the undetermined APG) seemed to be a major component of the strain 8 cell membrane (91% of the quantified phospholipids).

Intact phospholipid molecular species analysis. The HPLC/ESI/MS chromatogram of strain 8 is shown in Fig. 4. At first, the whole intact phospholipid molecular species (IPMS) of PI and PG were eluted between 5 and 12 min. The IPMS of DPG and APG possessed higher retention times (>20 min). All of the molecular species are reported in **Table 4**. Both PG and PI contained 9 IPMS, whereas DPG was composed of only a single molecular species. Thus, both the low response and abundance of this phospholipid class could explain the apparent lack of diversity of the DPG IPMS. Otherwise, the number of molecular species of APG was considerably higher (>13), because there were several separated isomers [e.g., APG (16:1*2×18:1); Table 4, Fig. 4). This diversity of the APG molecular species is the consequence of the three fatty acyl chain distributions on either the glycerol backbone or the glycerol polar head group.

Insofar as the IPMS study is concerned, for PG we noticed that the shortest fatty acyl chain is generally linked to the *sn-2* position [the position was determined by the *sn-2/sn-1* ratio calculation; e.g., the intensity ratio of (16:0)$^-$ over (18:1)$-$ was ~2.2 for PG (18:1/16:0)]. Such a result is in accordance with the characterization of several Gram-positive bacteria IPMS (1). In contrast to PG, the *sn* position of PI, APG, and DPG could not be determined. In fact, APG and DPG contain three to four fatty acyl chains, whereas the PI *sn-2/sn-1* ratio retains a value of ~1. In addition, the main PLFAs of PG were 16:1Δ^{7Z}, 16:1Δ^{7E}, 16:0, 18:1Δ^{9Z}, 9-Me-18:1Δ^{10E}, and some x-Me-19:1. The 10-Me-18:0 was present only in a minority of IPMS [PG (19:0/16:0); 3.7%]. On the other hand, this PLFA, which is present in a small amount (3.6% of all PLFAs), is linked to one of the major IPMS of PI [PI (19:0*16:0); 35.0%]. Consequently, although the molecular species of PG and PI possess similar biosynthetic pathways (40, 41), the enzymes involved in the metabolic routes probably have specific activities. Thus, the IPMS study, completed by a PLFA analysis, allows the definition of new and efficient biomarkers that should be very useful for either bacteria chemotaxonomy or membrane property studies.

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TABLE 4. Intact phospholipid molecular species composition of *Corynebacterium* species strain 8

Compound Number	Phospholipid Molecular Species	Amount[a]	Compound Number	Phospholipid Molecular Species	Amount[a]
1	PG (32:1)	3.0	17	PI (19:0*16:0)	35.0
2	Σ PG (18:1*16:1)	3.7[b]	18	PI (19:1*18:1)	0.5
3	PG (18:1/16:0)	62.0	19	APG (2× 16:0*16:1)	0.8
4	PG (19:1/16:0)	17.7	20	APG (49:1)	0.5
5	PG (19:0/16:0)	3.7	21	APG (2× 16:1*18:1)	0.3
6	PG (2× 18:1)	4.8	22	Σ APG (50:2)	9.3[b]
7	PG (18:0*18:1)	3.7	23	APG (2× 16:0*18:1)	2.8
8	PG (19:1*18:1)	0.3	24	Σ APG (51:2)	4.2[b]
9	PG (37:1)	1.2	25	Σ APG (51:1)	3.7[b]
10	PI (32:1)	2.0	26	Σ APG (16:1*2× 18:1)	6.0[b]
11	PI (2× 16:0)	0.2	27	Σ APG (16:0*2× 18:1)	42.0[b]
12	PI (19:1*14:0)	3.0	28	Σ APG (16:0*18:1*19:1)	26.2[b]
13	PI (19:0*14:0)	1.8	29	APG (3× 18:1)	0.5
14	PI (18:1*16:1)	0.8	30	APG (54:2)	2.0
15	PI (18:1*16:0)	16.3	31	APG (54:1)	1.7
16	PI (19:1*16:0)	40.3	32	DPG (2× 16:1*18:1*19:1)	100

APG, acyl phosphatidylglycerol; DPG, diphosphatidylglycerol.

[a] Indicated values are expressed in relative percentages and correspond to means of three different analyses. The standard deviation did not exceed 12% of the mean. Relative percentage areas are given for the molecular species belonging to a sole phospholipid class (i.e., PG, PI, APG, or DPG).

[b] Sum of different separated isomers.

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