



# Lipidomic Analysis of Lower Organisms

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## Abstract

Current lipidomics is a modern method of analysis of lipids, important cell constituents found in all microbial cells and fulfilling vital roles as structural components of cell membranes, cell energy storage sources, and in some cases as signaling compounds. In either of its current branches, i.e., shotgun lipidomics and LC-MS lipidomics, it provides a fast and reliable information on the lipids present in microorganisms such as archaea, bacteria, cyanobacteria,

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algae, and yeast, including those inhabiting unusual (psychrophilic, halophilic, thermophilic, etc.) habitats. The number of lipids and more specifically molecular species of lipids ranges from hundreds to thousands, and lipidomics is thus expected to provide a huge amount of data to be processed and evaluated. Further development of lipidomic analysis can be expected to involve the use of new ionization techniques, e.g., atmospheric pressure photoionization MS or high-resolution mass spectrometry of time-of-flight MS analysis of lipids containing unusual head groups, very-long chain saturated, or very-long chain polyunsaturated fatty acids and the application and use of chemical compounds labeled with stable isotopes in the study of dynamic changes of metabolic pathways.

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## 1 Introduction

Lipidomics is a new research field, which has recently begun to be used in the study of lipids in biological systems based on the principles of analytical chemistry. The main tool is a mass spectrometric analysis of lipids, often implemented on devices with a high resolution, which allows determination of the molecular formula of analyzed compounds. There are two major and mutually complementary approaches in lipidomics. The first consists in the direct entry of the sample into the mass spectrometer and is called “shotgun lipidomics.” The second uses a classical connection of a chromatograph, almost exclusively liquid, with mass spectrometer.

The main advantage of shotgun lipidomics over liquid chromatography-mass spectrometry (LC-MS) is the fact that the mass spectrum of molecular ions of each molecular species of occurring lipid classes can be obtained at a constant concentration of the lipid solution during direct infusion. Another advantage is the short analysis time (several tens of seconds). Conversely, a huge drawback is the inability to separate and identify the regioisomers and enantiomers of the individual molecular species (see Table 1 for the number of triacylglycerols (TAGs)). Both approaches can of course use tandem mass spectrometry including neutral loss scans and precursor ion scans of one or more ionic reactions. An ideal way is the combination of both methods for sample analysis, see e.g., the lipidomic profile of snow algae (Rezanka et al. 2014).

Rapid development of lipidomic analysis began in essence 10 years ago with the advent of commercially available mass spectrometers using soft ionization techniques (electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), matrix-assisted laser desorption/ionization (MALDI), time-of-flight mass spectrometry (TOF)), etc.

Lipids are structurally diverse chemical compounds that perform many key biological functions, serving as structural components of cell membranes, reservoirs and sources of energy, or as signaling molecules. Lipids may be broadly defined as hydrophobic or amphipathic molecules which, at least in part, are formed by condensation of thioesters (fatty acids, polyketides, etc.) or isoprene units (prenols, sterols, etc.).

**Table 1** The number of possible TAGs from algal oil

| Description         | Number of possible TAGs   | Number of possible TAGs for y = 11 |
|---------------------|---------------------------|------------------------------------|
| Without isomers     | $x = (y^3 + 3y^2 + 2y)/6$ | 286                                |
| Without enantiomers | $x = (y^2 + y^3)/2$       | 726                                |
| All isomers         | $x = y^3$                 | 1331                               |

where x is the number of TAGs, y is the number of FAs in TAGs

Lipids are generally divided into “simple” and “complex” ones. Simple lipids are those which on hydrolysis provide at most two types of products, whereas complex lipids yield upon hydrolysis three or more products. Examples are shown in Fig. 1.

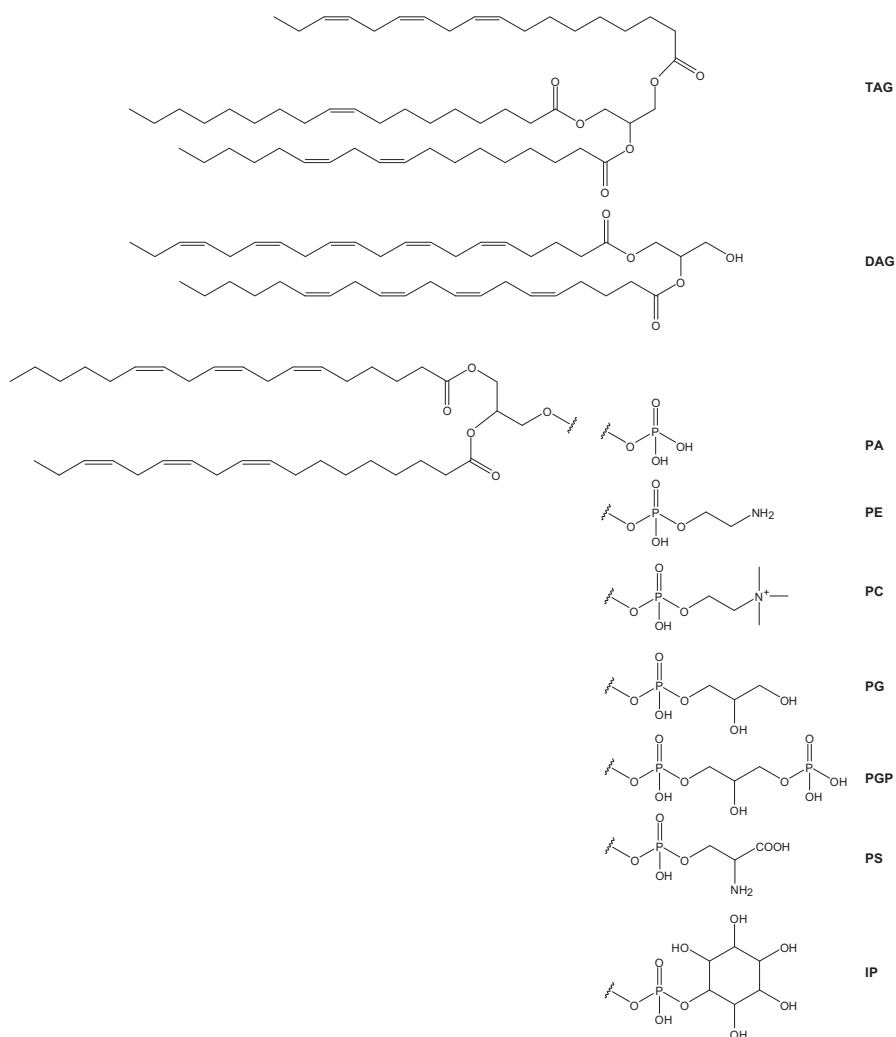
The variability of cell lipids reaches tens of thousands of molecular species, see, e.g., Table 1 showing the number of possible TAGs.

Obviously, all theoretically predicted molecular species may not be present or even detected. Many of them are below the detection limit of the instrumentation used. Even so, several thousand molecular species of phospho- and glycolipids have, for instance, been identified in *Staphylococcus aureus* (Hewelt-Belka et al. 2014).

## 2 Fatty Acids

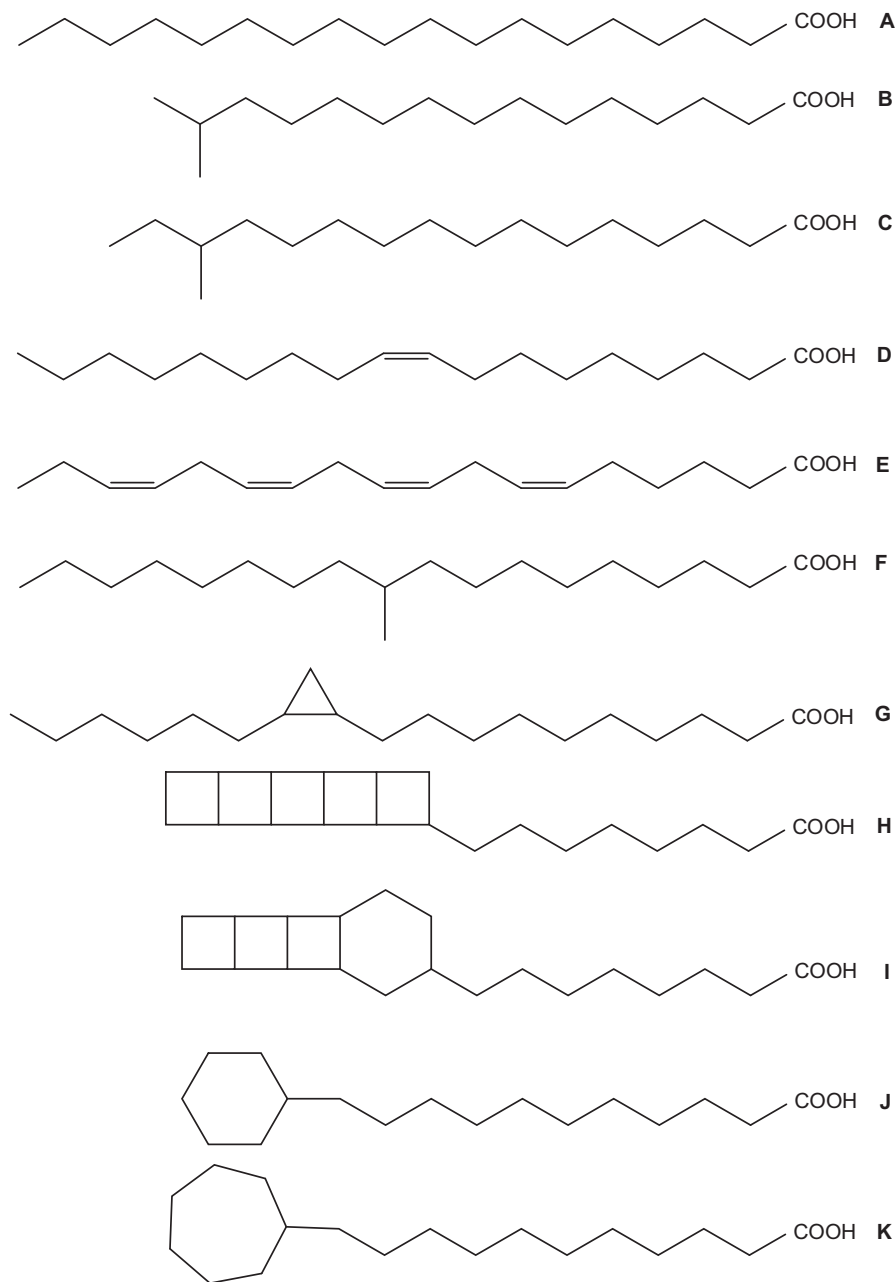
Though as such they are not a subject of lipidomic analysis, fatty acids (FAs) play an important and irreplaceable role in the analysis of lipids. Their analysis has been performed for more than 50 years and the number of analyzed microorganisms therefore exceeds several times the number of organisms that have been analyzed as to their lipidomic profile. Furthermore, analysis of FAs, whether concerning total FAs or individual classes of lipids, is far easier to implement, and at a much lower cost per analysis. With certain exceptions (see below) routine determination of FAs presents no problem. Therefore, an introduction to this chapter describes the types of fatty acids (Fig. 2) that can be encountered in different groups of organisms.

In bacteria, the situation is different and is characterized by a huge variety and diversity of FAs. Besides the common bacteria such as *Escherichia coli* (gram-negative bacteria) that contains saturated FAs, often with a cyclopropane ring in the middle of the alkyl chain, or *Bacillus subtilis* (gram-positive bacteria) which are characterized by the presence of iso- and anteiso-FAs having amino acids Val, Leu, or Ile as biosynthetic precursors, bacteria contain also less common FAs. These are mainly polyunsaturated fatty acids (PUFAs) of a structure that may be identical with those of PUFAs of eukaryotes (algae, mammals). These PUFAs were surprisingly identified primarily in extremophilic bacteria, e.g., the genus *Shewanella* and many others (Russell and Nichols 1999). Their biosynthesis is also quite different; unlike the biosynthesis of PUFAs in animals, plants, fungi, and cyanobacteria, which are biosynthesized by a combination of elongation and oxygen-dependent desaturation of existing fatty acids catalyzed by fatty acid synthetase, in these bacteria they are biosynthesized using polyketide synthases, i.e., very much like antibiotics such as tetracyclines.



**Fig. 1** Structures of “simple” and “complex” lipids (explanation of abbreviations, see below). *DAG* diacylglycerol, *TAG* triacylglycerol, *PA* phosphatidic acid, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *PGP* phosphatidylglycerolphosphate, *PI* phosphatidylinositol, *PS* phosphatidylserine

As mentioned above, bacterial FAs may contain a cyclopropane ring, with mycobacteria containing even more rings. Anaerobic ammonium oxidizing (anammox) bacteria belonging to the phylum Planctomycetes, which oxidize ammonium to  $N_2$  with nitrite as the terminal electron acceptor, contain ladderanes, which are compounds containing cyclobutane ring(s) (see below). Cyclopentane fatty acids were identified in, e.g., *Gibberella fujikuroi* which is a fungal plant pathogen, or in



**Fig. 2** Types of fatty acids (**A** – saturated, i.e., stearic acid, **B** – *iso*-branched, i.e., isopalmitic acid, **C** – anteisobranched, i.e., anteismargaric acid, **D** – oleic acid, **E** –  $\alpha$ -linolenic acid, **F** – tuberculostearic acid, **G** – lactobacillic acid, **H, I** – ladderane fatty acids, **J** – 11-cyclohexylundecanoic acid, **K** – 11-cycloheptylundecanoic acid)

the plant family Flacourtiaceae.  $\omega$ -Cyclohexyl and  $\omega$ -cycloheptyl FAs have been identified in thermoacidophilic bacteria of the genus *Alicyclobacillus* (Rezanka et al. 2009).

It can thus be said that bacteria contain all known types FAs with the exception of those with a cyclopentane ring.

Eukaryotic organisms from algae through lower plants (mosses, ferns, lichens) to flowering plants contain mainly straight-chain FAs, either saturated or monounsaturated, and also PUFAs. Many plants contain so-called very-long-chain fatty acids, which are considered to include FAs with a chain longer than 22 carbon atoms. Typical examples are wax esters on the surface of vascular plants, which are important biomarkers found in sediments.

By contrast, specialized tissues (organs) of mammals, e.g., sperm, brain, or eye, contain very-long-chain polyunsaturated fatty acids (VLCPUFAs) of the type of C28-C36 FAs belonging to the n-3 and n-6 families and containing 4–6 double bonds. It is surprising that similar FAs, although only up to the C<sub>28</sub>, but with up to eight double bounds are found in dinoflagellates, which are a large group of flagellate protists, mostly from the marine plankton that constitute the phylum Dinoflagellata.

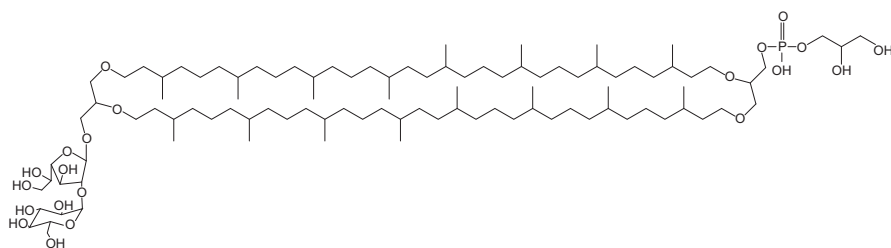
This brief introduction about the types of fatty acids and their presence or absence in various organisms is intended to show the enormous diversity of their structure. According to Chemical Abstracts (SciFinder database), there are reportedly around one thousand known naturally occurring FAs.

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### 3 Archaea

Archaea are a large domain of prokaryotic unicellular organisms whose independence from bacteria and eukaryotes was recognized in 1977. First, one should note that the Archaea (formerly archaeobacteria) form a large kingdom of unicellular prokaryotic organisms that is independent of other domains of life (bacteria and eukaryotes). They differ from bacteria and eukaryotes in the structure of their cell membrane, cell wall, the genome, and certain metabolic processes. These differences include, for example, the presence of different stereochemistry of the archaeal glycerol moiety (another enantiomer) and the fact that none of the hitherto analyzed archaeal microorganisms contains fatty acids. Complex phospholipids contain isoprenoid chains with multiple side-branches coupled to the glycerol backbone by ether bond (see below). Archaea might be found in areas with extremely high temperatures, extreme pH, or high salt content.

The chemical structure of archaeal membrane is unique. As mentioned above, their lipids do not contain FAs. Archaeal phospholipids are unusual in several respects; above all, they consist of glycerol-ether lipids (De Rosa et al. 1986). The ether linkages are highly stable and this enables Archaea to inhabit extreme environments (Albers et al. 2000). Chains are not straight but mostly branched and are based on isoprene units (Damste et al. 2002) and therefore have no double bond(s) (Koga and Morii 2005). As stated above, the complex lipids contain



**Fig. 3** The structure of diglycosyl phosphatidylglycerol tetraether (hexose2-PG-T) from marine hyperthermophilic archaeon *Pyrococcus furiosus*

L-glycerol, which is the enantiomer of D-glycerol occurring in all other organisms (Koga and Morii 2005). The main problems with Archaea are the difficult analysis and their non-culturability. This problem, however, far exceeds the scope of this chapter. For more details, see, e.g., Woese et al. (1990).

The use of lipidomics for Archaea is illustrated by several studies reporting on the possibilities of lipidomic analysis. HPTLC and MALDI-TOF/MS of the archaeon *Pyrococcus furiosus*, which grows at 100 °C, were used to identify polar lipids of the type of archaeol (diethers) and caldarchaeol (tetraethers) (Lobasso et al. 2012). MALDI with 9-aminoacridine- like matrix identified the structure shown in Fig. 3.

Lipidomic analysis of two extremely haloalkaliphilic archaea, *Natronococcus occultus* and *N. amylolyticus*, combined the use of TLC and MALDI-TOF/MS analysis (Angelini et al. 2012). The major lipids were phosphatidylglycerol and phosphatidylglycerophosphate methyl ester, including cardiolipin that contained four isoprenoid chains. This lipid was also hypothesized to play a crucial part in the adaptation to high pH and high salinity.

MALDI-TOF/MS was again used in the lipidomic analysis of the halophilic archaeon *Halobacterium salinarum* (Angelini et al. 2010) which identified many glyco- and phospholipids up to a molecular weight of 2,000 Da, among them, e.g., (3'-sulfo)Galpβ1-6Manpα1-2Glcα1-1-[sn-2,3-di-O-phytanylglycerol] or (3'-sulfo)Galpβ1-6Manpα1-2Glcα1-1-[sn2,3-di-O-phytanylglycerol]-6-[phospho-sn-2,3-di-O-phytanylglycerol].

In two halophilic microorganisms, *Halorubrum trapanicum* and *Haloferax volcania* isolated from a salt lake near Malaga (Spain), Lobasso et al. (2015) identified unusual sulfated bis-diglycosyl diphytanylglyceroldiethers with m.w. around 1000 Da using a TLC-MALDI-TOF/MS and discussed their effect on the bacterial resistance to high salinity.

The frequently studied archaeon *Sulfolobus islandicus* was found to contain common archaeal lipids, i.e., dialkyl glycerol diethers and tetraethers substituted with polar phosphate groups often further substituted by, e.g., inositol, glycerol, or from one to four monosaccharides (Jensen et al. 2015a). In another study, Jensen et al. (2015b) investigated the influence of temperature on the number of cyclopentane rings in the lipid molecule.

Gagen et al. (2016) described a change in lipids (mainly tetraethers) in *Thermococcus kodakarensis* during cultivation; the lipids were separated by UHPLC and identified by tandem ESI/MS.

RP-HPLC with tandem QTOF-ESI-MS was used to analyze the lipids of the thaumarchaeon *Nitrosopumilus maritimus* (Elling et al. 2014). The value of the organic paleothermometer (TEX<sub>86</sub>) was found to depend on membrane dibiphytanyl glycerol tetraether lipids (GDGTs).

The above examples constitute a mere fraction of published works and should allow readers to get familiar with the analysis of archaeal lipids. The main problem in the field does not seem to be the actual analysis of lipids but the collection and especially cultivation of these microorganisms (Blum 2008), or their contamination by other organisms.

A very nice example of a connection of cultivation and analysis of Archaea is given in the study performed on *T. kodakarensis* by (Gagen et al. 2016; Meador et al. 2014); it should be however noted that the research team has extensive experience with cultivation of hardly cultivable microorganisms.

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## 4 Bacteria

Bacteria are a separate domain of unicellular prokaryotic organisms and are the most widespread living organisms. This is reflected in the large number of different classes of lipids, see Fig. 1.

It is believed that the lipids of a single bacterial cell can include many thousands of molecular lipid species (Breslow et al. 2008; Schuldiner et al. 2005; Yetukuri et al. 2008). As mentioned above, bacteria contain in addition to conventional-type straight-chain FAs (saturated, unsaturated, and polyunsaturated) (Russell and Nichols 1999) also branched (iso and anteiso) fatty acids and FAs with cycles, see Fig. 2 (Lanekoff and Karlsson 2010).

Data on the diversity of bacterial lipid structures were published in several reviews (Parsons and Rock 2013; Sohlenkamp and Geiger 2016). Lipidomic analysis of these lipid structures has also been published (Leray 2012; Řezanka et al. 2012).

*Escherichia coli* and *Bacillus subtilis* are two typical representatives of gram-negative and gram-positive bacteria, and it is therefore not surprising that one of the first studies dealing with lipidomic analysis of bacteria was devoted to them (Gidden et al. 2009). MALDI-TOF/tandem MS showed that both fatty acids and lipids of the two species differ widely. For instance, *B. subtilis* contains lysyl-PG and diglucosyl diglycerides that are missing in *E. coli*.

Zhang et al. (2011) analyzed lipids up to m.w. 1,000 Da in 2 gram-positive and 14 gram-negative bacteria by DESI and ESI and used principal component analysis to determine the taxonomy of the bacteria.

Analysis of *S. aureus*, one of the best known human pathogens, was performed by HPLC-QTOF-MS (Hewelt-Belka et al. 2014). The authors identified over 7000 molecular species belonging to 18 major classes and 36 subclasses of lipids.

This provided the possibility to compare strains with different phenotypic characteristics and hence different sensitivity to antibiotics.

Garrett et al. (2012) used NP-LC/ESI-MS to identify molecular species of cardiolipin in *E. coli* grown at different temperatures; the content of these lipids was found to vary depending on temperature.

Lipidomic analysis of *Pseudomonas aeruginosa*, a known pathogen that forms biofilms, showed changes in the inner and outer membrane of the cells depending on their age (Benamara et al. 2014). Kondakova et al. (2015) described lipidomic analysis of *P. fluorescens* by HPTLC-MALDI-TOF/MS, which detected PC otherwise contained in eukaryotes.

Lipids in *B. subtilis*, *Streptomyces coelicolor*, *Mycobacterium smegmatis*, and *P. aeruginosa* were determined using nanospray MS-DESI directly from Petri dishes without performing the extraction of lipids (Watrous et al. 2012).

Identification of different species of *Bacillus* was performed by MALDI-TOF/MS and the cells were found to contain phospholipids – PE, PC, PG, DGDG (Shu et al. 2012).

Hansen et al. (2015) investigated the plasma membrane of the probiotic *Lactobacillus acidophilus* La-5 using high-resolution shotgun lipidomics. They described changes in the composition of fatty acids in plasma membrane lipids, mainly in cardiolipin and monolysocardiolipin, after addition of Tween 20, a polysorbate surfactant like Tween 80, but primarily containing lauric and myristic acids as well as linoleic and alpha-linolenic acids.

## 4.1 Plasmalogens

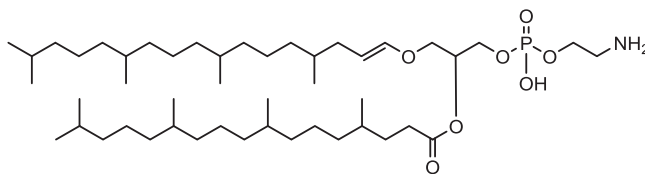
One of the most interesting groups of lipids is plasmalogens. These compounds are glycerol derivatives wherein alcohol is bound to the *sn*-1 position via ether bonds and a fatty acid esterifies the *sn*-2 position. The alcohol binds as vinyl ether, the acid by an ester linkage, see Fig. 4 for the structure of plasmalogen-phosphatidyl ethanolamine (pPE).

The alcohol usually has 16 or 18 carbon atoms, whereas the acid is always characteristic for the given group of organisms, for instance, straight chain or branched chain saturated (iso or anteiso) acid in bacteria (Rezanka et al. 2012), or polyunsaturated acids in mammals.

At the 3-position of the glycerol, backbone is a phosphate group, so that plasmalogens include plasmalogen-phosphatidylserine, plasmalogen-phosphatidylglycerol, plasmalogen-phosphoethanolamine, etc.

Much more interesting than their structure is their distribution in nature. They are found only in anaerobic bacteria and in animals (Braverman and Moser 2012; Magnusson and Haraldsson 2011) and have not been found in aerobic bacteria, fungi, and plants, including algae (Felde and Spiteller 1994). Their presence in fungi is highly debatable (Horrocks and Sharma 1982).

Plasmalogens were analyzed in recent years almost exclusively by LC-MS. Other methods are time-consuming and inaccurate and usually do not provide information



**Fig. 4** The structure of plasmalogen-phosphatidyl ethanolamine (pPE)

about native plasmalogens and their molecular species. Identification of plasmalogens is usually not complicated, see the paper of Hsu et al. (2003) which describes very well the analysis and provides excellent background information.

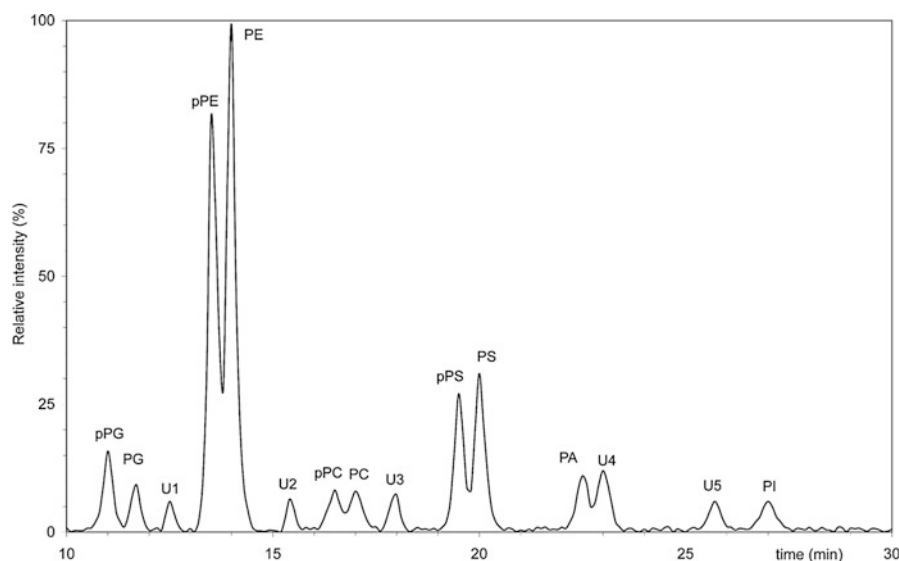
Lipidomic analysis can be used in industrial practice for instance in the brewing industry to identify the contamination of beer by anaerobic bacteria of the genus *Pectinatus* and *Megasphaera* (Rezanka et al. 2015). Analysis of *Pectinatus frisingensis* was performed by LC-MS, see Fig. 5, and it was found that the major plasmalogen is cyclo-plasmenyl-19:0/17:1 PE. Alanyl, lysyl-, and glucosyl-phosphatidylglycerols and CLS have been identified in thermophilic bacteria of the genus *Anoxybacillus* using HILIC-LC / ESI-MS/MS (Rezanka et al. 2012). Bacteria of genus *Clostridium* have been found to contain plasmalogens (Goldfine and Guan 2017; Kolek et al. 2015).

Basically the same facts that were said about cultivability and/or noncultivability of Archaea also apply to bacteria. The review by Alain and Querellou (2009) stated that more than 90% of bacteria found in nature are noncultivable. Their presence can obviously be proved in substrates such as geothermal water, forest soil, active sludge from wastewater treatment, but during subsequent culture of bacteria from these substrates only some are able to multiply and grow. The overall population is thus overgrown by several fastest propagating bacteria, which reduces the species diversity of the population.

## 4.2 Mycobacteria

As suggested by their name, mycobacteria were for many years considered to belong to fungi, among others for the unusual structure of their lipids, particularly mycolic acids and mycolates (Fig. 6). Many of them are human pathogens (*M. tuberculosis* or *M. leprae*). The lipids of mycobacteria include both nonpolar lipids, such as phthiocerol dimycocerosates, and polar ones (phosphatidylinositol mannosides). Lipidomic analysis identified over 5000 molecular species (Layre et al. 2011). Three databases of these lipids were created, i.e., MTB LipidDB, MycoMass, and MycoMap (Layre et al. 2011; Madigan et al. 2012; Sartain et al. 2011).

Lipids can form over half of cell mass and, in addition to the already mentioned mycolic acids, they include complex lipids and FAs such as palmitic, oleic and tuberculostearic acids. The complex lipids have not been found to include sphingolipids, PE, PC, PG, and diphosphatidylglycerol, whereas the presence of



**Fig. 5** HILIC/APCI-MS chromatogram of the phospholipids from *Pectinatus frisingensis* DSM 20465

phosphatidylinositol and phosphatidylinositol mannosides is common (Hsu et al. 2007a, b).

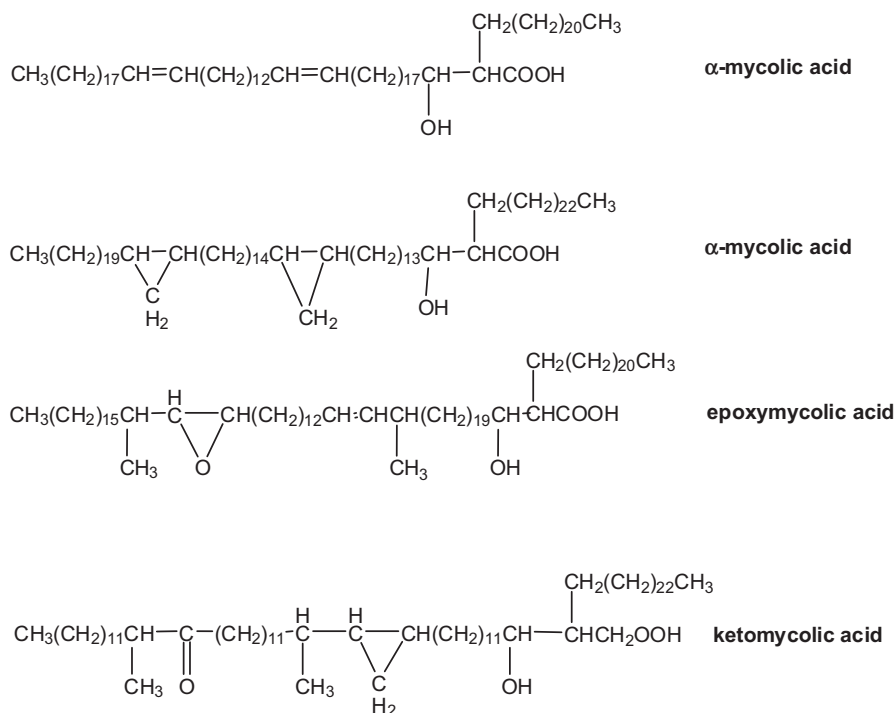
Mycolic acids, which have a totally unique structure and have not been found in other bacteria, consist of  $\alpha$ - and  $\beta$ -hydroxymycolic chains differing in length and chain branching, unsaturation, and substitution of polar groups (Guenin-Mace et al. 2009). They provide essential structure information that can be used in taxonomy (Butler and Guthertz 2001).

The use of isoniazid, an important drug which inhibits the biosynthesis of mycobacterial lipids, led to the understanding of the structure of cell membranes (Layre et al. 2014).

Lipidomic analysis of bacteria including mycobacteria revealed the great variability of complex lipids, the use of which can be seen especially in chemotaxonomy, but also in the elucidation of resistance to antibiotics including the knowledge of the behavior of bacteria in the biofilm.

## 5 Yeast

Yeast is one of the most important microorganisms used in biotechnology. Yeasts are basically single-celled fungi that usually reproduce by budding or fission and are used primarily for the production of beer, wine, and bread. However, some yeasts, e. g., *Candida albicans*, are pathogenic.



**Fig. 6** Unusual structure of mycolic acids

Lipidomics of yeast was the subject of several major studies that mostly analyzed the famous yeast species *Saccharomyces cerevisiae*. Shotgun lipidomic analysis of two million cells allowed the identification of 21 classes of lipids and more than 250 molecular species. Changes were found in the lipid content of yeast cultured at 24 °C and 37 °C (Ejsing et al. 2009).

Lipidomic studies using UHPLC-MS/MS performed on a wild and a recombinant strain of *S. cerevisiae* showed a correlation between PI metabolism of xylose and glucose (Xia et al. 2011).

A study of the dependence of the 21 classes of lipid in *S. cerevisiae* on the duration of cultivation showed a change in the contents of 34:2-PC versus 32:2-PC or 34:2-PA versus 32:2-PA (Casanovas et al. 2015).

Cultivation of two yeasts, *S. cerevisiae* and *Zygosaccharomyces bailii*, on an atypical carbon source (acetic acid) was investigated by lipidomic analysis, and it was found that the tolerance to acetic acid is related to the increase in the content of sphingolipids (Lindberg et al. 2013).

Da Silveira dos Santos et al. (2014) studied the effect of deletion of nonessential genes encoding kinases or phosphatases on lipid content. The results showed changes in some molecular species, e.g., 32: 2 and 34: 2-PC.

The absence of YBR141C and YJR015W genes, whose function is unknown, was studied in mutants of *S. cerevisiae* (Tarasov et al. 2014).

Lipidomic profile of *S. cerevisiae*, *S. bayanus*, *Kluyveromyces thermotolerans*, *Pichia angusta*, and *Yarrowia lipolytica* showed that the yeast contain 9 classes of phospholipids (mainly CL, PE, PI PC, PS, and PG) with more than 100 molecular species (Hein and Hayen 2012).

Lipidomic analysis of the wild type strain of *S. cerevisiae* and its mutants cultured at four different temperatures (15 °C, 24 °C, 30 °C, and 37 °C) was performed in order to investigate changes in phospholipids (Klose et al. 2012). The analysis showed that increased temperature enhances PI content and reduces the content of TAG and PE.

Comparison of lipidomic profiles of homogenate and microsomes in methylotrophic yeast *Pichia pastoris* showed changes in the content of TAG, PC, and PI (Klug et al. 2014).

As stated above, with a few exceptions the authors examined only *S. cerevisiae*. Further developments in this area can be seen particularly in the lipidomic analysis of less and less easily cultivable yeasts, for example, psychrophilic yeasts (Fig. 7) (Rezanka et al. 2016).

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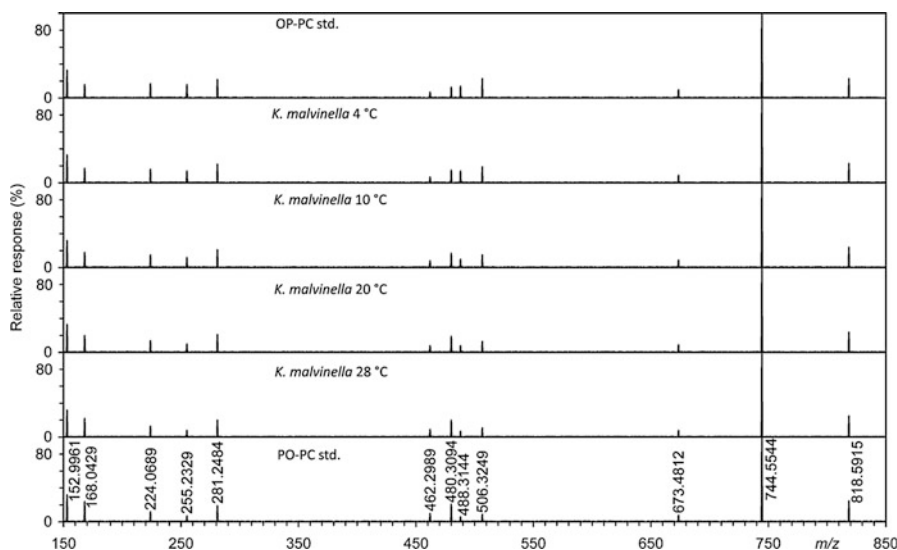
## 6 Cyanobacteria and Algae

### 6.1 Cyanobacteria

Algae and cyanobacteria are taxonomically very different, but they biosynthesize basically very similar lipids. Also both their occurrence and cultivation are very much the same. That is why they are here discussed together, although algae are eukaryotes while cyanobacteria are prokaryotes.

Cyanobacteria were found to contain glycolipids (MGDG, DGDG, and SQDG) as well as phospholipids, with PG as a major lipid. In this cyanobacterial lipids resemble lipids of algae and are different from most bacteria, which are taxonomically much closer.

Marques et al. (2016) examined the effects of As (III) on the lipidomic profiles of two cyanobacterial species (*Anabaena* and *Planktothrix agardhii*) using LC-MS with simultaneous processing of the results by the multivariate curve resolution alternating least squares. They found that As (III) induced significant changes in the lipid composition of the cyanobacteria. The biggest changes occurred primarily in the content of pigments (chlorophyll *a* and its degradation product pheophytin *a*, as well as in carotene compounds such as 3-hydroxycarotene and 3-carotene-3,3'-dione) and in the content of MGDG.



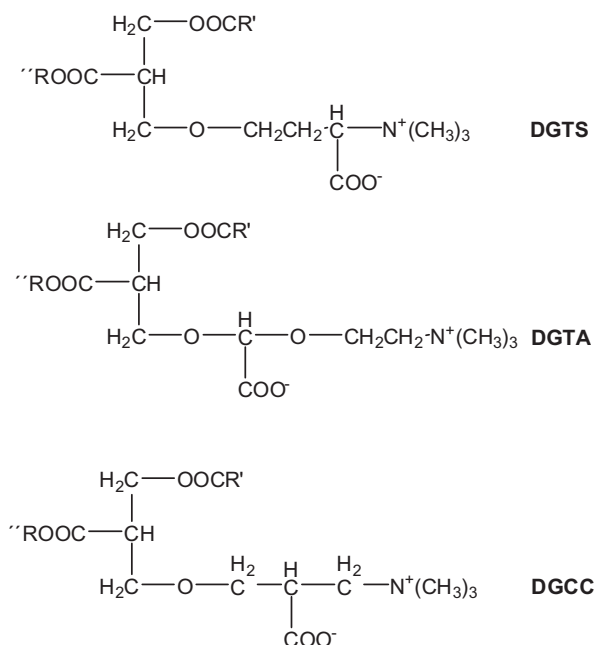
**Fig. 7** Tandem mass spectrum of natural PC of *K. malvinella* cultivated at different temperatures and two commercial standards, i.e., molecular species 1-palmitoyl-2-oleylphosphatidylethanolamine and (PO-PC) and 1-oleyl-2-palmitoyl-phosphatidylethanolamine (OP-PC), respectively

## 6.2 Algae

Algae are lower plants, both unicellular and multicellular (see thallus). They live in fresh or salt water, or in symbiosis in lichens. Although algae are typical photosynthetic organisms, they can be cultivated exclusively heterotrophically, which is widely used in various biotechnological applications. Algae contain a variety of lipids, including the less common ones, e.g., betaine lipids or sulfolipids. Typical examples are diacylglyceryltrimethylhomoserine (DGTS), diacylglyceryl hydroxymethyl trimethylalanine (DGTA), and diacylglycerylcarboxyhydroxymethylcholine (DGCC), see Fig. 8.

Unlike bacteria and yeasts, algal dry weight often contains more than 50% PUFAs, for instance, 18:5, 20:5, and 22:6 acids that are missing in higher plants. To produce PUFAs, algae are commonly cultured in fermenters with volumes of tens of thousands liters (e.g., the commercially available preparation containing docosahexaenoic acid from different strains of algae).

Lipidomic analysis has often been performed in order to determine the behavior of algae under abnormal cultivation conditions. One of the stressful conditions is a salt content higher than that at which algae usually grow. For freshwater algae, it is, e.g., cultivation in sea water. Lu et al. (2012, 2013) studied the alga *Chlamydomonas nivalis* (snow alga that lives in the snow, which is basically almost pure water) for its content of lipid biomarkers (DGTS, MGDG, DGDG, or SQDG) using ESI in positive- and negative-ion mode. They identified a noncommonly occurring hexadecatetraenoic acid and identified lipids in algae cultured under different conditions using multivariate statistical analysis.



**Fig. 8** Examples of structures of diacylglyceryltrimethylhomoserine (DGTS), diacylglyceryl hydroxymethyl trimethylalanine (DGTA) and diacylglycerylcarboxyhydroxymethylcholine (DGCC)

Two geographic varieties of the alga *Nannochloropsis oceanica*, which are morphologically and taxonomically (via 18S rRNA) indistinguishable, showed, using UHPLC-Q-TOF-MS, a completely different taxonomically pertinent lipid profile (Li et al. 2015). As biomarkers have been identified, e.g., 20:4/20:5-DGTS, 20:5/14:0-MGDG, 20:5/16:1-DGDG, and 16:1/20:5/20:5-TAG.

Geographic varieties of the snow alga *Chloromonas pichinchae* were analyzed by silver LC/APCI-MS and LC-NARP/APCI-MS (Rezanka et al. 2014), see Fig. 9, who identified uncommon molecular species of 16:4/16:4/18:4-TAGs, 16:3/16:3/18:4-TAGs or 18:4/18:4-SQDG.

The dependence of the lipidomic profile on culture conditions of the red alga *Galdieria sulphuraria* growing at low pH was determined by LC-MS (Vitova et al. 2016). Cultivation was carried out at pH 1–4 and 14 classes of lipids were identified including many tens of molecular species of lipids, including regioisomers. Low pH promotes the biosynthesis of betaine lipids and causes variation in the ratio of regioisomers.

Analysis of the psychrophilic alga *Chlamydomonas reinhardtii* (Yang et al. 2015) using positive and negative mode ESI identified polar lipids, and their molecular species, e.g., 16:0/18:4-DGTS, 16:0/18:3-SQDG, and 16:1/18:3-PG.

Danielewicz et al. (2011) studied the possibility of using lipidomic analysis for potentially oleaginous saltwater microalgae *Phaeodactylum tricornutum*,



The alga *Nannochloropsis salina* was investigated using several ionization techniques (ESI, APCI, APPI, and MALDI) and showed differences in the representation of individual lipid classes (Lee et al. 2013). This study pointed out how important it is to use internal standards for the quantification. It is to be regretted that not all necessary standards are commercially available.

Investigation of the influence of temperature changes on the lipidome of red alga *Pyropia haitanensis* revealed that the alga contains 39 lipids that can serve as lipid biomarkers (Chen et al. 2016).

MacDougall et al. (2011) used UHPLC-MS to identify and quantify the contents of TAGs in 6 algae of the genus *Botryococcus*, *Nannochloropsis*, *Neochloris*, *Phaeodactylum*, *Porphyridium*, and *Scenedesmus*. They detected the presence of 28:1/28:2/18:1 TAG or 28:2/28:2/18:1-TAG belonging to TAGs with the longest known chain found in nature. In addition, the alga *Scenedesmus obliquus* contains polyunsaturated TAGs, e.g., 20:5/18:3/16:3-TAG. Although the publication is 6 years old, it points to further possibilities of lipidomic analysis of algae and other organisms.

In their excellent review, da Costa et al. (2016) summarized the findings obtained by lipidomic analysis of glycolipids of many tens of microalgae.

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## 7 Research Needs

We believe that further development of lipidomic analysis is expected in four directions.

**New ionization techniques.** The first is the use of new ionization techniques, e.g., atmospheric pressure photoionization MS which is connected to tandem MS, or the use of high-resolution mass spectrometry of time-of-flight MS, connected with ultra-performance liquid chromatography.

**Wider range of samples.** The second direction is the analysis of a much wider range of samples, particularly from the group of microorganisms not commonly found, such as extremophilic Archaea, bacteria, yeast or cyanobacteria, and algae harvested from unusual (psychrophilic, halophilic, thermophilic, etc.) habitats. Shotgun and LC-MS lipidomics has so far been rarely used for analysis of lipids containing unusual head groups, very-long chain or very-long chain polyunsaturated FAs.

**Chemical compounds labeled with stable isotopes.** The third direction involves the application and use of chemical compounds labeled with stable isotopes in the study of dynamic changes of metabolic pathways. This approach is at the beginning and suffers so far from the lack of suitable and commercially available precursors and metabolites and their high price. Nevertheless, the use of labeled compounds leads to a complex analysis of lipid metabolism on the molecular level and a better understanding of the role of lipids in biotechnological applications.

**Single cell lipidomics.** The fourth area concerns the sensitivity of currently produced mass spectrometers, which has already reached the attomole ( $10^{-18}$ ) level, i.e., the concentration of lipids in a single cell. This creates a new highly promising discipline, which we may call single cell lipidomics.

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