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Identification and relative quantification of fatty acids in *Escherichia coli* membranes by gas chromatography/mass spectrometry

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The lipids that are essential to the functioning of the bacterial membrane exist in hundreds of different forms. The reasons for this diversity are far from clear but are presumably related to the roles of these lipids in both facilitating enzymic activities and generating proteolipid domains. A full understanding of bacterial physiology therefore requires characterization of lipids in different strains in a variety of environmental conditions. This characterization then becomes the basis for lipidomics, the lipid aspect of the growing field of metabolomics. To exploit the power of derivatization chemistry and of gas chromatography/mass spectrometry (GC/MS) and tandem mass spectrometry (MS/MS) for metabolomics studies, we report here the development of various GC/MS electron ionization (EI) and negative and positive chemical ionization (CI) methods for the identification and, for the first time, the relative quantification of fatty acids present in extracts from membranes of a laboratory strain of *Escherichia coli*. They consist of seven saturated fatty acids (C10:0, C12:0, C14:0, C15:0, C16:0, C17:0 and C18:0) and six unsaturated fatty acids (C16:1, cyC17:0 plus two isomers of C18:1, C18:2 and cyC19:0). Copyright © 2007 John Wiley & Sons, Ltd.

Lipids are not just passive, structural constituents of bacterial membranes but are believed to play active roles in processes as diverse as metabolism,[1] protein transport across membranes,[2] synthesis of the peptidoglycan wall,[3] and the initiation of chromosome replication and cell division.[4] It has recently been proposed that macromolecules, ions and lipids come together in large assemblies or hyperstructures to perform specific functions.[5] This proposal is supported by evidence that the overproduction of certain membrane proteins is accompanied by overproduction of specific lipids and that this can result in the formation of extended proteolipid structures.[6] Hence, the diversity of species of lipids and variations in this diversity take on a special importance: lipids become major players in determining the variety and functioning of hyperstructures and hence of the organization and activity of the cell. In this context, analysis of the full complement of lipids in the cell – the lipidome – is correspondingly important.

Mass spectrometry (MS) is a well-established technique for fatty acid (FA) analysis. It has contributed to the solution of many structural problems over the last 40 years using various ionization methods.[7,8] Electron ionization (EI)-MS in combination with gas chromatography (GC) allowed the analysis of derivatized fatty acids. Methyl (FAME) or trimethylsilyl TMS esters were the first to be studied but gave mass spectra that provided only a few structural features such as molecular weight and degree of unsaturation.[9] However, the EI-MS determination of the exact structures of FA molecules remained difficult since numerous intramolecular rearrangements occur during the fragmentation processes. To locate either double bonds or ring structures or substituents on the acyl chain, several approaches have been developed. The introduction of the desorption ionization technique of fast atom bombardment (FAB) coupled to tandem mass spectrometry (MS/MS) allowed direct analysis of free fatty acids (FAs) and their complete identification.[10–16] Charge remote fragmentation (CRF) processes were observed by collision-induced dissociation (CID) of either a carboxylate ion, or a cationized molecule of an FA salt, and the latter caused specific cleavages of the chain and gave the required structural information. Such fragmentation patterns were also observed for a cationized alkyl ester of a FA.[17] Although FAB has been extensively used, some electrospray ionization tandem mass spectrometry (ESI-MS/MS) studies have also been reported.[8,18,19] The high-energy CID of FAs and their derivatives gives more structurally informative product ion

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mass spectra than low-energy CID experiments. One of the limitations of FAB-MS/MS is that it cannot be directly applied to complex mixtures of FAs that include isobaric forms. For such samples, chromatographic separation is necessary prior to MS. Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) has also been used to study FAs[8] and GC/MS/MS methods have been developed that use electron capture negative chemical ionization (NCI) of pentafluorobenzyl ester derivatives.[20] As the electron capture is dissociative for these esters, the in-source generated carboxylate anions undergo the CRF process under high-collision energies.[21] Another approach that involved the derivatization of FAs into picolinyl esters was developed by Harvey who showed that EI-MS of these derivatives produces a series of ions from the CRF process from which structural information on chain branching or the position of double bonds can be deduced.[22–24] More recently, derivatization to picolinyl esters using a pyrolyzer as a thermochemical micro-reactor followed by GC/MS analysis allowed the characterization and identification of FAs from whole bacterial cells.[25]

The aim of the work presented here was to determine the lipid composition of membranes from LM 3118, a wild-type, K12 laboratory strain of *E. coli*. We focus here on the fatty acyl moiety (a report of the analysis of the composition of phospholipids is being submitted elsewhere[26]). Structural identification of individual FAs was achieved by compilation of data from GC/EI-MS analyses using Harvey's method as well as from GC/NCI-MS/MS using either B/E linked scan mass spectrometry or CID-MIKE (metastable ion kinetic energy) experiments. The FA compositions of membranes from this strain of *E. coli* were then compared by relative quantification of FAME derivatives using an internal standard method and positive chemical ionization (PCI) GC/MS.

EXPERIMENTAL

Reagents and chemicals

All reagents, i.e. potassium hydroxide, hydrochloric acid, diisopropylamine, pentafluorobenzyl bromide, thionyl chloride, 3-(hydroxymethyl)pyridine, stock solution of 14% BF_3 in methanol and standard of C17:1 (10-heptadecenoic acid) fatty acid, were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Stock solutions of the standard (1 $\mu g\,\mu L^{-1}$) were prepared in hexane.

HPLC-grade acetonitrile, methanol, chloroform, and hexane were supplied by VWR (Fontenay-sous-Bois, France). Deionized water (18 MΩ) was obtained from a Milli-Q apparatus (Waters, Saint-Quentin-Yvelines, France).

Bacterial organisms and culture conditions

LM 3118, a wild-type K12 strain of *E. coli* [27] was grown in nutrient broth (Luria Broth (LB)) at 37°C on a rotary shaker (15–20 mL culture) overnight. The LB medium[28] was from the recipe of Miller (5 g yeast extract, 10 g peptone tryptone, 10 g NaCl). The pH was automatically maintained at 6.95 by titration with 5% H_2SO_4 or 5% NaOH. The peptone tryptone and yeast extract were from Difco (Le Pont de Claix, France) and the NaCl from Sigma. The medium was made in distilled water and autoclaved under standard conditions. Cells were then harvested by centrifugation at 10 000 *g* for 10 min at 3°C. The pellet was recovered and dispersed in 1 mL of deionized water. Three independent sets of experiments were performed.

Sample preparation

Fatty acid extraction

Lipids were extracted from pelleted cells according to the method of Bligh and Dyer:[29] 2 mL of $CHCl_3/CH_3OH$ (2:1, v/v) were added to 1 mL of bacterial suspension, the samples were vortex mixed, centrifuged at 1000 *g* for 10 min and the chloroform-rich (bottom) phase containing the phospholipids was removed with a Pasteur pipette. The extraction step was repeated twice and the collected organic phase was evaporated. In order to release the FAs, alkaline hydrolysis was carried out: the dried residue was heated with 1 mol L^{-1} KOH (1 mL) for 1 h at 100°C. After cooling, aqueous HCl (3 mol L^{-1}) was added to adjust the value of the pH to 1. The FAs were extracted three times with 500 μL of hexane. The organic layers were separated after centrifugation (1000 *g*, 10 min), then combined and evaporated to dryness under vacuum. Then 500 μL of a mixture of $CH_3CN/CHCl_3$ (50/50) were added and aliquots of 50 μL were collected and evaporated. The obtained FAs were submitted to various derivatization procedures. A volume of 50 μL of a stock solution (1 $\mu g\,\mu L^{-1}$) of C17:1 FA was added to a series of culture before the hydrolysis step in order to perform the relative quantification.

Preparation of FA pentafluorobenzyl esters

Derivatization of FAs into pentafluorobenzyl esters was performed according to literature procedures:[20,21] 100 μL of CH_3CN and 1.5 μL of diisopropylamine were added to the dried aliquot of FA residue. After vortex mixing, 6 μL of pentafluorobenzyl bromide were added and the mixture was left for 1 h at room temperature, evaporated under vacuum and diluted in 500 μL of hexane.

Preparation of FA picolinyl esters

The FAs were derivatized by adapting the method of Harvey.[22–24] The dried aliquot of FAs was mixed with 100 μL of thionyl chloride (gently added) for 15 min at room temperature in the dark. The thionyl chloride was evaporated under a stream of N_2 and a solution of 20% 3-(hydroxymethyl)pyridine in CH_3CN (100 μL) was slowly added. The mixture was vortex mixed for 15 min and heated for 10 min at 70°C. The samples were diluted in hexane to a final volume of 1000 μL.

Preparation of FA methyl esters

The methyl esters were prepared by submitting dried FAs to 14% BF_3 in methanol (500 μL), mixed for 15 min at 80°C and then adding 500 μL of water. After vortex mixing, the methyl derivatives were extracted three times with hexane (500 μL).

Gas chromatography/mass spectrometry

GC/MS analyses were performed using a Hewlett-Packard 6890 series gas chromatograph coupled with an Autospec

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Table 1. Oven temperature gradients used for GC/MS experiments

	Temperature program of oven
Pentafluorobenzyl esters	Started at 170°C, maintained for 1 min, ramped to 200°C at 5°C min^{-1}, then ramped to 300°C at 2°C min^{-1}
Picolinyl esters	Initial temperature at 200°C, held for 1 min, raised to 240°C at 8°C min^{-1}, then ramped to 300°C at 2°C min^{-1}
Methyl esters	Isothermal for 1 min at 60°C, raised to 260°C at 5°C min^{-1}

mass spectrometer of EBE geometry (Micromass, Manchester, UK) equipped with an Opus 3.1 data system. Chromatographic separations were obtained using an Optima-5 MS capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness; Macherey Nagel, Hoerdt, France). Helium was the carrier gas at a flow rate of 0.8 mL min^{-1}. The temperature program was adjusted for each derivative sample (Table 1). The temperatures of the injector, the interface and the lines were 250°C. Injections of 0.5 μL were performed in splitless mode.

For picolinyl esters, EI mass spectra were recorded using an electron energy of 30 eV and a trap current of 200 μA as Harvey had demonstrated that an electron energy much lower than 70 eV yields higher quality picolinyl ester spectra.[22–24]

Negative (methane) and positive (isobutene) CI were performed for the pentafluorobenzyl and methyl esters, respectively, and the emission current was set at 1 mA.

For each pentafluorobenzyl derivative (i) CID-MIKE spectra were recorded that involved dissociations in the third field-free region of the mass spectrometer (between the magnet and the second electrostatic sector), and (ii) linked field scan measurements (constant B/E ratio) were made by analyzing the product ions formed in the first field-free region (between the source and the first electrostatic sector). Helium was the collision gas in both cases and the flow rate was adjusted so that the signal intensity of the precursor ion was reduced to 50%.

The acceleration voltage was 8 kV and the resolving power was 1000. The magnet scan rate was 1 s/decade over an m/z range 600–40. The temperature of the ion source was 250°C.

Relative quantification was performed using C17:1 FA as internal standard (IS). Each sample was injected six times. The measurements are averages and the relative standard deviation (% RSD) was determined. For quantification, the area ratios of protonated molecule of a given FAME versus the IS were calculated. Three separate cultures were grown on different occasions so that the variability of the biological material could be estimated.

RESULTS AND DISCUSSION

Bacterial membranes of the strain of *E. coli* were saponified and the released fatty acids (FAs) were collected into several aliquots in order to prepare pentafluorobenzyl, 3-picolinyl and methyl esters. From the GC/NCI-MS/MS and GC/EI-MS analyses of, respectively, pentafluorobenzyl (PFB) and picolinyl esters, eleven major FAs present in the wild-type strain were identified (Table 2). They consist of six saturated FAs (C12:0, C14:0, C15:0, C16:0, C17:0 and C18:0), three unsaturated FAs (C16:1 and two isomers of C18:1) and two cyclopropyl FAs (cyC17:0 and cyC19:0). These results are in agreement with previous reports[19,21,24] and this pattern of FAs, particularly cyclopropyl C17 and C19, has been reported to be characteristic of *E. coli*. However, traces of saturated C10:0 and unsaturated C18:2 FAs were also detected; to our knowledge, these compounds have never been reported for *E. coli*. The retention times of the PFB, picolinyl and methyl esters are reported in Table 2.

The fluorinated esters underwent dissociative electron capture ionization which generates abundant gas-phase carboxylate anions according to the process:[20,21]

$$R\text{–}COO\text{–}CH_2\text{–}C_6F_5 + e^- \rightarrow R\text{–}COO^- + CH_2\text{–}C_6F_5$$

CID-MIKE and linked field scan (constant B/E ratio) experiments were recorded for each carboxylate ion. The data produced characteristic CRF patterns,[8,18] which allow the unambiguous identification of the seven saturated FAs (from C10 to C18), three monounsaturated FAs (C16:1 and two C18:1 isomers) and one double-unsaturated FA (C18:2

Table 2. Identification of the fatty acids present in the membranes of *E. coli*

Fatty acid	Usual name	Retention time			Chemical formula	Molecular weight	Notation
		PFB esters	Picolinyl esters	Methyl esters			
Decanoic	Capric	10 min 16	nd	11 min 24	$C_{10}H_{20}O_2$	172	C10:0
Dodecanoic	Lauric	11 min 20	nd	16 min 12	$C_{12}H_{24}O_2$	200	C12:0
Tetradecanoic	Myristic	16 min 17	10 min 32	20 min 40	$C_{14}H_{28}O_2$	228	C14:0
Pentadecanoic	Pentadecylic	19 min 08	12 min 12	22 min 47	$C_{15}H_{30}O_2$	242	C15:0
9-Hexadecenoic	Palmitoleic	21 min 20	12 min 57	24 min 24	$C_{16}H_{30}O_2$	254	C16:1
Hexadecanoic	Palmitic	22 min 14	14 min 10	24 min 50	$C_{16}H_{32}O_2$	256	C16:0
9,10-Methylenehexadecanoic	/	24 min 59	15 min 23	26 min 43	$C_{17}H_{32}O_2$	268	$_{cy}$C17:0
Heptadecanoic	Margaric	25 min 21	16 min 14	27 min 03	$C_{17}H_{34}O_2$	270	C17:0
cis-9,*cis*-12-Octadecadienoic	Linoleic	27 min 38	nd	28 min 44	$C_{17}H_{32}O_2$	280	C18:2
cis-9-Octadecenoic	Oleic	27 min 59	17 min 59	28 min 53	$C_{18}H_{34}O_2$	282	C18:1
cis-11-Octadecenoic	Vaccenic	28 min 10	18 min 11	29 min 02	$C_{18}H_{34}O_2$	282	C18:1
Octadecanoic	Stearic	28 min 36	18 min 34	29 min 32	$C_{18}H_{36}O_2$	284	C18:0
11,12-Methyleneoctadecanoic	/	31 min 34	20 min 02	31 min 55	$C_{19}H_{36}O_2$	296	$_{cy}$C19:0

nd: non detected.

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or linoleic acid). Indeed, the classical series of 'C_i' ions, generated from successive carbon–carbon bond cleavages under high-collision energy, were observed (data not shown). The mechanisms responsible for the formation of this series are well understood.[8,10,11,15–18] In the case of the unsaturated FAs, these series are interrupted by the presence of a double bond; fragmentations of the allyl carbon–carbon bonds on both sides of the double bond occur (giving rise to a gap of 55 *m*/*z* units in the series) which permit localization of double bonds. This permitted identification of 9-octadecenoic acid and 11-octadecenoic acid. The specific isomer of the 9-octadecenoic acid in question was identified as oleic acid by comparing its retention time with the retention time of the PFB derivative of a commercial standard of oleic acid, while the 11-octadecenoic acid corresponded to vaccenic acid according to Oursel *et al*.[26]

This method allows the unambiguous identification of eleven FAs present in this strain of *E. coli*. However, it did not allow unambiguous determination of the exact structures of what turned out to be cyC17:0 and cyC19:0 FAs (see below). GC/MS analyses of the 3-picolinyl esters using 30 eV electron energy were therefore carried out: these provided additional evidence for most of the above structures and showed that the other two are the cyC17:0 and cyC19:0 FAs which contain a cyclopropane ring and correspond to 9,10-methylene-hexadecanoic and 11,12-methylene-octadecanoic acids. Mass spectral features characteristic of picolinyl esters were the same as those previously observed[22–24] and all our mass spectra showed quite abundant molecular ions and the expected fragment ions: abundant ions at *m*/*z* 92, 93 and 108 indicative of the pyridine moiety; *m*/*z* 151 obtained through a McLafferty rearrangement; *m*/*z* 164, the first of a series of ions typical of picolinyl esters generated by random hydrogen abstraction from the acyl chain, migration onto the nitrogen of the pyridine residue and chain cleavage according to Harvey.[22] The greater abundance of *m*/*z* 164, compared with fragment ions of higher *m*/*z* values, can be explained by the formation of a conjugated fragment ion. From the EI mass spectra, it appears that the fragmentation mechanism of the picolinyl esters is not dissimilar to the CRF mechanism proposed by Claeys and co-workers,[15,17] since the charge fixed by the pyridinium radical cation induced one random hydrogen abstraction as the initial step of fragmentation. The above series of ions, which are separated by 14 *m*/*z* units from one another,[22] have even *m*/*z* values and similar abundances; major variations in the abundances of the ions in this series correspond to double bond(s), chain branching or a cyclopropane ring. In the case of cyC17:0 and cyC19:0, existence of a cyclopropane ring was determined by the presence of ions specifically characteristic of this ring and, in addition, localization of this ring was determined by the particular *m*/*z* values of these ions (as previously described for a standard of cyC19:0[22–24]). Such fragment ions are produced by cleavage through the cyclopropane ring and this process involves distonic forms of the molecular ion. This pattern can be used to distinguish cyclopropane acids from their isobaric unsaturated counterparts and to locate the position of the ring on the acyl chain.[22–24] The EI mass spectra of C16:1 and C18:1 picolinyl esters also confirmed the location of the double bond previously determined by GC/NCI-MS/MS; i.e. the presence of 9-hexadecenoic, 9-octadecenoic and 11-octadecenoic acids was proved (data not shown) through several spectral features.

Taken together, these results permit the identification of thirteen FAs in this laboratory strain of *E. coli*. Relative quantification of these FAs was performed for the major

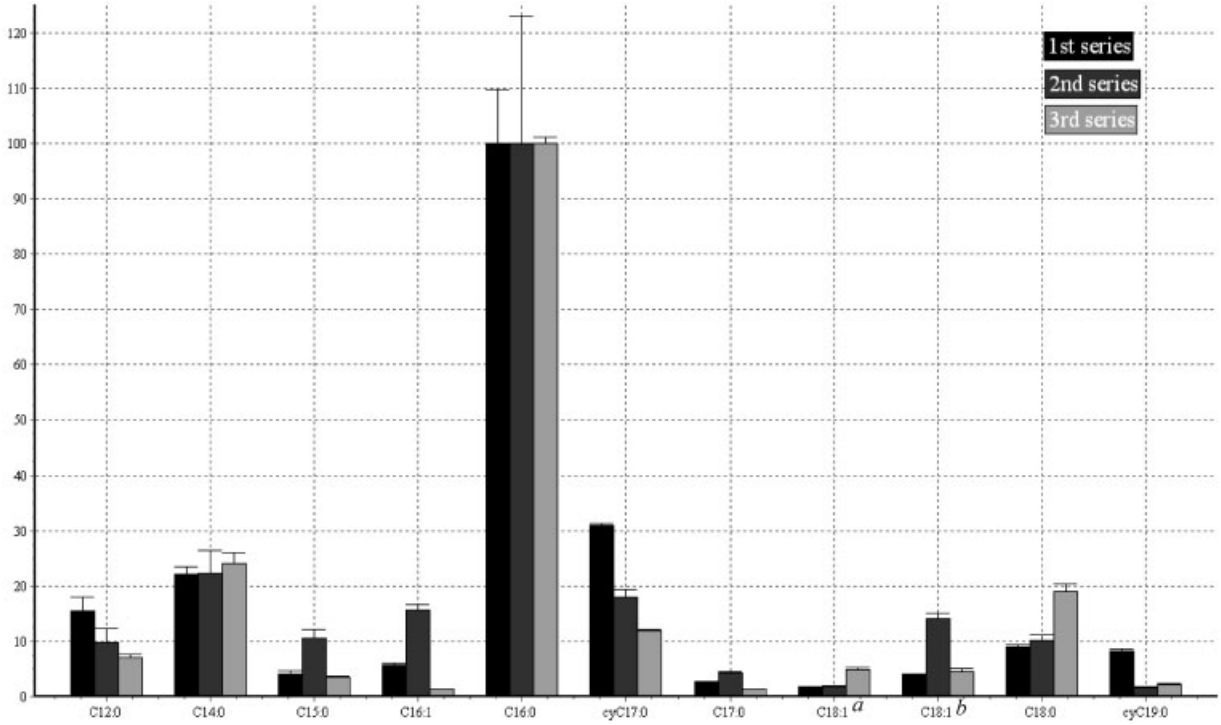

Figure 1. Relative composition of FAMEs in the K12 laboratory strain of *E. coli*. Values are means ± RSD. (a) and (b) correspond to oleic and vaccenic acids, respectively.

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species using 10-heptadecenoic acid (C17:1) as internal standard (IS). A known quantity of C17:1 FA was added to each sample of the *E. coli* colony before the extraction and derivatization steps. The relative quantification was performed by analyzing the methyl esters of FAs in GC/PCI-MS experiments. After optimization of the chromatographic conditions, the methyl ester of the IS ($t_R = 26$ min 34) eluted just before the cyC17:0 ($t_R = 26$ min 43) derivative so that no co-elution occurred. Using isobutene as reagent gas for PCI, abundant $[M+H]^+$ ions were observed for each derivative. The ratio between the relative abundance of the protonated molecule from a given methyl ester to that of the IS could then be determined (RA_{FAME}/RA_{IS}). In Fig. 1, these ratios are plotted as a percentage of the highest ratio obtained, namely $RA_{C16:0}/RA_{IS}$, providing a rapid comparison of the FA composition for this strain. Six *intra-day* injections were performed for each of the three cultures and the standard deviations in Fig. 1 show the variability in the analysis of the material once extracted which, in the best case, ranged from 1 to 12% with a mean RSD value of 5%. The profiles of the three cultures are very similar. It is worth noting that C16:0 FAME (palmitic acid methyl ester**)** is the most abundant species of this strain grown under the above conditions. The C14:0, C16:1, cyC17:0, C18:1 (oleic acid) and C18:0 derivatives are also abundant. Minor species consist of the C10:0, C17:0, C18:2 (linoleic acid), C18:1 (vaccenic acid) and cyC19:0 FAMEs. Apart from the C10:0 and C18:2, these results are in agreement with previous reports,[25,30,31] and are consistent with quantitative data obtained in our laboratory.[26] The small differences in the lipids extracted from the three cultures are presumably due to small differences in culture conditions arising from variations in composition of the media, quorum-sensing molecules, temperature and pH.

CONCLUSIONS

GC/NCI-MS/MS analyses of pentafluorobenzyl esters in conjunction with GC/EI-MS studies of picolinyl esters permit identification of fatty acids (FAs) in membrane extracts of the LM 3118 strain of *E. coli*. The ensemble of the results revealed thirteen FAs comprising seven saturated, four monounsaturated and two cyclopropane compounds. Unambiguous location of double bonds was achieved by MIKE-CID experiments on selected carboxylate precursor ions generated by dissociative electron capture of the pentafluorobenzyl esters. Moreover, diagnostic fragment ions were obtained from EI mass spectra of picolinyl esters. These ions led to structural elucidation of the fatty acyl moieties. It was therefore possible to identify all the fatty acids present and to determine the position of the double bond in C16:1, C18:2 and in two isomers of C18:1 as well as the position of the cyclopropane ring contained in the cyC17:0 and cyC19:0 FAs. One new finding was the presence in small amounts of C10:0 and C18:2. Relative quantification was performed by GC/PCI-MS analyses of the FAME derivatives using C17:1 FAME as internal standard. Fatty acid profiles were thus obtained for three separate cultures. A striking feature is that palmitic acid is the main component. The presence of cyC17:0 and cyC19:0 FAs, which are considered characteristic of *E. coli*, was also confirmed. The differences between the three cultures grown under similar conditions were reassuringly small thereby confirming the validity of this method for determining the lipidome and opening up the possibility that it may be used to reveal significant differences in lipid profiles resulting from important differences in genotypes and in growth conditions.

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