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Lipidomic analysis of two closely related strains of the microalga *Parietochloris* (Trebouxiophyceae, Chlorophyta)

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ABSTRACT

The analysis of ribosomal internal transcribed spacer 2 (ITS2) secondary structure and morphology of two strains of the green microalga *Parietochloris* (CCALA 1082 and 1084) confirmed that they represent one biological species. However, their fatty acid profiles showed slight differences, which was fully confirmed by a series of lipidomic analyses using both shotgun lipidomics and hydrophilic interaction liquid chromatography-tandem mass spectrometry with electrospray ionization. 26 classes of lipids were identified, including more than 430 molecular species of lipids. The analyses showed that the greatest differences are in three types of phospholipids - phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol. These phospholipids have different spatial configurations (cylindrical, conical, and conical inverted), especially for those molecular species containing at least one arachidonic acid. Based on the lipidomic analyses we were able to distinguish the two strains. This may be advantageous in cases when routine DNA analysis shows that two strains are nearly identical.

1. Introduction

Lipids are a very diverse and ubiquitous group of compounds that have many key biological functions. They play an important role in energy storage, as cell membrane structural components, and they are involved in many metabolic processes such as signal transduction, transcriptional and translational control, cell-cell interactions, morphogenesis, secretion, and vesicle trafficking [1–3].

Lipids are biosynthesized by multi-step reactions from glucose, mostly via acetyl-CoA (malonyl-CoA). Tens of different lipid classes differ in their head groups and some of them, e.g. phospholipids or glycolipids, contain two fatty acid moieties. This all leads to hundreds to thousands of possible molecular species. It should however be noted that not all theoretically possible structures are present and can be experimentally detected; some are not biosynthesized at all, others are present at low concentrations that are below the detection limit of the analytical method. Lipidomics, as a subset of metabolomics, is a suitable partner for metagenomic analysis [4].

The main benefit of lipidomic analysis is the use of soft ionization mass spectrometry, most frequently electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI), or atmospheric pressure chemical ionization (APCI). High resolution devices are now often used, which greatly facilitate the identification of individual molecular species. Lipidomics is based on a targeted metabolomic platform that allows comprehensive analysis of many kinds of cell lipids based on electrospray ionization tandem mass spectrometry. Shotgun lipidomic analysis requires a very small amount of sample. For instance, the detection limit of cyclopropane plasmalogen in beer-spoilage anaerobic bacteria was 1 pg, which was about 830 bacterial cells [5].

Like other organisms [6], all microalgae contain a number of different lipids [7,8] but individual species differ in the composition of both lipids and the component fatty acids (FAs) [9]. Due to this fact, the FA profiles of various microalgae, including unusual lipids and FAs, can be used as chemotaxonomic tools suitable for characterizing groups and classes of microalgae [10,11,12–15]. A number of studies have been concerned with using FA profiling as a chemotaxonomic tool [16] in cyanobacteria [17], microalgae [18,19], macroalgae [20,21] and higher plants [22]. The results of gas chromatography–mass spectrometry (GC–MS) studies combined with multivariate statistics showed that FAs can be used to provide insight into the chemotaxonomic relationships between different species of microalgae [23]. Extensive studies investigating FA profiles of several thousand microalgal strains [11] revealed that these profiles can be readily used as suitable chemotaxonomic markers to define higher-rank taxa while a cluster analysis of photosynthetic glycerolipids of three *Skeletonema* species [9] showed that lipids can also be used as biomarkers to distinguish

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individual microalgal species. It should be noted, however, that FA proportions can vary between both closely related species and even among isolates belonging to the same species. Even so, the technical progress in lipid extraction, isolation, and purification, along with mass spectrometry could aid in establishing lipid composition as a useful chemotaxonomical tool for the classification of microalgae.

The genus *Parietochloris* (Trebouxiophyceae, Chlorophyta) is characterized by uninucleate coccoid cells and naked zoospores with counterclock-wise absolute orientation of basal bodies [24]. Based on 18S rDNA sequence data, it was demonstrated that this genus is polyphyletic. Some species previously designated as *Parietochloris* belong to *Lobosphaera* clade, which is distinct from the lineage containing the type species *P. alveolaris* [25,26].

The Culture Collection of Autotrophic Organisms (CCALA) of the Institute of Botany CAS houses two strains of *Parietochloris* sp., i.e., strain CCALA 1082 isolated from soil in the Joshua Tree National Park, Pinto Wells, CA, USA, and CCALA 1084 isolated also from soil in the same park, but in a ca. 25 kilometer distant location (Pinto Basin) (33°55′ 38.409″ N and 115° 42′ 4.3014″ W versus 33°56′ 27.1248″ N and 115°25′ 1.2318″ W [27]). Their taxonomic designation was carried out on the basis of their morphological traits and 18S rDNA analysis (GenBank accession numbers JX446475 and JX446477). The partial sequence data placed the two strains in a cluster with two known members of the genus *Parietochloris*, including the type species of the genus, *P. alveolaris* (EU878373.1, strain UTEX 836 [26,27]. Whereas the strains of *Lobosphaera* are among the richest producers of arachidonic acid (20:4ω-6) [28], lipid composition of those from the genus *Parietochloris* sensu stricto is unknown.

In the present article, we focused on the identification of these two closely related strains of the green alga *Parietochloris* using lipidomic analysis including both shotgun lipidomics and hydrophilic interaction liquid chromatography coupled with high resolution electrospray mass spectrometry, and subsequent identification of triacylglycerols using chiral liquid chromatography. To confirm their close phylogenetic relationship, internal transcribed spacer 2 (ITS2) rRNA secondary structures were compared. Although the two strains are nearly identical based on morphological and molecular characteristics, lipidomic analysis of total lipids showed (in contrast to analysis of the total fatty acids) that the content of lipids, including fatty acids in individual lipid classes, was different.

2. Materials and methods

2.1. Cultivation of microalgal strains

Two strains of *Parietochloris* sp. (CCALA 1082 and 1084) were obtained from the Culture Collection of Autotrophic Organisms at the Institute of Botany CAS, Třeboň, Czech Republic. They are kept as growing cultures on test tube slants at about 15 °C, under fluorescent lighting with photosynthetically active radiation (PAR) intensity of 40 μmol m^{-2} s^{-1}. The cultures were used to pre-cultivate the strain in a batch stationary culture in liquid Z8 medium (Table 1S) in 2 L glass bottles containing initially 0.25 L medium. When the cultures became denser, its volume was increased to 2 L and the PAR intensity was 48 μmol m^{-2} s^{-1}. Stirring of the suspension and CO_2 saturation was ensured by bubbling an excess air with 2% CO_2 introduced to the bottom of the bottle via a tube; the gaseous mixture was filtered through membrane filters (Midisart 2000, 0.2 μm PTFE Sartorius). After reaching stationary phase, the cultures were harvested by centrifugation at 2000 rpm for 15 min and the samples were stored in aluminium foil until solvent extraction.

2.2. DNA extraction, PCR and sequencing

Total genomic DNA was extracted from algal cultures following the standard protocol in DNeasy Plant Mini Kit (Qiagen, Germany), with minor modifications. The internal transcribed spacer regions 2 (ITS2 rDNA) were amplified from DNA isolates by PCR using the primers TW-81 (5′- GGGATCCGTTTCCGTAGGTGAACCTGC-3′), AB-28 (5′- GGGATCCATATGCTTAAGTTCAGCGGGT-3′) [29], Al1500af (5′- GCGCGCTACACTGATGC-3′) [30] and LR3 (5′- GGTCCGTGTTTCAAGACGG-3) [31]. New sequences are available in the NCBI Nucleotide Sequence Database under accession numbers KY311560 and KY311561. For complete description of the methods, see Supplements.

Table 1
Fatty acid composition from three parallel cultivations of strains CCALA 1082 and CCALA 1084 (arithmetic means ± standard deviations (S. D.)).

FA	Abbreviations	CCALA 1082	± S.D.	CCALA 1084	± S.D.
14:0	M	0.04	0.00	0.11	0.02
14:1	Mo	0.04	0.01	0.10	0.02
16:0	P	13.98	0.10	14.06	0.44
16:1n-11	He	4.77	0.10	3.22	0.03
16:1n-7	Po	0.17	0.01	0.51	0.03
16:2n-6	Pl	1.70	0.04	1.78	0.05
16:3n-3	Pn	4.04	0.09	3.50	0.04
18:0	S	1.69	0.03	2.21	0.05
18:1n-9	O	6.57	0.10	12.59	0.37
18:1n-7	V	5.02	0.11	2.21	0.05
18:2n-6	L	13.23	0.16	16.34	0.06
18:3n-6	γ-Ln	1.49	0.05	1.57	0.01
18:3n-3	Ln	10.28	0.19	8.51	0.03
20:2n-6	Ad	0.02	0.02	0.22	0.03
20:3n-6	At	1.20	0.04	0.92	0.04
20:4n-6	A	34.01	0.25	30.08	0.67
20:4n-3	Et	0.04	0.00	0.68	0.01
20:5n-3	E	1.71	0.05	1.39	0.01

2.3. Secondary structure prediction of nuclear rDNA ITS2

Nuclear rDNA ITS2 regions of both strains were identified using a web interface at the ITS2 database [32]; http://its2.bioapps.biozentrum.uni-wuerzburg.de/cgi-bin/index.pl?annotator) and genus affiliation to *Parietochloris* was kept according to original strain designation in CCALA culture collection since the purpose of this study was lipidomic analysis of closely related species, not taxonomic revision of publicly available strains. These sequences were then folded with 5.8S-LSU stem regions using the Mfold server at http://mfold.rna.albany.edu/?q5mfold [33] to predict several secondary structure models, from which we selected the model that was consistent with the specific features of nuclear rDNA ITS2, U-U mismatch in helix II and the UGGU motif near the 59-end site apex of helix III [34]. The secondary structures were drawn using VARNA version 3.7 [35] and modified by Inkscape 0.91 (Free Software Foundation Inc., USA).

2.4. Isolation of lipids

Isolation and pretreatment of lipids was carried out according to [36], with modifications as described previously [37]. For full description of the experiments see the Supplements.

2.5. FAMEs analysis

Analysis of fatty acids in the form of methyl esters (FAME) was described previously [38,39]. A complete description of all conditions of FAME analysis by GC–MS is in the Supplements.

2.6. Lipidomic analysis by high-resolution electrospray ionization mass spectrometry

The high resolution hybrid mass spectrometer LTQ Orbitrap Velos was used; for detailed conditions see the Supplements.

2.7. HILIC-LC/MS-ESI

After purification by Sep-Pak Vac Silica cartridge, the lipids were identified and quantified by LC/MS. A detailed description of both positive and negative ESI is again in the Supplements.

2.8. Statistics

All experiments were repeated at least thrice. Three replicates (n = 3) of each cultivation were used for each sample. The software Statistica for Windows (version 12.0, Dell) was used for analyses. Data were presented as means ± S.D.

3. Results and discussion

3.1. Cell morphology and analysis of molecular data

The morphology of the strains CCALA 1082 and CCALA 1084 was consistent with the characteristics of the genus *Parietochloris*. The cells had lobed chloroplast with a pyrenoid covered with starch grains. No significant differences between the isolates were observed.

The phylogenetic analysis of 18S rDNA sequences confirmed that the strains CCALA 1082 and CCALA 1084 are closely related strains from the genus *Parietochloris* (Trebouxiophyceae) [27]. For a more detailed assessment of their relationship, we performed analysis of the secondary structure of ITS2 rDNA, which is a variable marker used for the definition of species according to the compensatory base change (CBC) concept [40]. The analysis showed some differences between the two isolates that were located in variable regions: several base changes in the terminal part of helix I, two changes at the end of helix II, one change in the region between helices II and III, one change at the end of helix III and one change in the most variable helix IV (Fig. 1). However, no CBC (double sided base change of a nucleotide pair in helix retaining secondary structure) was detected in helix III, the most conserved helix [34,41]. Therefore, we infer that based on the secondary structure of ITS2 rDNA, the strains CCALA 1082 and CCALA 1084 represent one biological species. Moreover, they were isolated from nearby sites with presumably very similar ecological conditions [27]. Complete ITS2 rDNA sequences of the other members of the genus *Parietochloris* are not freely available. The closest strains belonged to other genera from the class Trebouxiophyceae but the sequence similarity was very low (below 70%).

3.2. Identification and composition of fatty acids

The first problem to be clarified was the presence of several isomers of hexadecenoic acid. To finally decide which positional isomer it is, we performed derivatization of a mixture of total fatty acids. The resulting dimethyl disulfide adducts and 3-pyridyl carbonyl (previously called picolinyl) esters were analyzed by GC–MS. Fig. 2 shows the mass spectrum of dimethyl disulfide adduct of hexadecenoic acid. From this spectrum, and mainly on the basis of abundant ions having the values of 245.2, 213.2 and 117.1 Da (see also the inserted figure) it is obvious that this is a derivatized *cis*-11-hexadecenoic acid. To completely eliminate the possibility of misinterpretation, we performed a GC–MS of other derivatives, i.e., 3-pyridyl carbonyl esters, see Fig. 3. This mass spectrum again shows that this is 11–16:1 or 16:1ω5 acid. The spectrum (see also intermediate structure in Fig. 3) contained diagnostic ions (at *m/z* 262.2 and at *m/z* 288.2, and at *m/z* 262.2 or at *m/z* 302.2, respectively) forming gaps of 26 or 40 Da. Therefore, we assume that the given acid is *cis*-11-hexadecenoic acid or 16:1ω5.

In *Lobosphaera* (formerly *Parietochloris*) *incisa*, Cohen's group [28,42,43] identified 16:1ω7 and 16:1ω11 acids but the position of the double bond was obtained only by GC–MS of methyl esters of FAs. The 16:1ω7 acid or 9–16:1 (palmitoleic acid) is known and widespread in many algae [11]. The other isomer, i.e. the positional isomer 16:1ω11 (5–16:1) is much more rare and is present in seed oils of *Carlina corymbosa* or *C. acaulis* from the Compositae family, which contain *cis*-5-hexadecenoic acid, with trace amounts of 7–16:1 and 9–16:1. The position of the double bond was determined by reductive ozonolysis [44] or by means of dimethyl disulfide (DMDS) derivatives in the seed oil of meadowfoam (*Limnanthes alba*) and other *Limnanthes* species, where Δ5-FAs including 5–16:1 are present [45].

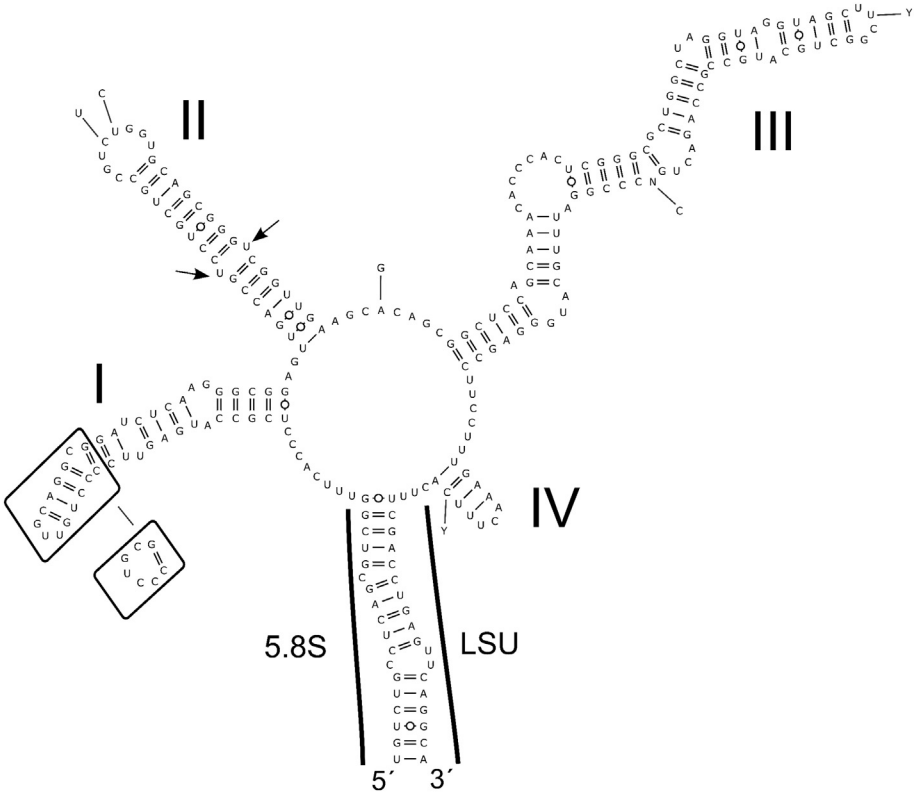

Fig. 1. Comparison of secondary structure of ITS2 rDNA transcript between the strains CCALA 1084 (accession number: KY311561) and CCALA 1082 (accession number KY311560). Note the U-U mismatch in helix II (arrowheads). Differences characteristic for the strain CCALA 1082 are shown by nucleotides outside the secondary structure.

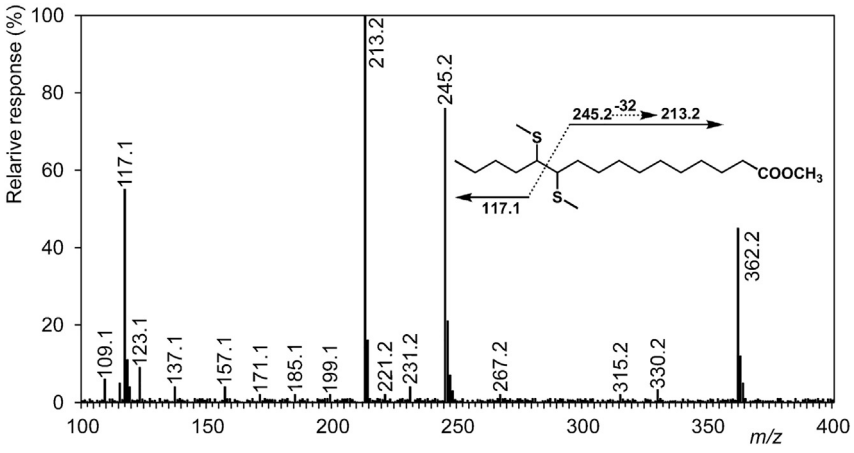

Fig. 2. Mass spectrum of dimethyl disulfide adduct.

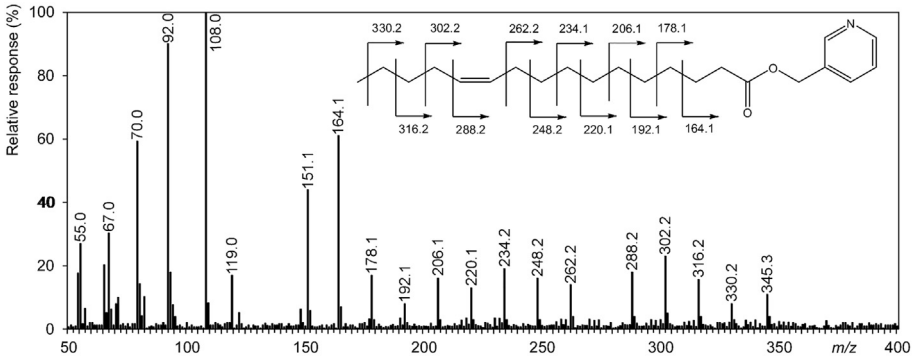

Fig. 3. Mass spectrum of 3-pyridyl carbonyl esters.

Another positional isomer, i.e., 11–16:1 (16:1ω5), was repeatedly shown to be present in other algae, e.g., in *Pterocladia capillacea* [46], *Scenedesmus acuminatus*, *Nannochloropsis* sp., *Anabaena* sp., *Chlorella* sp. and *Oscillatoria* sp. [47], including identification of a fatty acid-Δ11 desaturase from the microalga *Thalassiosira pseudonana* [48], where the position of the double bond was obtained using the DMDS adduct of FAME. The 16:1ω5 isomer was also found in the cyanobacterium *Nostoc* sp. [49]. Based on both our analyses, we can say with certainty that the present hexadecenoic acid is *cis*-11-hexadecenoic acid or 16:1ω5.

At first sight, analysis of total FAMEs, see Table 1, does not show any substantial differences in the content of total fatty acids of the two strains, which is understandable given the results of genetic analysis, see above. Even so we attempted to perform the statistical processing of the data from Table 1. We tested whether the selections have the same mean values against sided alternative hypothesis. We used *t*-test for samples containing arachidonic acid with different variances. The resulting p-value is 0.008, which means that the null hypothesis of conformity of mean values is rejected at 1% significance level and we conclude that these selections have different mean values.

The data in Table 1 are the means of three cultivations including standard deviations (± S.D.). Table 2S (Supplements) then gives the results for particular cultivations, again with ± S.D. These data stand for differences between individual analyses carried out by GC–MS with this particular cultivation. On comparing the results of Table 1 and Table of 2S it is clear that the standard deviations for individual cultivations are more than an order of magnitude higher than those for individual analyses. This was to be expected since the biological variability (the difference in the composition of the biomass) is always greater than the difference between the individual analyses performed on today's advanced devices. Therefore in further text we always show ± S.D. between cultivations and not between analyses.

As mentioned above, the content of FAs has been repeatedly described in *Parietochloris* species, which were moved to the genus *Lobosphaera* [25]. According to [11], the content of arachidonic acid in *L. incisa* and *L. interrupta* is 13.2% or 1.5% of total FAs. Bigogno et al. [28,42,50] and [43] give the contents of arachidonic acid in *L. incisa* in the interval from 22.5 to 46.2% total FAs, which establishes this organism as a prominent producer of arachidonic acid. Based on the extensive database of FA profiles published by Lang et al. [11] only some red algae and a limited number of other green algal genera reach higher values. Therefore, we focused on the two less studied *Parietochloris* strains, which contain substantial amount of arachidonic acid and could be suitable candidates for its production. In our three parallel cultivations of the strains CCALA 1082 and CCALA1084 (see Table 1 and Table 2S) the amounts of arachidonic acid ranged from 29.5 to 34.2% of total FAs, which corresponds well with the results obtained by Cohen's group. Other major identified acids included palmitic, oleic, linoleic and linolenic acids, minor acids comprising, e.g., 16:2, 16:3, 18:3, 20:3 and 20:5. In conclusion we can say that the strains from the clades *Lobosphaera* and *Parietochloris* (sensu stricto) have a very similar representation of FAs regardless of where they come from.

3.3. Lipidomic analysis by shotgun high resolution mass spectrometry

We used shotgun lipidomic analysis of total lipids to explain the minor differences in the content of arachidonic acid based on the *t*-test. To our surprise, however, some molecular species, preferably those containing arachidonic acid, significantly differed in abundance.

Lipid extracts of both strains (CCALA 1082 and CCALA 1084) obtained by the methodology of [36] were analyzed by the shotgun method. The mass spectra are shown in Fig. 4. We have identified over 430 molecular species using LIPID MAPS [51,52] while, Lee et al. [53] have identified only 48 molecular species of lipids. If the appropriate molecular species were not found in Lipidmass, we performed manual identification using ChemDraw (ChemDrawUltra version 10, Cambridge Soft) based on analogies. For instance, the molecular species 16:1–20:5/PE, but not 16:2–20:4/PE (isobaric molecule), can be found in Lipidmass. Based on the similarity of 16:1–20:5/PE and 16:1–20:4/

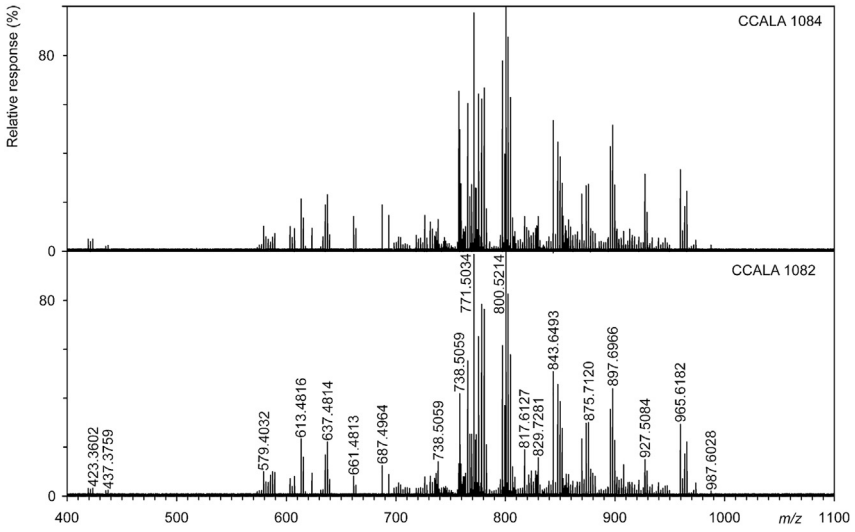

Fig. 4. Shotgun lipidomic analysis of two strains (CCALA 1084 - top and CCALA 1082 - bottom) of *Parietochloris* sp.

PE one can predict the tandem mass spectrum of 16:2–20:4/PE. The number of such compounds did not reach 10% of total molecular species, since Lipidmass essentially lacks only 16:2 and 16:3 acids and their corresponding molecular species of lipids. Lu et al. [54] predicted that the number of possible molecular species can amount to thousands, but they identified only a few dozen individual compounds.

The use of HRMS offers considerable advantages mainly in the range of higher *m/z* values. An example concerns the four molecular species for ions of type $[M + Na]^+$ at *m/z* 915 (low resolution), i.e., ions at *m/z* 915.5251 (chlorophyll *a* with molecular formula $C_{55}H_{72}MgN_4NaO_5$), at *m/z* 915.5263 (SQDG 40:7 with molecular formula $C_{49}H_{80}O_{12}SNa$), at *m/z* 915.6015 (DGDG 32:0 with molecular formula $C_{47}H_{88}O_{15}Na$), and at *m/z* 915.6473 (TAG 56:13 with molecular formula $C_{59}H_{88}O_6Na$). Unfortunately, identification of regioisomers, enantiomers or positional isomers (e.g., lipids with oleic or vaccenic acids, etc.) cannot be performed and should be done only after their separation by chromatographic methods. Analysis using shotgun technique is very fast; dozens of samples can be analyzed within 1 h and it is, thus, very easy to sort samples for further analysis.

Because our interest was to identify those molecular species which have in the molecule at least one arachidonic acid, we used two other methods, neutral loss scanning (NLS) for triacylglycerols and precursor ion scanning (PIS) for polar lipids, see Fig. 5. The largest differences were observed in those molecular species that had 4 or more double bonds in the molecule. As an example, we have chosen 36:4 and 36:5 molecular species of phospholipids. The measured values (see Fig. 6) show that the two strains differ in the representation of molecular species having the same molecular weight, i.e., compounds that have the same number of carbon atoms and the same number of double bonds and differ only in the length and unsaturation of individual acyl chains, but the differences are not significant. This is caused by the fact that 36:4-phosphatidylethanolamine (PE) contains two highly different PE, i.e.16:0/20:4-PE and 18:2/18:2-PE. When the peak of the appropriate molecular weight is made up mostly or exclusively of a single compound, as is the case for 40:8-PE, phosphatidylcholine (PC) and phosphatidylinositol (PI) (the peak of this molecular weight comprises substantially only chains with arachidonic acid, the contribution of eicosatrienoic and eicosapentaenoic acids being negligible, see below), then the differences are evident at a glance. And so it was possible to determine from Fig. 6 that the largest differences were observed in lipids occupying different spatial arrangement, i.e. cylindrical lipids, with PC as the main representative, conical lipids (e.g. PE), and inverted conical (cone shaped) lipids with majority representation of PI.

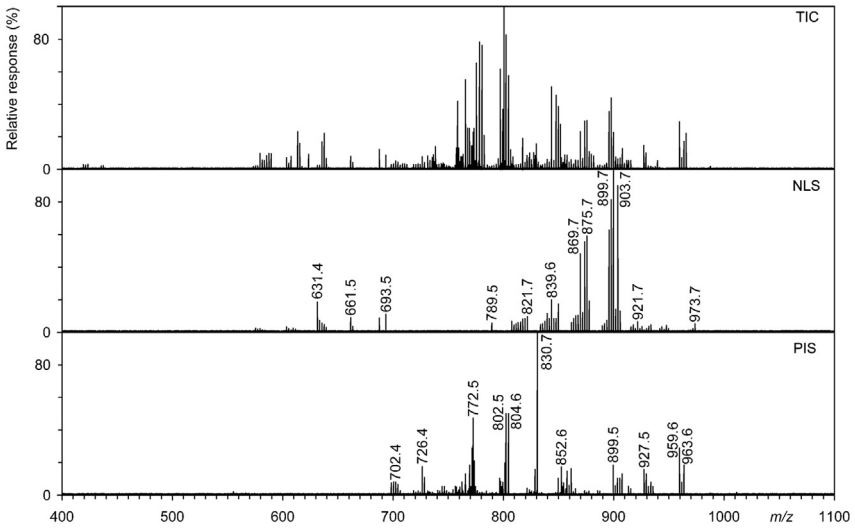

Fig. 5. Identification of molecular species having in the molecule at least one arachidonic acid by neutral loss scanning (NLS) and precursor ion scanning (PIS).

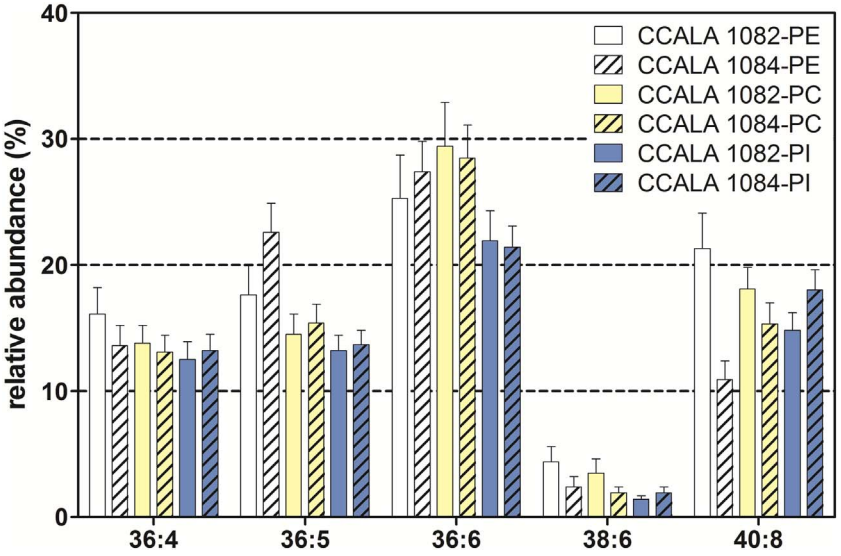

Fig. 6. The difference in relative abundance of phospholipids (phosphatidylethanolamine (PE) phosphatidylcholine (PC) and phosphatidylinositol (PI)) between both *Parietochloris* strains (CCALA 1082 and CCALA 1084).

3.4. Separation of polar lipids

Because the results of shotgun analysis are not immediately apparent, we used for further analysis hydrophilic interaction liquid chromatography (HILIC-LC/MS). Fig. 7 shows a chromatogram and data of Table 2 of commercially obtained standards and of the two strains, i.e. CCALA 1082 and CCALA 1084.

The values (mean and S.D.) again pertain only to the differences between the three cultivations. The differences between different analyses performed on a single cultivation, e.g., for the quantification of PC from the cultivation of CCALA 1082, reached 5.958 ± 0.012 (S.D.), this S.D. value being again more than an order of magnitude lower than that between the cultivations (± S.D. = 0.2).

Lipids were identified both on the basis of retention times with commercially obtained standards and their mass spectra, especially those of PE, PC and PI. PIS and NLS in positive ion mode allowed the unambiguous identification of individual phospholipid classes, i.e. a mass fragment 184 Da for PC and neutral loss of 141 and 277 Da fragments for PE and PI, respectively. Accurate determination of the differences between the two strains was further performed by LC-tandem MS (MS^2) analysis. Tables 3a,b show data from LC-MS analysis of PE, PC, and PI from both *Parietochloris* strains. Fig. 8 shows high resolution tandem mass spectra of $[M - H]^-$ ions at *m/z* 734.4766 (molecular species 16:1–20:5/16:2–20:4/16:3–20:3/18:3–18:3) and at *m/z* 786.5079 (molecular species 20:4–20:4/20:3–20:5) from strains CCALA 1082 and CCALA 1084. For MS/MS of compounds providing isobaric ions, e.g. ion at *m/z* 734.4766 which contained the following four molecular species, 16:1–20:5/PE, 16:2–20:4/PE, 16:3–20:3/PE, and 18:3–18:3/PE, the identification was based on two types of ions selected based on their abundance, i.e., loss of acyl chain as ketene (R′CH═C═O) from $[M - H]^-$ and $RCOO^-$ ion. The values for these ions are listed in Table 6S. Other isobaric molecular species listed in Tables 3a and 3b were similarly identified. Regiosiomers, for example, *sn*-20:3/20:5-PE vs. *sn*-20:5/20:3-PE were not separated and therefore not identified by MS.

In *L. incisa*, Bigogno et al. [40,48] give the respective PC content of 6.8 and 7.6% of total lipids. We found that the content of PC is 5.0 or 4.7%, which is in good agreement. On the other hand, our data on the proportions of other classes of lipids differ from the literature since our strains contain about 10-times less TAGs than the above-mentioned strain. While Cohen's group identified in *L. incisa* more than 10 lipid classes, we found 25 classes, 6 of which we could not identify (they are marked U_1–U_6). We believe that the difference in the number of separate classes of lipids is mainly due to the equipment used, i.e., TLC in

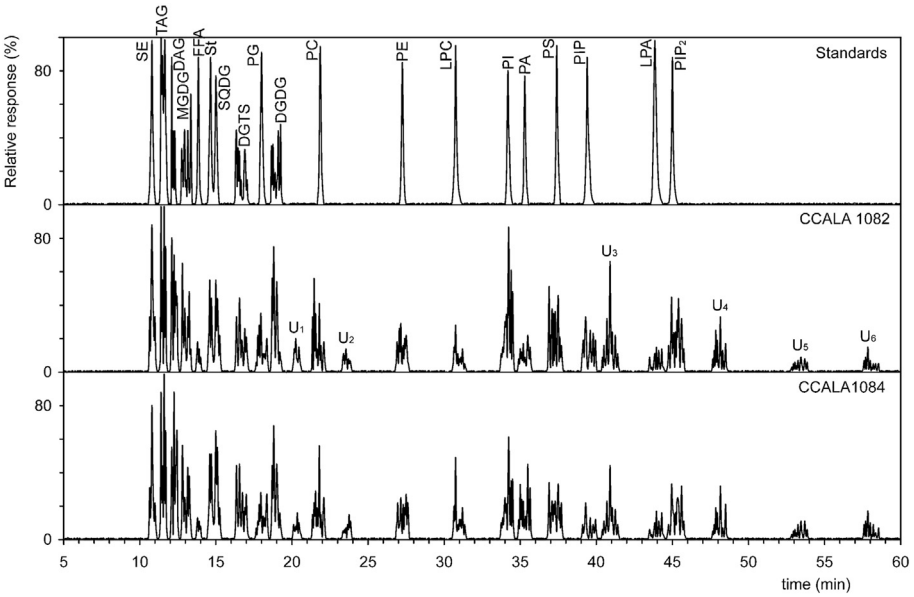

Fig. 7. HPLC/ESI-MS separation of standards (top) and major lipid classes of two strains (CCALA 1084 (middle) and CCALA 1082 (bottom)). For abbreviations, see Table 2.

Table 2
Major lipid classes of strains CCALA 1082 and CCALA 1084 (arithmetic means from three cultivations ± standard deviations (S. D.)).

Lipid classes	Abbreviations	CCALA 1082	± S.D.	CCALA 1084	± S.D.
Sterol esters	SE	3.1	0.2	3.5	0.2
Triacylglycerols	TAG	6.7	0.3	6.5	0.3
Diacylglycerols	DAG	6.8	0.3	6.5	0.3
Monogalactosyldiacylglycerols	MGDG	5.4	0.2	5.2	0.2
Free fatty acids	FFA	1.0	0.1	1.1	0.1
Sterols	St	3.3	0.2	3.0	0.2
Sulfoquinovosyl diacylglycerols	SQDG	4.1	0.3	4.0	0.3
Diacylglycerylhydroxymethyltrimethylalanine/diacylglyceryltrimethylhomoserine	DGTA/DGTS	4.4	0.3	4.7	0.3
Phosphatidylglycerol	PG	3.7	0.2	3.6	0.2
Digalactosyldiacylglycerols	DGDG	6.1	0.3	6.2	0.3
Unknown 1	U_1	1.4	0.1	1.8	0.1
Phosphatidylcholines	PC	6.0	0.2	4.7	0.2
Unknown 2	U_2	1.4	0.1	1.4	0.1
Phosphatidylethanolamines	PE	4.9	0.2	3.8	0.2
Lysophosphatidylcholines	LPC	2.6	0.1	2.5	0.2
Phosphatidylinositols	PI	6.0	0.2	7.7	0.3
Phosphatidic acids	PA	4.6	0.2	3.9	0.2
Phosphatidylserines	PS	5.5	0.2	6.4	0.2
Phosphatidylinositol phosphates	PIP	3.4	0.1	4.4	0.2
Unknown 3	U_3	3.3	0.1	2.6	0.1
Lysophosphatidic acids	LPA	2.0	0.1	1.9	0.1
Phosphatidylinositol diphosphates	PIP2	5.4	0.2	5.3	0.2
Unknown 4	U_4	1.5	0.1	1.2	0.1
Unknown 5	U_5	1.9	0.1	1.5	0.1
Unknown 6	U_6	1.7	0.1	2.1	0.1

Cohen's data and HILIC-LC/ESI-MS^2 in our analyses. Nevertheless, we can note some patterns; for example, as is clear from both the literature data and from our results, PC content was always higher than that of PE, which was lower than that of PI, Table 2.

3.5. Structure of polar lipids

Negative-ion ESI tandem MS was used to characterize the structure of each class of phospholipids and to identify the molecular species including regioisomers.

The major phospholipid ions in the negative ESI MS^2 include mainly the deprotonated molecular ion $[M - H]^-$ and three other types of ions, i.e., fragment ions arising from loss of fatty acyl group as ketene $[M\text{-}H\text{-}R'CH{=}C{=}O]^-$, loss of free FA ($[M\text{-}H\text{-}RCOOH]^-$) and FA carboxylate ion ($[RCOO]^-$). If we want to identify the regioisomers of FA bound to the *sn*-1 or *sn*-2 position, then we compare the abundance of ions of the type $[M\text{-}H\text{-}R'CH{=}C{=}O]^-$ or $[M\text{-}H\text{-}RCOOH]^-$.

Tables 3S, 4S and 5S (Supplements) summarize the *m/z* values, the abundance of ions and description of their structures for molecular species of 20:3/20:5 of three phospholipids, i.e., PE, PC and PI. Other molecular species given in Tables 3a and 3b were similarly identified.

When determining the molecular species of phospholipids, the differences between individual cultivations were again more than 10-fold higher than those between analyses. Thus the S.D. for the difference

Table 3a
Abundances of molecular species of phosphatidylethanolamine (PE) phosphatidylcholine (PC) and phosphatidylinositol (PI) from both *Parietochloris* strains (CCALA 1082 and CCALA 1084) determined by mass spectrometry (arithmetic means from three cultivations ± standard deviations (S. D.)).

Molecular species[a]	CCALA 1082-PE	± S.D.	CCALA 1084-PE	± S.D.	CCALA 1082-PC	± S.D.	CCALA 1084-PC	± S.D.	CCALA 1082-PI	± S.D.	CCALA 1084-PI	± S.D.
Pl-Pl/He-Pn	0.7	0.2	0.7	0.3	2.0	0.5	1.5	0.4	6.4	0.7	3.1	0.7
Pl-Pn	0.9	0.3	1.5	0.6	3.5	0.7	3.7	0.7	4.3	0.3	4.2	0.4
Pn-Pn	0.3	0.2	0.5	0.2	4.1	0.9	4.4	0.8	9.2	0.9	4.9	0.5
Ln-He/L-Pl/O-Pn/M-A/Mo-Ad	0.5	0.2	0.7	0.4	0.6	0.3	0.9	0.3	2.5	0.3	3.1	0.6
Ln-Pl/L-Pn/Mo-A	0.6	0.3	1.5	0.7	0.7	0.3	1.2	0.4	3.2	0.4	4.3	0.5
Ln-Pn	6.4	0.8	12.9	1.2	0.3	0.2	0.4	0.2	3.5	0.3	5.1	0.7
P-A/Po-At/Pl-Ad/O-Ln/L-L	16.1	1.6	13.6	1.1	13.8	1.7	13.1	1.4	12.5	1.2	13.2	1.3
Po-A/Pl-At/Pn-Ad/P-E/L-Ln	17.6	1.5	22.6	1.9	14.5	1.4	15.4	1.8	13.2	1.4	13.7	1.4
Pl-A/Pn-At/Po-E/Ln-Ln	25.3	2.2	27.4	2.0	29.4	1.9	28.5	1.9	21.9	1.7	21.4	1.9
Pn-A/Pl-E	1.0	0.4	1.5	0.3	1.9	0.4	3.1	0.4	2.1	0.5	1.6	1.2
Pn-E	0.5	0.2	0.5	0.3	3.4	0.7	6.0	0.5	0.8	0.4	0.7	0.3
S-A/O-At/L-Ad	0.1	0.1	0.2	0.2	0.4	0.2	0.4	0.2	0.3	0.2	0.3	0.2
S-E/O-A/L-At/Ln-Ad	0.2	0.1	0.3	0.1	0.3	0.2	0.6	0.2	0.6	0.3	0.5	0.2
O-E/L-A/Ln-At/	4.4	0.6	2.4	0.5	3.5	0.5	1.9	0.3	1.4	0.4	1.9	0.4
L-E/Ln-A	0.3	0.2	0.1	0.1	0.9	0.3	0.5	0.2	1.0	0.3	1.1	0.3
Ln-E	0.3	0.1	0.5	0.2	0.7	0.2	1.1	0.5	0.6	0.2	0.4	0.2
Ad-A	1.1	0.5	0.9	0.1	0.2	0.1	0.6	0.2	0.2	0.1	0.3	0.1
At-A/Ad-E	2.1	0.9	1.2	0.4	0.1	0.1	0.4	0.2	0.4	0.2	0.8	0.4
A-A/At-E	21.3	1.8	10.9	0.8	18.1	1.3	15.3	1.1	14.8	0.9	18.0	1.3
A-E	0.3	0.2	0.1	0.1	1.6	0.8	1.0	0.3	1.1	0.3	1.4	0.4

[a] Position of acyl at the *sn*-1 and/or *sn*-2 positions has not been determined.

Table 3b
Abundances of molecular species of phosphatidylethanolamine (PE) phosphatidylcholine (PC) and phosphatidylinositol (PI) from both *Parietochloris* strains (CCALA 1082 and CCALA 1084) determined by tandem MS (arithmetic means from three cultivations ± standard deviations (S. D.)).

Molecular species[a]	CCALA 1082-PE	± S.D.	CCALA 1084-PE	± S.D.	CCALA 1082-PC	± S.D.	CCALA 1084-PC	± S.D.	CCALA 1082-PI	± S.D.	CCALA 1084-PI	± S.D.
P-A	8.5	1.2	4.2	0.6	6.1	1.3	6.8	1.3	4.9	0.8	5.4	0.8
L-A	4.1	0.8	2.0	0.5	3.0	0.9	1.4	0.5	1.2	0.4	1.5	0.4
A-A	19.1	1.9	8.6	0.9	16.8	1.6	12.5	1.4	11.4	1.6	15.6	1.7
L-L	6.8	0.9	8.1	1.2	6.4	1.0	5.9	0.9	7.0	1.1	6.1	1.3
L-Ln	14.9	1.7	20.1	1.8	11.8	1.8	12.6	1.6	11.3	1.3	10.6	1.9
Ln-Ln	22.4	1.8	24.8	1.9	27.1	1.9	26.9	1.0	19.7	1.8	18.6	2.1

[a] Position of acyl at the *sn*-1 and/or *sn*-2 positions has not been determined.

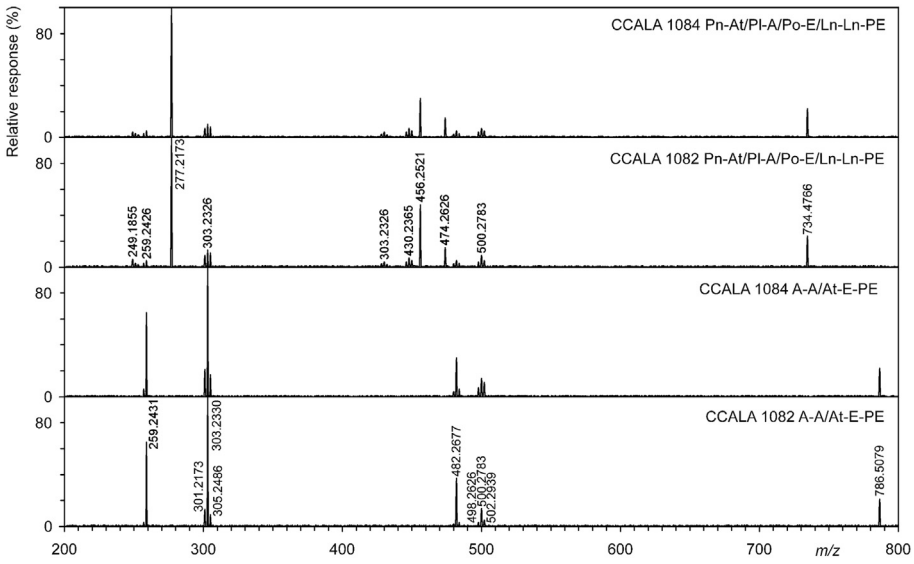

Fig. 8. High resolution tandem mass spectra of $[M - H]^-$ ions at *m/z* 734.4766 (molecular species of phosphatidylethanolamine Pl-A/Pn-At/Po-E/Ln-Ln) and at *m/z* 786.5079 (molecular species of phosphatidylethanolamine A-A/At-E) from strains CCALA 1084 and CCALA 1082.

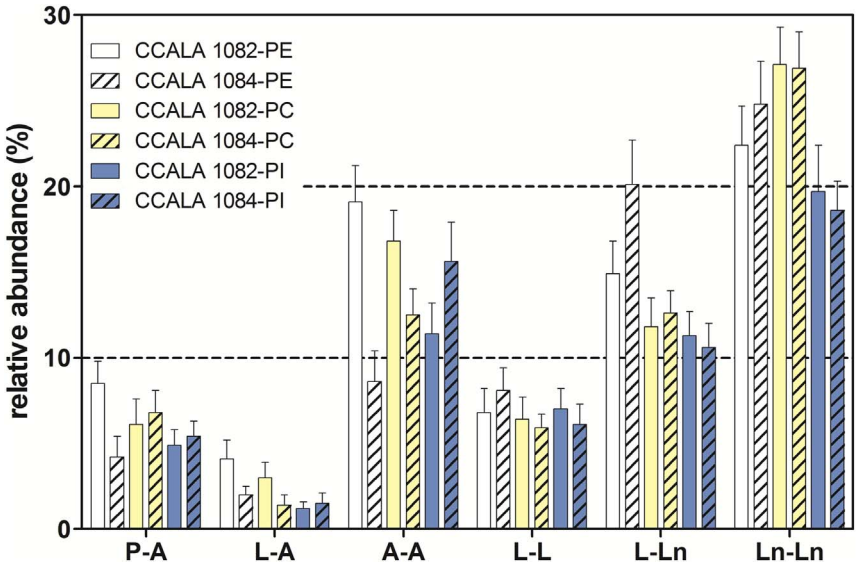

Fig. 9. The difference in relative abundance of molecular species of phospholipids (phosphatidylethanolamine (PE) phosphatidylcholine (PC) and phosphatidylinositol (PI)) between the two *Parietochloris* strains (CCALA 1082 and CCALA 1084).

between individual analyses for the molecular species 20:4/20:4 PE was 0.142, whereas the S.D. between individual cultivations of strain CCALA 1082 for the same molecular species was 1.9.

A total of 25 classes of lipids were separated, 19 of them being fully characterized. Unfortunately, unknown lipids designated U_1–U_6 defied identification. The biggest differences in the representation of lipid classes in the two strains, see Table 2, were found in PE, PC and PI.

The strains from the CCALA collection differ only in the sampling site (see above) and the analysis of secondary structure of ITS2 rDNA showed that they belong to the same (as yet undescribed) species. We therefore used lipidomic analysis for their resolution and, based on this, we have proved that the strains CCALA 1082 and CCALA 1084 differ from each other. The first signs of differences, although hardly noticeable at first glance, were the contents of total FAs. However, only shotgun lipidomic indicated that the two strains differ in the content of those lipids that consist primarily of arachidonic acid. Incidentally, this

FA is the most represented fatty acid of all the acids present (see also [28]). Further analysis by NLS and PIS made it possible to identify three phospholipids, i.e., PE, PC and PI, the contents of which varied the most. Full confirmation that these two strains differ precisely in these phospholipids occupying a different spatial arrangement - cylindrical (PC), conical (PE), and inverted conical (cone shaped) lipids (PI) was achieved by LC-MS/MS. Based on this analysis, it was confirmed that the largest differences concern molecular species having in the molecule two PUFAs, e.g., 18:3/18:3 or 20:4/20:4 (Fig. 9).

The content of molecular species 20:4/20:4 in strain CCALA 1082 decreases with increasing volume of the polar head, while the opposite is true in strain CCALA 1084. Quite the opposite trend was observed with the molecular species 18:3/18:3, where the lipid content in strain CCALA 1084 increases with the growing volume of the polar head while in strain CCALA 1082 it drops. We do not yet have a comprehensive hypothesis for this dependence, determined as a mean of three independent and parallel cultivations carried out under the same conditions, three measurements being performed for each culture. Nevertheless, we believe that it could be the influence of ecological parameters at the site of collection that is encoded at a site in the genome, which has not been analyzed.

4. Conclusion

Lipidomic analysis allowed us to distinguish between two closely related strains of the same species. This offers a possibility of future use of this method for fine-grained taxonomy in cases (as with our two strains) where routine DNA analysis shows that the strains belong to one species. Lipidomic analysis combined with proteomic, metagenomic and traditional morphological analysis can, thus, bring new insights into the diversity of microorganisms and their metabolic pathways.

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Appendix A. Supplementary data

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