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# Effects of pure *n*-alkanes and crude oil on bacterial phospholipid classes and molecular species determined by electrospray ionization mass spectrometry

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**Abstract**

Phospholipids are major components of bacterial membrane. Furthermore, the growth in vitro on xenobiotics such as *n*-alkanes, aromatic compounds or alkanols bring about to a bacterial membrane adaptive response. Concerning this work, we studied the membrane lipid composition of a hydrocarbon-degrading gram-positive bacterium (*Corynebacterium* sp.) on a soluble substrate and we detected four different phospholipid classes: phosphatidylglycerol, phosphatidylinositol, cardiolipin and acyl phosphatidylglycerol. In addition, a study of the lipid composition was performed after an in vitro culture on either pure *n*-alkane or crude oil. The growths on such hydrophobic substrates showed major qualitative and quantitative modifications. In the case of a growth on either heneicosane or crude oil, an increase of odd-numbered fatty acids was observed. Furthermore, the phospholipid polar head group composition was highly influenced by the crude oil addition. These modifications were, respectively, interpreted as the consequence of hydrocarbon assimilation and membrane fluidity adaptation. Finally, *Corynebacterium* sp. was taken back on the initial ammonium acetate substrate in order to determine its restoration abilities after a petroleum contamination.


*Keywords:* RP-HPLC/ESI/MS; Tandem ESI/MS/MS; Petroleum; Hydrophobic substrates; Gram-positive bacteria; *Corynebacterium* sp

*Abbreviations:* RP-HPLC/ESI/MS, reversed-phase high performance liquid chromatography/electrospray ionization/mass spectrometry; TLC, thin-layer chromatography; GC/MS, gas chromatography/mass spectrometry; TBABr, tetrabutyl ammonium bromide; PLFAs, phospholipid ester-linked fatty acids; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; FAMEs, fatty acid methyl esters; DMDS, dimethyl disulfide; HDAI, hydrocarbon-degrading activity index = (odd-numbered straight chain SFAs + branched SFAs + odd-numbered straight chain MUFAs + branched MUFAs)/even-numbered MUFAs); PG, phosphatidylglycerol; PE, phosphatidylethanolamine; DPG, diphosphatidyglycerol or cardiolipin; APG, acylphosphatidylglycerol; PI, phosphatidylinositol; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-glycerol; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol; IPCs, intact phospholipid classes; IPMS, intact phospholipid molecular species; *n*-$C_{20}$, eicosane; *n*-$C_{21}$, heneicosane; BAL 250, Blend Arabian Light petroleum topped at 250 °C

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## 1. Introduction

Microorganisms characterization are of particular interest in natural environmental samples so as to estimate biomasses and to describe bacterial community structures. There are two different groups of methods in order to obtain such information. The first group involves microbial isolation and microbiological techniques. However, these techniques are time consuming [1], selective and not quantitative [2,3] since most prokaryotes in the environment are viable but not cultivable [4,5]. The second group concerns a variety of biochemical methods that does not need any bacterial cultures or isolations. Among these methods, we can name different approaches such as the molecular biology techniques [6] or the detection of some bio-organic compounds [7]. Concerning this last approach, some compounds, like lipids for instance, were early proposed as biomarkers [8–12]. The most common method is related to phospholipid ester-linked fatty acids (PLFAs) analysis by GC/MS as fatty acid methyl esters derivatives [2,13–16]. Such an analysis provided precious information about cultures of isolated bacteria and especially concerning the effects of xenobiotics such as ethanol and toluene [17–20] or *n*-alkanes [21–24] on PLFA profiles. In the same way, the analysis of PLFAs extracted from in vitro bacterial community grown on crude oil revealed the effects of this complex hydrophobic substrate [21]. Nevertheless, the use of PLFAs possesses some limitations. In one hand, methanolysis lead to a PLFA mixture that corresponds to different phospholipid classes [25] and it results in an important loss of information. In other hand, regarding to in situ samples, the usual lipid extract is a complex matrix that contains various non-phospholipid fatty acid-bearing biomolecules [12]. Thus, these two drawbacks could obscure the sedimentary microbial characterization. More recently, some works were related to the direct intact bacterial phospholipid analysis [26,27,25,28–32]. Afterward, several authors performed intact bacterial phospholipid analysis by HPLC/ESI/MS with a soft ionization technique implying an electrospray interface coupled with a normal phase [30,31] or a reverse phase liquid chromatography column [25,28,29,32].

In this paper, we reported the use of an ion-pairing RP-HPLC/ESI/MS technique so as to determine the intact phospholipid classes (IPCs) and molecular species (IPMS) of *Corynebacterium* sp., which possess hydrocarbon-degrading abilities [33,34]. The hydrocarbon-degrading abilities of many prokaryotes are well known [35], but there is very few studies about Corynebacterium genus [33,34]. Besides, a large part of the works, which are related to the effects of xenobiotics on bacterial membrane, focused on gram-negative bacteria studies [17–19,36]. Consequently, we investigated the lipid membrane composition of *Corynebacterium* sp. since few information are available as concerns the gram-positive bacteria. *Corynebacterium* sp. was cultured either on a soluble substrate such as ammonium acetate or on hydrophobic substrates such as pure *n*-alkanes (either *n*-$C_{20}$ or *n*-$C_{21}$) or crude oil. The effects of the three different hydrophobic media on PLFA, IPC and IPMS composition as well as on fatty acyl chain parity were monitored in order to differentiate the nature of the xenobiotics. Afterward, *Corynebacterium* sp. was taken back on the initial ammonium acetate substrate so as to determine its restoration abilities. Furthermore, a study of the phospholipid fatty acyl chain stereochemistry by tandem ESI/MS/MS provided useful information concerning the membrane properties of *Corynebacterium* sp.

## 2. Experimental

### 2.1. Chemicals and materials

Acetone, dichloromethane, methanol, heptane, water (chromasolv grade), diethyl ether (puriss. p.a.) and tetrabutyl ammonium bromide (ion-pair reagent, purity ≥99%) were purchased from Fluka (Germany). TLC Si 60 F254, Silica gel 60 (0.063–0.200 mm) were purchased from Merck (Germany). GF/F filters (47 mm Ø) and 1 g Silica gel plus Sep-Pak™ cartridges were purchased from Whatman (England) and Waters (Ireland), respectively. Boron trifluoride (10% in methanol, m/v), dimethyl disulfure (99%), iodine (99.99%) and pyrrolidin (99%) were purchased from Aldrich (USA). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-glycerol P-6956 (POPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine P-5203 (POPE), 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol P-6412 (DMPG), cardiolipin or diphosphatidylglycerol sodium salt from bovine heart C-0563 (DPG) and L-α-phosphatidylinositol sodium salt from bovine liver P-5766 (PI) were purchased from Sigma (USA).

### 2.2. Culture media

The mineral salt medium (MSM) was composed of 23 g $l^{-1}$ of NaCl, 0.75 g $l^{-1}$ KCl, 5 g $l^{-1}$ of tris(hydroxymethyl)aminomethane, 1 g $l^{-1}$ $NH_4Cl$, 3.9 g $l^{-1}$ $MgSO_4$, 5 g $l^{-1}$ $MgCl_2$, 1.5 g $l^{-1}$ $CaCl_2$, 0.12 g $l^{-1}$ $K_2HPO_4$, 0.002 g $l^{-1}$ $FeSO_4{\cdot}7H_2O$. The ammonium acetate medium was obtained by an addition of 3 g of ammonium acetate to 1 l of MSM. The eicosane and heneicosane media were 1 g $l^{-1}$ MSM solutions of *n*-$C_{20}$ and *n*-$C_{21}$, respectively. The BAL 250 medium was a 2 g $l^{-1}$ MSM solution of Blend Arabian Light petroleum topped at 250 °C (BAL 250).

### 2.3. Bacterial cultures

The gram-positive hydrocarbon-degrading bacterium *Corynebacterium* sp., previously named *Pseudomonas* sp. Strain P8 [37], was isolated from a sub-surface sediment coming from the Hycarfos site (Golf de Fos, France). *Corynebacterium* sp. was pre-cultured in the darkness at 20 °C, under aerobic conditions and in 500 ml inverted T-shaped flasks containing 200 ml of MSM supplemented with 3 g $l^{-1}$ of

ammonium acetate. Aeration was provided by agitation on a reciprocal shaker (96 rpm).

An experimental protocol has been worked out in order to estimate the effects of a massive crude oil addition as well as for monitoring degradation and remediation abilities after a petroleum contamination [21].

Firstly, a cell inoculum from the ammonium acetate pre-culture was transferred on the ammonium acetate medium and grown for 72 h (late exponential growth phase) under the same conditions described above. Then, cells were centrifuged (3500 × *g* for 30 min), washed and frozen until lipid extraction. This sample was called *Reference*. In the same way, three others cell inoculae from the acetate pre-culture were transferred on three different hydrophobic media that were eicosane, heneicosane and BAL 250 solutions, respectively. The cultures were carried out during 21 days (aerobic conditions, 20 °C, late exponential growth phase). Afterward, cells were transferred on the same hydrophobic substrates (eicosane, heneicosane or BAL 250) and grown 21 days again (as previously described) so as to prevent any growth of the microorganisms on ammonium acetate traces remaining from the pre-culture medium. Only cells from the second growth were centrifuged (3500 × *g* for 30 min), washed and frozen until lipid extraction. These samples were called *n*-$C_{20}$, *n*-$C_{21}$ and BAL, respectively. Finally, three cell inoculae from *n*-$C_{20}$, *n*-$C_{21}$ or BAL samples were brought back on ammonium acetate, grown twice on this medium (72 h, aerobic conditions, 20 °C) so as to prevent any growth of the microorganisms on pure alkanes or petroleum traces. Only cells from the second growth were centrifuged (3500 × *g* for 30 min), washed and frozen until lipid extraction. These last samples were called *Return* (*n*-$C_{20}$), *Return* (*n*-$C_{21}$) and *Return* (BAL), respectively.

### 2.4. Lipid extraction and fractionation

Bacterial cells were extracted as wet pellets using a modified Bligh and Dyer method [38]. Cell pellets (0.5–1 g) were extracted at 4 °C for 24 h by agitation with a mixture of chloroform/methanol/water (10:20:8, v/v). After centrifugation at 3500 × *g* for 5 min, the mixture was filtered and divided into two phases by adding 16 ml chloroform and 20 ml water. After settling (24 h), the lower phase was collected and the aqueous phase was re-extracted with 26 ml chloroform. The two organic phases were added and then concentrated using rotary evaporation followed by a blow-down under a gentle stream of nitrogen.

Lipid extract (about 100 mg) from BAL was fractionated on a column (1.5 cm i.d., 30 cm length) packed with 6 g of silica gel 60 deactivated with 5% of distilled water. Neutral lipids including saturated hydrocarbons and polyaromatic unsaturated hydrocarbons were eluted with chloroform (100 ml), glycolipids with acetone (100 ml) and the polar fraction, containing phospholipids (and some non phospholipid polar lipids as impurities), with methanol (300 ml).

Lipid extracts (5–10 mg) from *Reference*, *n*-$C_{20}$, *n*-$C_{21}$, the polar fraction previously isolated from BAL, *Return* (*n*-$C_{20}$), *Return* (*n*-$C_{21}$) and *Return* (BAL) were fractionated on 1 g silica gel Sep-Pack™ cartridges. Each concentrated extract was loaded on the top of a cartridge and eluted successively with chloroform (10 ml), acetone (10 ml), acetone/methanol (10 ml, 95:5, v/v) and methanol (30 ml). The methanolic fractions, which contained purified phospholipids, were evaporated and dried as described above until direct LC/ESI/MS analysis or derivatizations prior to GC/MS analysis.

### 2.5. Analysis of fatty acid methyl esters (FAMEs) and derivatives by GC/MS

Phospholipid ester-linked fatty acids were transmethylated ($BF_3$/MeOH) to produce FAMEs [39,21]. The double-bond position and the geometry of monounsaturated fatty acids were determined by forming dimethyl disulfide (DMDS) adducts [40]. Elucidation of the methyl branching position was achieved according to the Andersson and Holman method [41]. The *N*-acylpyrrolidine derivatives were prepared by FAMEs treatment and purified by TLC (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 (HP, Geneva, Switzerland) equipped with a AT-5 MS (Alltech, USA), 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 all 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 $min^{-1}$. For FAMEs and DMDS derivative analyses, the oven program was 30 °C for 1 min, then 50 °C $min^{-1}$ up to 70 °C, 10 °C $min^{-1}$ up to 120 °C, 2 °C $min^{-1}$ up to 290 °C, and finally held for 10 min. For pyrrolidine derivative analyses, the column was held at 30 °C for 1 min, ramped to 100 °C at 50 °C $min^{-1}$, ramped to 200 °C at 20 °C $min^{-1}$, then to 290 °C at 2 °C $min^{-1}$ and finally held for 20 min.

### 2.6. Semi-quantitative analysis of phospholipids by RP-HPLC/ESI/MS

For phospholipid analysis an HP 1100 series LC/MSD system (Agilent technologies, USA) was used. The phospholipids were separated on a Microsorb $5C_8$, 150 mm × 4 mm (5 μm) column with a Microsorb $5C_8$ (5 μm) precolumn (VARIAN, USA). The HPLC system was coupled with a single quadrupole mass spectrometer equipped with an electrospray ionization source (ESI). 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 $min^{-1}$ and 300 °C, respectively. The electrospray needle was at ground

Table 1
Linear gradient composition (%) for the separation of phospholipids on a reverse phase column (Microsorb $5C_8$, 150 mm × 4 mm, 5 μm) coupled to an ESI/MS source (A: $CH_2Cl_2$, B: MeOH, C: 30 mM TBABr solution)

| Time (min) | %A | %B | %C | Flow rate (ml min$^{-1}$) |
|---|---|---|---|---|
| 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 |

potential, whereas the capillary tension was held at 4000 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 1600 Da. A ternary solvent system was used for the phospholipid elution: dichloromethane (phase A), methanol (phase B) and a 30 mM tetrabutyl ammonium bromide (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 of TBABr. The gradient profile is given in Table 1. The flow rate was 0.5 ml min$^{-1}$ and the separations were carried out at the room temperature. All samples and standards were dissolved in dichloromethane/methanol/water (25:60:15, v/v/v) prior to the 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$^{-1}$ for PI and DMPG and 50 μg ml$^{-1}$ for DPG. The molecular species contained in each standards were not present within the samples. For instance there was no natural occurrence of DMPG (i.e. PG (2 × 14:0)) in all bacterial extracts. Quantitative analyses were carried out in single ion monitoring mode. For each samples, $[M-H]^-$ ions of phospholipid molecular species of each classes were determined by a direct injection within the electrospray ionization source. Afterward, semi-quantitative analyses were achieved after a separation on a reverse phase column.

### 2.7. Qualitative analysis of phospholipids by tandem ESI/MS/MS

Electrospray mass spectra were acquired on an API III Plus:AB triple quadrupole (SCIEX, Canada) in negative ionization mode. Samples were directly introduced into the electrospray ionization source at a flow rate of 9 μL min$^{-1}$ using a syringe pump (Harvard Apparatus, USA). The temperature of the nebulization gas ($N_2$) and the drying gas ($N_2$) was kept at 48 °C. Their respective flow rates were set to 0.8 L min$^{-1}$ and 0.4 L min$^{-1}$. Ion spray voltage was set at 4800 V. The full scan mass ranges were *m*/*z* 700–800, *m*/*z* 800–900 and *m*/*z* 950–1050 for the pseudo-molecular ions of PG, PI or APG classes, respectively. CID ions mass spectra were acquired by colliding the Q1 selected precursor ions with Argon gas at a collision target gas thickness of (5–7) × $10^{14}$ molecules cm$^{-2}$ and applying collision energy of 30 eV in Q2. Scanning range of Q3 was *m*/*z* 200–320 with a step size of *m*/*z* 0.1 and a dwell time of 4 ms. Both Q1 and Q3 were operated at unit resolution. The qualitative analysis in tandem ESI/MS/MS allowed us to confirm the fatty acyl chain stereochemistry of the molecular species of each sample previously determined by LC/ESI/MS.

### 2.8. FAME 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. octadecenoic acid is 18:1). The position of the double bond is indicated with 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 phosphatidylinositol or a glycerol for the phosphatidylglycerol). 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 ($\Sigma C{:}\Sigma n$) where PL corresponds to the polar head group and $\Sigma C$ refers to the sum of the total number of carbon atoms and $\Sigma n$ is related to the total number of double bonds of the whole fatty acids. If the distribution and the number of carbon atoms or double bonds of each fatty acyl chain are unknown, then the phospholipid is designated as PL ($\Sigma C{:}\Sigma n$) (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 mentioned as PL (*C*:*n***C*:*n*). Finally, when the acyl chains composition and distribution are both determined, then the phospholipids are noted as PL ($C{:}n_1/C{:}n_2$). $C{:}n_1$ 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 the 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol).

## 3. Results

### 3.1. Phospholipid ester-linked fatty acid analysis

#### 3.1.1. Reference sample

The PLFA composition of *Corynebacterium* sp. Strain 8 grown on the initial substrate (ammonium acetate) is given in Table 2. This sample exhibited a large proportion of monounsaturated fatty acids (MUFAs, 58.5%), mainly composed of $18{:}1\Delta^{9Z}$ (44.4%). The group of saturated fatty acids (SFAs) contained no odd-numbered straight chain compounds. These

Table 2
GC/MS relative amounts of the PLFAs extracted from *Corynebacterium* sp. grown on ammonium acetate (*Reference*, *Return* (*n*-$C_{20}$), *Return* (*n*-$C_{21}$) and *Return* (BAL)), eicosane (*n*-$C_{20}$), heneicosane (*n*-$C_{21}$) or crude oil (BAL)

| PLFAs[a] | PLFAs[a] relative amounts (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Reference*[b] | *n*-$C_{20}$ | *n*-$C_{21}$ | BAL | *Return* (*n*-$C_{20}$) | *Return* (*n*-$C_{21}$) | *Return* (BAL) |
| 14:0 | 0.9 | 1.4 | 0.4 | 0.9 | 2.1 | 2.3 | 1.5 |
| 15:0 | | | 11.2 | 5.2 | | | |
| 16:1$\Delta^{7Z}$ | 2.0 | 0.7 | | | 2.1 | 1.8 | 4.9 |
| 16:1$\Delta^{7E}$ | 1.1 | | 0.4 | | 1.9 | 1.5 | 1.1 |
| 16:0 | 32.4 | 35.5 | 3.0 | 27.1 | 40.3 | 38.7 | 28.6 |
| Σbr-17:0[c] | | | | 5.9 | | | |
| Σ17:1[c] | | | 10.1 | | | | |
| 17:1$\Delta^{9Z}$ | | | | 4.2 | | | |
| 17:0 | | | 21.5 | 6.7 | 1.0 | | |
| Σbr-18:0[c] | | | 1.2 | 4.5 | | | |
| 18:1$\Delta^{9Z}$ | 44.4[e] | 43.5[e] | 7.4 | 27.2[e] | 45.5[e] | 44.2[e] | 56.8[e] |
| 18:0[d] | 5.4 | 5.3 | 1.9 | 1.8 | 3.6 | 4.5 | 3.8 |
| 9-Me-18:1$\Delta^{10E}$[d] | 7.6 | 7.0 | 1.6 | 3.4 | 1.2 | 4.2 | 0.3 |
| Σbr-19:1[c] | 1.3 | | | | | 0.3 | |
| Σbr-19:0[c] | | | | 8.4 | | | |
| 10-Me-18:0 | 2.9 | 5.7 | 0.9 | | 2.3 | 2.1 | 3.2 |
| Σ19:1[c] | 2.1 | | 25.6[e] | | | 0.4 | |
| 19:1$\Delta^{10Z}$ | | | | 4.7 | | | |
| 19:0 | | | 1.9 | | | | |
| 20:0 | | 0.8 | | | | | |
| 21:0 | | | 12.9 | | | | |
| SFAs[f] | 41.6 | 48.7 | 54.9 | 60.5 | 49.3 | 47.6 | 37.0 |
| Even SFAs[f] | 38.7 | 43.0 | 5.3 | 29.8 | 46.0 | 45.5 | 33.8 |
| Odd SFAs[f] | | | 47.5 | 11.9 | 1.0 | | |
| br SFAs[f] | 2.9 | 5.7 | 2.1 | 18.8 | 2.3 | 2.1 | 3.2 |
| MUFAs[g] | 58.5 | 51.2 | 45.1 | 39.5 | 50.7 | 52.4 | 63.1 |
| Even MUFAs[g] | 47.5 | 44.2 | 7.8 | 27.2 | 49.5 | 47.5 | 62.8 |
| Odd MUFAs[g] | 2.1 | | 35.7 | 8.9 | | 0.4 | |
| br MUFAs[g] | 8.9 | 7.0 | 1.6 | 3.4 | 1.2 | 4.5 | 0.3 |
| SFAs[f]/MUFAs[g] | 0.71 | 0.95 | 1.22 | 1.53 | 0.97 | 0.91 | 0.59 |
| HDAI[h] | 0.29 | 0.29 | 11.03 | 1.58 | 0.09 | 0.15 | 0.06 |

See Section 2 for abbreviations.
[a] Phospholipid ester-linked fatty acids.
[b] Indicated values are the means of three different cultures.
[c] Sum of non-identified isomers.
[d] 18:0 and 9-Me-18:1$\Delta^{10E}$ are co-eluted. Relative amounts were calculated from characteristic ions.
[e] Major compounds.
[f] SFAs: saturated fatty acids; Even SFAs: even-numbered straight chain SFAs; Odd SFAs: odd-numbered straight chain SFAs; Br SFAs: branched SFAs.
[g] MUFAs: monounsaturated fatty acids; Even MUFAs: even-numbered straight chain MUFAs; Odd MUFAs: odd-numbered straight chain MUFAs; Br MUFAs: branched MUFAs.
[h] Hydrocarbon-degrading activity index (Aries et al., 2001). HDAI = (Σ odd-numbered straight chain SFAs + Σ branched SFAs + Σ odd-numbered straight chain MUFAs + Σ branched MUFAs)/Σ even-numbered MUFAs.

observations were in accordance with different previous results showing that bacterial PLFAs are characterized by the predominance of 14:0, 16:1, 16:0, 18:1, 18:0 [42,8]. However, some uncommon PLFAs were found in *Corynebacterium* sp. strain 8 such as 10-Me-18:0 (2.9%) and a rare fatty acid (9-Me-18:1$\Delta^{10E}$ (7.6%)). Both of these PLFAs were previously observed among several *Corynebacterium* species [27].

### 3.1.2. Samples from the growth on a pure n-alkane (n-$C_{20}$ or n-$C_{21}$) or a crude oil (BAL)

The PLFAs extracted from the eicosane sample showed a fatty acid composition close to the *Reference* (Table 2). In fact, the MUFAs group represented 51.2% of the total PLFAs with the 18:1$\Delta^{9Z}$ as major compound (43.5%). Furthermore, the amount of the 16:0 is similar on both substrates (32.4 and 35.5% for *Reference* and *n*-$C_{20}$, respectively). On the other hand, the main differences between the two samples concerned the loss of br-19:1 and 19:1 PLFAs but also the occurrence of 20:0 (0.8%) after a growth on eicosane. These observations were confirmed by the hydrocarbon-degrading activity index (HDAI) proposed by Aries et al. [21] since we found 0.29 for the two samples.

Concerning the growth on an odd-numbered alkane (*n*-$C_{21}$) or a crude oil (BAL), we observed some major differences with the *Reference* PLFA composition. Actually, there was an increase of both odd-numbered straight chain and

branched SFAs (49.6, 30.7 and 2.9% for *n*-$C_{21}$, BAL and *Reference* samples, respectively) as well as both odd-numbered and branched MUFAs (37.3, 12.3 and 11.0% for *n*-$C_{21}$, BAL and *Reference* samples, respectively). Like above, the HDAI was applied and it returned the following values: 11.03, 1.58 and 0.29 for *n*-$C_{21}$, BAL and *Reference* samples, respectively. Furthermore, several branched SFAs (Σbr-17:0, Σbr-18:0 and Σbr-19:0) were observed only on BAL 250 medium. Lastly, the growth on pure *n*-alkane and crude oil induced an increase of the saturation degree (SFAs/MUFAs was equal to 0.95, 1.22 and 1.53 for *n*-$C_{20}$, *n*-$C_{21}$ and BAL, respectively).

### 3.1.3. *Return (n-$C_{20}$), Return (n-$C_{21}$) and Return (BAL) samples*

The returns on ammonium acetate after a growth on pure alkane (*n*-$C_{20}$ or *n*-$C_{21}$) produced PLFA compositions particularly close to this one related to the reference sample. Thus, we observed a high proportion of even-numbered MUFAs (49.5 and 47.5% for *Return* (*n*-$C_{20}$) and *Return* (*n*-$C_{21}$), respectively) with a large predominance of the 18:1$\Delta^{9Z}$ (45.5 and 44.2% for *Return* (*n*-$C_{20}$) and *Return* (*n*-$C_{21}$), respectively). Concerning the SFAs, the 16:0 was again the second major compound during the two returns (40.3 and 38.7% for *Return* (*n*-$C_{20}$) and *Return* (*n*-$C_{21}$), respectively). Moreover, the proportion of this last PLFA was slightly higher compared to the *Reference* (32.4%). As far as *Return* (BAL) is concerned, like both previous samples, a restoration was globally obtained for this sample. However, we noticed an increase of the 18:1$\Delta^{9Z}$ relative amount (56.8%).

## 3.2. *Intact phospholipid class analysis*

The negative ion mass spectrum of the *Reference* sample, obtained from a direct injection, is reproduced in Fig. 1. At first, we noticed the prominent peaks of the main fatty acyl chains with ion-to-charge ratios of *m/z* = 227.2, 255.1, 281.2 and 295.2 that corresponded to 14:0, 16:0, 18:1 and 19:1 PLFAs, respectively. Afterward, we observed several $[M-H]^-$ ions from *m/z* = 719.5 to 787.5 (mainly *m/z* = 719.5, 747.5 and 761.5; Table 4) which were related to the molecular species of the phosphatidylglycerol (PG) class. In the same way, the molecular species of the phosphatidylinositol (PI) class were characterized by different $[M-H]^-$ from *m/z* = 807.5 to 875.5 (mainly *m/z* = 849.5; Table 5). The molecular species of the acyl phosphatidylglycerol (APG) class were represented by the $[M-H]^-$ ions from *m/z* = 957.8 to 1039.8 (mainly *m/z* = 1011.8 and 1025.8; Table 6). Finally, only one molecular species (*m/z* = 1370.8) of diphosphatidylglycerol (DPG) was detected. The negative ion mass spectra of the other samples (data not shown) were qualitatively similar concerning the phospholipid classes and differed only in the molecular species composition.

The relative amounts of the intact phospholipid classes (IPCs) were determined by LC/ESI/MS with the use of internal standards excepted for acyl phosphatidylglycerol since no commercial product was available as far as we were aware. The linear dynamic range was from 1 to 200 μg $ml^{-1}$ for either PG or PI. The sensitivity of DPG was about 10 fold less important compared to PG and PI (linear dynamic range: 20 to 500 μg $ml^{-1}$). A triplicate LC/ESI/MS quantitative analysis of the same sample was performed and a variation ≤12% was obtained. Further details are given in a previous work [32]. The Semi-quantitative results are reported in Table 3. The *Reference* sample was characterized by a large predominance of PG (about 91%) over the other IPCs (9% of PI and less than 1% of DPG). The growth on pure *n*-alkane (*n*-$C_{20}$ or *n*-$C_{21}$) essentially ended up to an increase of the PG class (98% for *n*-$C_{20}$ and more than 99% for *n*-$C_{21}$) associ-

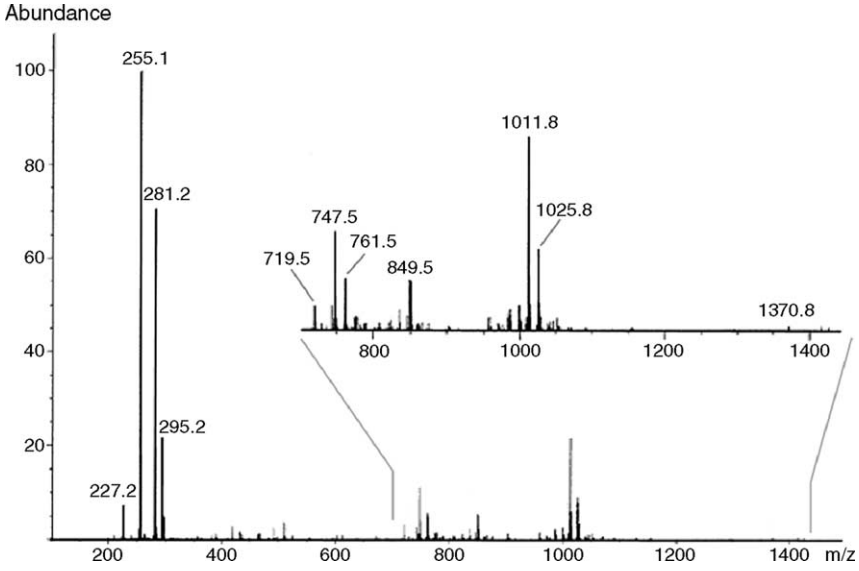


Fig. 1. ESI/MS negative ionization of the *Reference* sample: mass spectrum of the phospholipid molecular species ions and their corresponding fatty acyl chains fragments.

Table 3
LC/ESI/MS relative amounts of the intact phospholipid classes extracted from *Corynebacterium* sp. grown on ammonium acetate (*Reference*, *Return* ($n$-$C_{20}$), *Return* ($n$-$C_{21}$) and *Return* (BAL)), eicosane ($n$-$C_{20}$), heneicosane ($n$-$C_{21}$) or crude oil (BAL)

| IPCs[a] | IPCs[a] relative amounts (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Reference*[b] | $n$-$C_{20}$ | $n$-$C_{21}$ | BAL | *Return* ($n$-$C_{20}$) | *Return* ($n$-$C_{21}$) | *Return* (BAL) |
| PI | $9 \pm 1$ | 2 | N.D.[d] | 1 | 1 | 30 | 3 |
| PG | $91 \pm 1$ | 98 | >99 | 24 | 99 | 70 | 79 |
| DPG | <1[c] | <1[c] | <1[c] | 75 | <1[c] | <1[c] | 18 |

See Section 2 for abbreviations.
[a] Intact phospholipids classes.
[b] Indicated values are the means (±1 S.D.) of three different cultures.
[c] Diphosphatidylglycerol was present in very low amount.
[d] Non detected.

ated to a decrease of PI class (2% for $n$-$C_{20}$ and non-detected for $n$-$C_{21}$). Besides, the petroleum (BAL) growth produced a really different phospholipid class composition. In fact, DPG appeared as the major class (75%) instead of PG (24%). In this experiment, only the fate of PI (1%) for this sample was comparable with the loss of this IPCs observed during the cultures on pure *n*-alkanes. Insofar the returns are considered, the three samples exhibited three different compositions. Actually, *Return* ($n$-$C_{20}$) was characterized by a very high amount of PG (99%) whereas *Return* ($n$-$C_{21}$) contained about 70% of PG and 30% of PI and *Return* (BAL) was made up of 79% of PG, 3% of PI and a noticeable quantity of DPG (18%). Finally, DPG constituted a very minor IPC within the whole samples (excluding BAL and *Return* (BAL)) and, therefore, only few or even no molecular species were detected. Consequently, we studied exclusively the IPMS related to PG, PI and APG.

### 3.3. Intact phospholipid molecular species analysis

#### 3.3.1. Determination of the sn fatty acyl chain position from standards

We first elucidate the intact molecular species (IPMS) stereochemistry (*sn* fatty acyl chain position) after a separation on reverse phase column. Moreover, we used a tandem ESI/MS/MS so as to confirm the structural determination. All the analyses were achieved in negative ionization mode. Some workers have studied the variation of the *sn*-1 to *sn*-2 abundance ratio according to the carbon chain length and the number of double bonds [43,44]. Their results, with regards to PE, phosphatidylserine (PS) and phosphatidylcholine (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 <20 and the number of double bonds is <4. Thus, since the aim of this work concerned bacterial phospholipid analysis (mainly characterized by 14:0, 16:0, 16:1, 18:0 and 18:1 fatty acids) [42,8], we studied the *sn*-2/*sn*-1 ratio of different standards (POPG, POPE and PI) that we considered as representative of the prokaryotic phospholipids. As described in a previous work [32], we found, concerning POPG and POPE that the intensity of the carboxylate anion arising from the *sn*-2 position (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. $[C_{16}H_{31}O_2]^-$, $m/z = 255.2$) (data not shown). 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 [43,45,46]. The *sn*-2 position is in general known as the preferential cleavage position [47]. However, unlike both POPG and POPE, we found for phosphatidylinositol equivalent intensities of *sn*-1 and *sn*-2 carboxylate anions. Consequently, it was not possible to define the fatty acyl chain *sn* position of PI molecular species within the whole samples.

#### 3.3.2. Overall description of LC/ESI/MS chromatographic patterns

The IPMS relative amounts within the different bacterial extracts were determined by LC/ESI/MS. Fig. 2 repre-

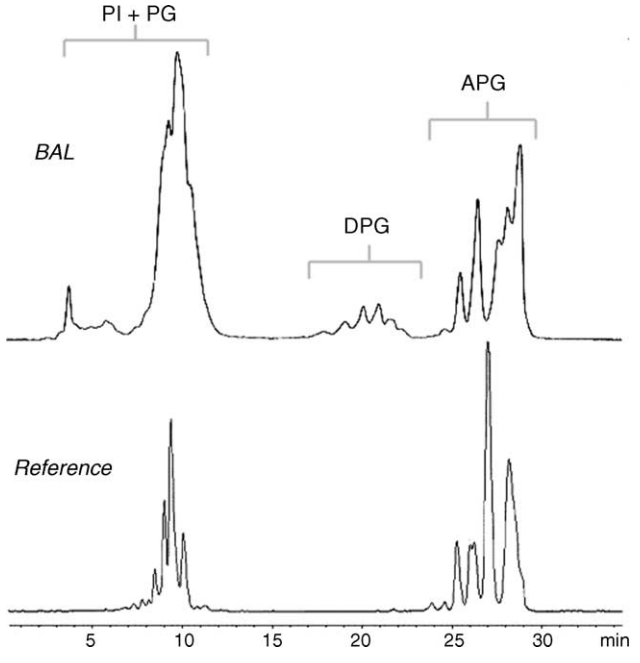


Fig. 2. Single ion monitoring of the phospholipid molecular species extracted from *Reference* and BAL cultures. LC/ESI/MS conditions are described in Section 2.6.

Table 4
LC/ESI/MS relative amounts of the phosphatidylglycerol intact molecular species extracted from *Corynebacterium*sp. grown on ammonium acetate (*Reference*, *Return* (*n*-$C_{20}$), *Return* (*n*-$C_{21}$) and *Return* (BAL)), eicosane (*n*-$C_{20}$), heneicosane (*n*-$C_{21}$) or crude oil (BAL)

| IPMS[c] | IPMS[c] relative amounts (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *m/z* | *Reference*[a] | *n*-$C_{20}$ | *n*-$C_{21}$ | BAL | *Return* (*n*-$C_{20}$) | *Return* (*n*-$C_{21}$) | *Return* (BAL) |
| PG (32:1) | 719.5 | | | | 6.2 | | | |
| PG (18:1/14:0) | 719.5 | } 3.2[b] | 1.1 | | | } 2.6[b] | } 4.7[b] | } 1.8[b] |
| PG (16:1/16:0) | 719.5 | | | | | | | |
| PG (17:1/15:0) | 719.5 | | | 5.4 | | | | |
| PG (17:1/16:0) | 733.5 | | | | } 11.0[b] | | | |
| PG (18:1/15:0) | 733.5 | | | | | | | |
| PG (34:2) | 745.5 | | | | 7.5 | | | |
| PG (18:1/16:1) | 745.5 | 6.6 | 2.2 | | | 5.6 | 7.3 | 14.7 |
| PG (2 × 17:1) | 745.5 | | | 1.3 | | | | |
| PG (18:1/16:0) | 747.5 | 61.7[d] | 50.9[d] | | 20.2 | 64.6[d] | 59.3[d] | 53.3[d] |
| PG (17:1/17:0) | 747.5 | | | } 28.5[b] | | | | |
| PG (19:1/15:0) | 747.5 | | | | | | | |
| PG (19:1/16:0) | 761.5 | 18.9 | 31.8 | | } 24.8[b,d] | 17.1 | 18.4 | 2.8 |
| PG (18:1/17:0) | 761.5 | | | 19.6 | | | | |
| PG (35:0) | 763.5 | 0.8 | 5.9 | | 9.0 | | | |
| PG (19:0/16:0) | 763.5 | | | } 0.6[b] | | 2.2 | 1.5 | 1.8 |
| PG (18:0/17:0) | 763.5 | | | | | | | |
| PG (36:2) | 773.5 | | | | 8.5 | | | |
| PG (2 × 18:1) | 773.5 | 5.0 | 4.7 | | | 5.0 | 4.5 | 17.7 |
| PG (19:1/17:1) | 773.5 | | | 4.2 | | | | |
| PG (36:1) | 775.5 | 3.3 | 1.4 | | | 2.0 | 3.0 | 8.0 |
| PG (19:1/17:0) | 775.5 | | | 39.1[d] | 12.7 | | | |
| PG (37:2) | 787.5 | 0.6 | 2.0 | 1.3 | | 0.9 | 1.3 | |
| ΣPG (ΣC:2)[e] | | 12.2 | 8.9 | 6.8 | 16.0 | 11.5 | 13.1 | 32.4 |
| ΣPG (ΣC:1)[f] | | 87.1 | 85.2 | 92.6 | 74.9 | 86.3 | 85.4 | 65.9 |
| ΣPG (ΣC:0)[g] | | 0.8 | 5.9 | 0.6 | 9.0 | 2.2 | 1.5 | 1.8 |

See Section 2 for abbreviations.
[a] Indicated values are the means of three different cultures.
[b] PG (18:1/14:0) and PG (16:1/16:0), PG (17:1/17:0) and PG (19:1/15:0), PG (19:0/16:0) and PG (18:0/17:0), PG (17:1/16:0) and PG (18:1/15:0) or PG (19:1/16:0) and PG (18:1/17:0) were co-eluted and identified within the same sample.
[c] Intact phospholipid molecular species.
[d] Major compounds.
[e] Sum of PG containing two monounsaturated fatty acyl chains.
[f] Sum of PG containing one monounsaturated fatty acyl chain.
[g] Sum of PG containing no monounsaturated fatty acyl chains.

sent the LC/ESI/MS single ion monitoring of the *Reference* and BAL samples, respectively. Concerning the two cultures (Fig. 2), we observed the whole molecular species of PG and PI between 3 and 13 min but also some molecular species of DPG (from 17 to 23 min) and APG (over 23 min). The chromatograms of *n*-$C_{20}$, *n*-$C_{21}$ and the three returns were particularly similar to the *Reference* and are not shown here. In fact, only the BAL chromatographic pattern was different because we could clearly distinguish at least six molecular species of DPG. In addition, the unresolved complex mixture corresponding to PG and PI revealed an increase of the molecular species diversity. Actually, the BAL sample contained a slightly higher number of IPMS (22 for both PG and PI; Tables 4 and 5) with a lower abundance of the major compounds (24.8% for both PG (19:1/16:0) and PG (18:1/17:0) as well as 23.6% for PI (35:0); Tables 4 and 5) compared to the *Reference* (17 IPMS with 61.7% for PG (18:1/16:0) and 50.2% for PI (19:1*16:0); Tables 4 and 5).

### 3.3.3. Phosphatidylglycerol molecular species

Concerning the *Reference* and the three return samples (Table 4), we noticed that the major IPMS was PG (18:1/16:0) (61.7, 64.6, 59.3 and 53.3% for *Reference*, *Return* (*n*-$C_{20}$), *Return* (*n*-$C_{21}$) and *Return* (BAL), respectively). This result was in good match with the combinations of the two main PLFAs (Table 2): 16:0 (32.4, 40.3, 38.7 and 28.6% for *Reference*, *Return* (*n*-$C_{20}$), *Return* (*n*-$C_{21}$) and *Return* (BAL), respectively) and 18:1$\Delta^{9Z}$ (44.4, 45.5, 44.2 and 56.8% for *Reference*, *Return* (*n*-$C_{20}$), *Return* (*n*-$C_{21}$) and *Return* (BAL), respectively).

Table 5
LC/ESI/MS relative amounts of the phosphatidylinositol intact molecular species extracted from *Corynebacterium*sp. grown on ammonium acetate (*Reference*, *Return* (*n*-$C_{20}$), *Return* (*n*-$C_{21}$) and *Return* (BAL)), eicosane (*n*-$C_{20}$) or crude oil (BAL)

| IPMS[c] | IPMS[c] relative amounts (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *m/z* | *Reference*[a] | *n*-$C_{20}$ | BAL | *Return* (*n*-$C_{20}$) | *Return* (*n*-$C_{21}$) | *Return* (BAL) |
| PI (32:1) | 807.5 | 2.6 | | 2.7 | | | |
| PI (18:1*14:0) | 807.5 | | | | } 5.3[b] | } 9.5[b] | } 3.2[b] |
| PI (16:1*16:0) | 807.5 | | | | | | |
| PI (32:0) | 809.5 | | 10.4 | 2.5 | 12.1 | 2.9 | 2.0 |
| PI (33:1) | 821.5 | | | 2.6 | | 1.0 | |
| PI (19:1*14:0) | 821.5 | 3.9 | | | | | |
| PI (33:0) | 823.5 | 1.6 | | 4.7 | | 0.6 | |
| PI (34:2) | 833.5 | 1.3 | | | | 4.2 | 8.9 |
| PI (34:1) | 835.5 | | 7.7 | 5.9 | | | |
| PI (18:1*16:0) | 835.5 | 18.8 | | | 46.6[d] | 50.9[d] | 49.3[d] |
| PI (34:0) | 837.5 | | | 13.1 | 5.9 | 5.9 | |
| PI (35:1) | 849.5 | | 5.6 | 8.3 | | | |
| PI (19:1*16:0) | 849.5 | 50.2[d] | | | 6.7 | 10.1 | 2.4 |
| PI (35:0) | 851.5 | | 68.8[d] | 23.6[d] | | | |
| PI (19:0*16:0) | 851.5 | 21.1 | | | 19.7 | 12.8 | 18.0 |
| PI (36:2) | 861.5 | | | | | 1.4 | 7.7 |
| PI (36:1) | 863.5 | | | 8.8 | | 0.7 | 7.7 |
| PI (36:0) | 865.5 | | 7.5 | 16.8 | 3.7 | | |
| PI (37:2) | 875.5 | 0.5 | | | | | |
| PI (37:1) | 877.5 | | | 4.6 | | | 0.8 |
| PI (37:0) | 879.5 | | | 6.4 | | | |
| ΣPI (ΣC:2)[e] | | 1.8 | | | | 5.6 | 16.6 |
| ΣPI (ΣC:1)[f] | | 75.5 | 13.3 | 32.9 | 58.6 | 72.2 | 63.4 |
| ΣPI (ΣC:0)[g] | | 22.7 | 86.7 | 67.1 | 41.4 | 22.2 | 20.0 |

See Section 2 for abbreviations. N.B.: There was no IPMS corresponding to *n*-$C_{21}$ since there was none phosphatidylinositol IPC detected.
[a] Indicated values are the means of three different cultures.
[b] PI (18:1*14:0) and PI (16:1*16:0) were co-eluted and identified within the same sample.
[c] Intact phospholipid molecular species.
[d] Major compounds.
[e] Sum of PI containing two monounsaturated fatty acyl chains.
[f] Sum of PI containing one monounsaturated fatty acyl chain.
[g] Sum of PI containing no monounsaturated fatty acyl chains.

The growth on eicosane (*n*-$C_{20}$ sample; Table 4) produced a similar composition compared to the whole cultures on ammonium acetate since PG (18:1/16:0) (50.9%) and PG (19:1/16:0) (31.8%) were largely dominant. As opposed, the growth on heneicosane (*n*-$C_{21}$ sample) led to the formation of different IPMS made up of one or two odd-numbered fatty acyl chain combination: PG (19:1/17:0) (39.1%), PG (17:1/17:0) and PG (19:1/15:0) (28.5%), PG (18:1/17:0) (19.6%). This result was in accordance with the odd-numbered straight chain SFAs and MUFAs increase observed previously on this *n*-alkane (Table 2). At a lesser extent, a similar fatty acyl chain imparity rising was obtained after growth on BAL 250 (BAL sample; Table 4): PG (19:1/16:0) and PG (18:1/17:0) (24.8%), PG (19:1/17:0) (12.7%), PG (17:1/16:0) and PG (18:1/15:0) (11%). Besides, whichever sample that we considered and as far as the structure elucidation is complete, we could notice the preferential occurrence of the shortest and/or saturated fatty acyl chain at the *sn*-2 position. For instance, concerning *Reference*, this trend was confirmed with the detection of PG (18:1/16:1), PG (18:1/16:0) and PG (19:1/16:0). Another example, we observed PG (17:1/16:0), PG (18:1/15:0), PG (18:1/16:0), PG (19:1/16:0), PG (18:1/17:0) and PG (19:1/17:0) within the BAL sample. Finally, similarly to PLFAs, phosphatidylglycerol IPMS compositions (Table 4) were globally restored during the two returns following the cultures on pure *n*-alkane. Concerning *Return* (BAL), the IPMS proportion of some compounds such as PG (18:1/16:1), PG (18:1/16:0), PG (19:1/16:0) and PG (2 × 18:1) (14.7, 53.3, 2.8 and 17.7%, respectively) was particularly different compared to the *Reference* (6.6, 61.7, 18.9 and 5.0%, respectively).

### 3.3.4. Phosphatidylinositol molecular species

With regard to *Reference* (Table 5), we observed that the two most abundant Phosphatidylinositol IPMS (PI

(19:1*16:0) (50.2%) and PI (19:0*16:0) (21.1%)) consisted of combinations of 16:0 (32.4%) with minor PLFAs (9-Me-18:1$\Delta^{10E}$ (7.6%), br-19:1 (1.3%), 19:1 (2.1%) and 10-Me-18:0 (2.9%); Table 2). Such a result was really different to the initial PG molecular species composition. Actually, concerning PG, we observed that the fatty acyl chains of the major IPMS were correlated with predominant PLFAs.

At first, we should mention that the abundance of PI is generally low. Consequently, the structures of some minor molecular species remained unclear in the different cultures on hydrophobic substrates. After a growth on pure *n*-alkane (only *n*-$C_{20}$ because both IPCs and IPMS of PI were not detected as concerns *n*-$C_{21}$), we noticed an important modification of the IPMS composition (Table 5). In fact, there was an occurrence of PI (32:0) (10.4%) but also a substitution of PI (19:1*16:0) (50.2% for *Reference*) by PI (35:0) (68.8% for *n*-$C_{20}$). This result revealed an important rising of PI fatty acyl chain saturation (ΣPI (ΣC:0) (22.7% for *Reference* and 86.7% for *n*-$C_{20}$) despite SFAs group (Table 2) increase slightly (42.9% for *Reference* and 47.9% for *n*-$C_{20}$). Furthermore, the PLFAs analysis did not permit us to differentiate

Table 6
LC/ESI/MS relative amounts of the acylphosphatidylglycerol intact molecular species extracted from *Corynebacterium* sp. grown on ammonium acetate (*Reference*, *Return* (*n*-$C_{20}$), *Return* (*n*-$C_{21}$) and *Return* (BAL)), eicosane (*n*-$C_{20}$), heneicosane (*n*-$C_{21}$) or crude oil (BAL)

| IPMS[c] | IPMS[c] relative amounts (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *m/z* | *Reference*[a] | *n*-$C_{20}$ | *n*-$C_{21}$ | BAL | *Return* (*n*-$C_{20}$) | *Return* (*n*-$C_{21}$) | *Return* (BAL) |
| APG (48:1) | 957.8 | 1.2 | | | 1.5 | | | |
| APG (2 × 16:0*16:1) | 957.8 | | | | | | 6.4 | 0.5 |
| APG (49:2) | 969.8 | | | | 1.5 | | | |
| APG (2 × 17:1*15:0) | 969.8 | | | 3.8 | | | | |
| APG (49:1) | 971.8 | 0.5 | | | 2.0 | | | |
| APG (50:2) | 983.8 | | 3.1 | | 4.4 | | | |
| APG (18:1*16:1*16:0) | 983.8 | 10.5 | | | | 12.8 | 15.8 | 5.9 |
| APG (18:1*17:1*15:0) | 983.8 | | | 9.6 | | | | |
| APG (50:1) | 985.8 | 3.6 | | 1.3 | 3.2 | | | |
| APG (18:1*2 × 16:0) | 985.8 | | | | | 11.0 | 15.4 | 3.1 |
| APG (51:2) | 997.8 | 5.1 | | | 12.7 | | 4.7 | 0.6 |
| APG (19:1*17:1*15:0) | 997.8 | | | | | | | |
| APG (2*17:1*17:0) | 997.8 | | | } 24.4[b,d] | | | | |
| APG (2 × 18:1*15:0) | 997.8 | | | | | | | |
| APG (51:1) | 999.8 | 3.4 | | | | | | |
| APG (17:1*2 × 17:0) | 999.8 | | | 3.3 | | | | |
| APG (19:1*2 × 16:0) | 999.8 | | | | | | 4.9 | |
| APG (52:3) | 1009.8 | 5.4 | 3.2 | | 1.7 | 5.0 | 5.3 | 16.0 |
| APG (52:2) | 1011.8 | | | | 25.2 | | | |
| APG (2 × 18:1*16:0) | 1011.8 | 40.3[d] | 51.8[d] | | | 48.3[d] | 36.0[d] | 52.2[d] |
| APG (19:1*18:1*15:0) | 1011.8 | | | 18.6 | | | | |
| APG (53:2) | 1025.8 | | | | 28.8[d] | | | |
| APG (19:1*18:1*16:0) | 1025.8 | 29.3 | 36.7 | | | 20.6 | 11.5 | 4.9 |
| APG (19:1*17*1*17:0) | 1025.8 | | | 23.0 | | | | |
| APG (53:1) | 1027.8 | | | | 1.5 | | | |
| APG (19:0*18:1*16:0) | 1027.8 | | 2.6 | | | | | 2.4 |
| APG (53:3) | 1037.8 | | | | 1.5 | | | 7.1 |
| APG (54:2) | 1039.8 | 0.8 | 2.6 | | 13.1 | 2.3 | | 5.3 |
| APG (19:1*18:1*17:0) | 1039.8 | | | 8.9 | | | | |
| APG (54:1) | 1041.8 | | | | | | | 1.4 |
| APG (55:2) | 1053.8 | | | | 2.9 | | | 0.5 |
| APG (2 × 19:1*17:0) | 1053.8 | | | 7.0 | | | | |

See Section 2 for abbreviations.
[a] Indicated values are the means of three different cultures.
[b] APG (19:1*17:1*15:0), APG (2*17:1*17:0) and APG (2 × 18:1*15:0) were co-eluted and identified within the same sample.
[c] Intact phospholipid molecular species.
[d] Major compounds.

easily the *Reference* and *n*-$C_{20}$ samples. A similar result was obtained concerning PG and APG molecular species. However, the information provided by PI molecular species allowed us to discriminate the two samples. Consequently, the IPMS study of a specific class could be used for monitoring accurately the effect of a hydrophobic substrate. The addition of a crude oil (BAL sample) induced an increase of the whole IPMs (12 for BAL and 8 for *Reference*) that could be interpreted as the result of an increase of the fatty acyl chain complexity and combinations. Furthermore, like *n*-$C_{20}$, this sample is characterized by a higher number of saturated fatty acyl chains: PI (32:0), PI (33:0), PI (34:0), PI (35:0), PI (36:0) and PI (37:0) represented 67.1% of the entire BAL PI molecular species.

During the three returns (Table 5), it appeared that the IPMS composition was clearly not restored. However, the three samples (*Return* (*n*-$C_{20}$), *Return* (*n*-$C_{21}$) and *Return* (BAL)) were made up of the same two most abundant IPMS: PI (18:1*16:0) (46.6, 50.9 and 49.3%, respectively) and PI (19:0*16:0) (19.7, 12.8 and 18.0%, respectively).

#### 3.3.5. Acyl phosphatidylglycerol molecular species

Similarly to PG, the molecular species of APG after a growth on eicosane (Table 6) were relatively comparable to these one obtained on ammonium acetate (*Reference*). Thus, APG (2 × 18:1*16:0) and APG (19:1*18:1*16:0) were the two major IPMS on both substrate: 40.3 and 29.3% for *Reference*, and 51.8 and 36.7% for *n*-$C_{20}$, respectively.

Another similarity with PG, the growth on heneicosane (Table 6) produced several IPMS with two or three odd-numbered fatty acyl chains (e.g. APG (2 × 17:1*15:0), APG (18:1*17:1*15:0), APG (19:1*17:1*15:0), APG (2*17:1*17:0), APG (17:1*2 × 17:0), APG (19:1*18:1*15:0), APG (19:1*17*1*17:0), APG (19:1*18:1*17:0) and APG (2 × 19:1*17:0)) that constituted at least 74.2% of total IPMS. In addition, as for PI, the number of IPMS related to the growth on petroleum (BAL) was slightly higher (13 compounds) than the initial acetate culture (10 compounds).

The last point of comparison between APG and PG molecular species referred to the return samples. At first, the three samples (Table 6) were characterized by the occurrence of APG (2 × 18:1*16:0), which was the major molecular species (48.3, 36.0 and 52.2% for *Return* (*n*-$C_{20}$), *Return* (*n*-$C_{21}$) and *Return* (BAL), respectively). Moreover, this last lipid corresponded to the major IPMS observed for the *Reference* sample (40.3%). Nevertheless, the second most abundant IPMS of *Reference* (APG (19:1*18:1*16:0), 29.3%) was found only within the *Return* (*n*-$C_{20}$) sample (20.6%). In other samples, it was replaced by APG (18:1*16:1*16:0) (15.8%) and APG (52:3) (16.0%) for *Return* (*n*-$C_{21}$) and *Return* (BAL), respectively. On the whole, the IPMS restoration was better in the case of PG. This could be justified by the fact that APG contains three fatty acyl chains, and therefore, a higher possibility of combinations is conceivable.

## 4. Discussion

### 4.1. Phospholipid ester-linked fatty acid study

According to the results (Table 2), it appeared that a growth of *Corynebacterium* sp. on heneicosane (*n*-$C_{21}$ sample) implied an odd-numbered PLFAs formation increase. Such a phenomenon was comparable to those one observed by Doumenq et al. [22] concerning a growth of *Marinobacter hydrocarbonoclasticus* 617 on various pure or mixed odd-numbered *n*-alkane. Thus, as regard to PLFA composition, we could suppose that odd-numbered *n*-alkanes should have similar effects on both a gram-positive bacterium like *Corynebacterium* sp. and gram-negative bacterium like *Marinobacter hydrocarbonoclasticus* 617. In addition, the hydrocarbon degrading activity index [21] revealed an important biotransformation of heneicosane into odd-numbered SFAs and MUFAs (HDAI = 11.03; Table 2). Conversely, this index showed no variation after a growth on eicosane (*n*-$C_{20}$ sample) despite a real biotransformation and biodegradation of this *n*-alkane occurred within the 21 days of growth. In fact, Syakti et al. [33] observed a biotransformation of 38 ± 4% of the eicosane carried out by *Corynebacterium* sp. after 21 days with similar culture conditions. The fact that the HDAI was not able to reveal the degradation of an even-numbered *n*-alkane (*n*-$C_{20}$) could be explained by the mechanism involved during the *n*-alkane biotransformation. Thus, after a monoterminal oxidation eventually followed by one or several β-oxidation and a double bond incorporation [48,49], an even-numbered *n*-alkane should essentially lead to even-numbered SFAs or MUFAs biosynthesis. Insofar the growth on petroleum is concerned, the HDAI returned values of 1.58. This value could be interpreted as consequence of both odd-numbered and branched PLFAs increase. Among the branched SFAs, Σbr-17:0 and Σbr-18:0 groups were previously identified as 11-Me-, 12-Me-, 13-Me-, 14-Me- and 15-Me-16:0; 10-Me-, 11-Me-, 12-Me-, 13-Me-, 14-Me-, 15-Me and 16-Me-17:0. These last PLFAs were considered as characteristic of a growth *Corynebacterium* sp. on BAL 250 [32]. Similar results were obtained by Aries et al. [21] concerning a bacterial community that contained *Corynebacterium* sp. and grown on the same hydrophobic substrate.

Besides, an increase of the saturation degree (SFAs/MUFAs ratio; Table 2) was observed during the growth on the hydrophobic substrates (especially concerning the *n*-$C_{21}$ and BAL 250 cultures). This result could be interpreted as an adaptive response of the *Corynebacterium* sp. membrane fluidity [50,51]. In fact, Sinensky [50] proposed the "homeoviscous adaptation" theory that has linked up the PLFAs saturation degree and the membrane viscosity. This theory postulated that the optimal membrane fluidity is maintained by a variation of the PLFAs saturation degree. The membrane fluidity could be influenced by the growth temperature but also by the presence of xenobiotics such as alkanes and aromatic compounds [51–53]. Hydrophobic compounds and solvents partition into membranes and could accumulate

between bilayers [51,53]. This accumulation should affect the lipid ordering and increase the fluidity. Consequently, similarly to the effects of high growth temperature [50], the accumulation of hydrocarbons into membranes involves an increase of the SFAs proportion that allow bacteria to keep an optimal fluidity [51,53]. Furthermore, the increase of SFAs was more important as regards to the BAL 250 culture (SFAs/MUFAs = 1.53; Table 2). This result should be justified by the presence of some hydrocarbon classes (i.e. PAH) that could not be assimilated by *Corynebacterium* sp. As assumed by Syakti et al. [33], these compounds could be toxic to *Corynebacterium* sp.

### 4.2. Intact phospholipid class study

IPCs qualitative and quantitative composition on the initial ammonium acetate medium (Table 3) was in accordance with the previous works of Niepel et al. [27]. In fact, the authors observed four phospholipid classes (PG, APG, DPG and PI) as concerns two strains of *Corynebacterium variabilis* cultured on rich medium. In addition, they found equivalent proportions of PG and APG (both phospholipids constituted about 95% of total IPCs). PI and DPG appeared as minor ($\leq$5 and $<$1%, respectively). On the other hand, Yagüe et al. [54] studied IPCs of *Corynebacterium amycolatum*. Although they found the four previous phospholipid classes (APG and PG still being the major IPCs), they detected also the presence of phosphatidylcholine and mannophosphoinositides. Concerning mannophosphoinositides (PIMs), PI and APG, elevated proportions of these IPCs are considered as characteristic of the *Corynebacterium* genus [55]. Actually, Brennan and Lehane [55] showed that lipids of PIMs and PI type were prominent when they studied the IPCs of *Corynebacterium diphteriae*, *Corynebacterium xerosis*, *Corynebacterium equi* and *Corynebacterium bovis*.

Usually, phospholipid class analysis provides some important information about membrane properties such as the fluidity [51]. As far as the membrane fluidity is concerned, it is commonly assumed that this property is rather dependant on PLFA composition [50,56,57] than on the polar head group of each IPC. However, after a growth on BAL 250, a large increase of DPG was observed. A similar change was previously reported by Ingram [52] concerning the effects of ethanol on the phospholipid class composition of *E. coli*. The rise of DPG is generally known to result in lower membrane fluidity [58]. Actually, among all phospholipids, DPG is characterized by the highest temperature phase transition at neutral pH [59]. Consequently, the variation of DPG could compensate the change of lipid ordering induced by the accumulation of hydrocarbons into the bilayers [51]. The increase of DPG proportion should have similar effect on membrane fluidity than the increase of SFAs. Thus, it appeared that the membrane fluidity, as assumed by [36] Ramos et al. concerning the effects of toluene on *P. putida* DOT-T1, could be altered by both the phospholipid head groups and the PLFA composition. Concerning the two others hydrophobic substrates (eicosane and heneicosane), only a decrease of PI was observed and PG was still the major phospholipid class after a growth on pure *n*-alkane (Table 3). Consequently, we assumed that *Corynebacterium* sp. membrane fluidity was less altered after a growth on pure *n*-alkane than on a petroleum. Such a result was confirmed by a higher degree of the PLFA saturation (SFAs/MUFAs ratio; Table 2) and was previously justified by the BAL 250 complex composition (presence of PAH) compared to the pure *n*-alkanes.

As concerns the three returns, we observed three different IPCs composition. Nevertheless, on the whole, the PG class appeared as the major phospholipid and a trend to a restoration of the polar head group composition could be assumed.

### 4.3. Intact molecular species study

As far as the *Reference* is concerned, the result showed that the major phosphatidylglycerol IPMS (Table 4) corresponded to the combination of major fatty acyl chain whereas it was not the case of the phosphatidylinositol IPMS (Table 5). Also, as regard to bacterial phospholipid metabolism, these two lipid classes possess two distinct biosynthesis pathways that involve two different enzymatic activities [60]. Thus, the two turn-over associated to either PG or PI molecular species biosynthesis could explain the fact that PI is a minor class in addition to the peculiar fatty acyl chain composition of its IPMS.

After the returns on the initial substrate, we noticed that the PI molecular species composition differed with the IPMS related to *Reference* (Table 5) and especially the important loss of PI (19:1*16:0) that could be explained by the decrease of the 9-Me-18:1$\Delta^{10E}$ during the whole returns (Table 2). Thus, an eventual alteration of the de novo fatty acid biosynthesis, after a growth on an hydrophobic substrate, could be the reason. According to this hypothesis, a lower occurrence of the 9-Me-18:1$\Delta^{10E}$ should lead to the decrease of the whole IPMS including a combination of a 19:1 and another fatty acyl chain.

As conclusion, when we studied the fatty acyl chain distribution of PG (Table 4), it appeared that the shortest and saturated acyl group was preferentially linked to the *sn*-2 position. This particularity concerning the IPMS of PG was in accordance with the observation previously formulated about the gram-positive bacteria [61–65] and especially for *Corynebacterium* sp. strain LMG 3820 [27]. In addition, concerning PG and APG, Yagüe et al. [66] observed the occurrence of octadecenoyl at the *sn*-1 position of the glycerol. On the other hand, an opposite distribution of the fatty acyl chains was commonly observed in gram-negative bacteria [8,29,61,65,67]. Thus, the information provided by the IPMS study concerning the fatty acyl chain stereochemistry added to the polar head group specificity (the important proportion of PE and its methylated derivatives within the gram-negative membranes [68]) could constitute a useful tool for bacterial identification [25,28] or bacterial community characterization [30].

## 5. Conclusions and future research

- In the case of *Corynebacterium* sp., a quantitative analysis of the IPCs did not provide any clear information about *n*-alkane or petroleum biotransformation. However, the variation observed on crude oil could be related to a regulation of the membrane fluidity.
- An increase of odd-numbered PLFAs and IPMS fatty acyl chains was observed after a growth on heneicosane or crude oil.
- The IPMS composition of PG and APG were globally restored during two returns (after *n*-$C_{20}$ and *n*-$C_{21}$). Concerning PI, the IPMS compositions of the three returns were comparable and particularly different to the IPMS composition of the *Reference* sample. As a consequence, the IPMS study of a specific class could be used for monitoring accurately the effect of a hydrophobic substrate.
- The IPMS of PG were characterized by the preferential occurrence of the shortest and saturated fatty acyl chain at the *sn*-2 position. This result confirmed the gram-positive membrane of *Corynebacterium* sp. Further applications involving fatty acyl chain stereochemistry studies should provide some information concerning bacterial community structures.
- Ongoing research concerns the intact phospholipid study of a gram-negative Bacterium cultured on ammonium acetate and petroleum. Afterward, the LC/ESI/MS technique will be applied to sediments for characterizing and monitoring the effects of crude oil on in situ bacterial communities.

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