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Lipidomic analysis of the extremophilic red alga *Galdieria sulphuraria* in response to changes in pH

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ABSTRACT

Analysis of polar lipids from the red micro-alga *Galdieria sulphuraria* by means of the HILIC-LC/high resolution ESI-tandem MS determined 14 classes of lipids including identification of tens of molecular species of lipids and their regioisomers. The extremophilic alga *G. sulphuraria* uses several mechanisms for adaptation to low pH and high temperature, the major change being a replacement of cellular phospholipids by betaine lipids. We also found a change in the ratio of regioisomers of the polar lipids, i.e. phospho-, glyco- and betaine lipids, in dependence on the culture medium pH. We hypothesize that biosynthesis of polar lipids via the prokaryotic and/or eukaryotic pathway is dependent on ambient pH.

1. Introduction

Organisms inhabiting extreme biotopes are mostly prokaryotic microorganisms, but also some eukaryotes. These include e.g. the eukaryotic unicellular red micro-alga *Galdieria sulphuraria*, which may even be dominant under certain conditions. This extremophile alga, living in hot (up to 56 °C) but also in cold sulfur springs, i.e. environments having a pH in the range 0–4, is able to grow photoautotrophically, photoheterotrophically, or chemoheterotrophically. This species is colored yellow-green to dark blue-green when grown heterotrophically and yellow or green during autotrophic growth. Cell size is from about 3 to 9 μm, and it has a highly proteinaceous cell wall, a single chloroplast and Floridean starch as a storage substance. Its taxonomic position has been revised by Ciniglia and colleagues [1]. *G. sulphuraria* is a highly interesting model organism for the study of adaptation to extreme environments.

Lipids and fatty acids (FA) in *G. sulphuraria* have so far received little attention. Although hundreds of articles have been published about this alga, the number of publications on fatty acids is negligible. Lang and colleagues [2] analyzed two strains of *G. sulphuraria* (SAG collection, i.e. culture collection of algae of the University of Göttingen) and found just a narrow spectrum of fatty acid methyl esters (FAME), i.e. palmitic (P), oleic (O), linoleic (L), and linolenic (Ln) acids. Depending on the type of cultivation (heterotrophic or autotrophic), Graziani and colleagues [3] identified in addition to the already mentioned FA a small amount (in units of percent of total FA) of myristic (M), palmitoleic (Po), and stearic (S) acids. Finally, Gross and colleagues [4] identified in both thermophilic and mesophilic strains also virtually the same FAs, i.e. P, S, O, L and Ln.

However, taking into account the changes in the taxonomy (cf. [5,6,7,8]), different authors identified in *G. sulphuraria*, (originally *Cyanidium caldarium* M-8 and/or *C. caldarium* [4] Allen), apart from common FA such as P, O, L and Ln, also minor odd-numbered (tridecanoic and pentadecanoic), and even-numbered minor FA (M and Po), but also polar lipids, e.g. monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfolipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) [8].

According to our current knowledge, analysis of both simple (triacylglycerols — TAG) and complex glycerolipids (phospho- and glycolipids) has not yet been published.

We analyzed the lipids of this alga cultivated at different pH (from 1 to 4). Using LC-MS analysis we identified different classes of lipids, i.e. phospho- and glycolipids, including betaine lipids, and hundreds of different molecular species, and performed tandem MS analysis of regioisomers of many of these lipids.

2. Material and methods

2.1. Chemical and standards

Acetonitrile, 2-propanol, hexane and dichloromethane were purchased from Sigma-Aldrich (Prague, CR). 1,2-Dipalmitoyl-

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diacylglyceryltrimethylhomoserine (DGTS) and 1,2-dioleoyl PI (ammonium salt) was purchased from Avanti Polar Lipids, Inc., Alabaster, AL, USA, and DGDG, MGDG, and sulfoquinovosyldiacylglycerol (SQDG) (plant leaf lipids), 1,2-dioleoyl- phosphatidic acid (PA), 1,2-dioleoyl-PC, 1,2-dioleoyl-PE, 1,2-dioleoyl-phosphatidylglycerol (PG) (Na-salt), and 1,2-dimyristoyl-phosphatidylserine (PS) (Na-salt) from Larodan (Malmö, Sweden). Because there is no commercially available diacylglyceryl hydroxymethyltrimethyl-β-alanine (DGTA), the concentration of this lipid was estimated by comparing the peak area (total ion current in the full scan data in tandem positive ESI-MS) with those of DGTS, which has a similar structure.

2.2. Cultivation

The unicellular rhodophyte *G. sulphuraria* 002 (Galdieri) Merola was obtained from Algal Collection of Dipartimento delle Scienze Biologiche, Section of Plant Biology, University "Federico II" of Naples, Italy (http://www.acuf.net/index.php?lang=en). The inoculum of *G. sulphuraria* for this experiment originated from a cultivation at pH 2.5, in standard medium and constant light regime. Laboratory cultivation units consisted of glass cultivation photobioreactors placed in a thermostatic bath and continuously illuminated by white fluorescent light tubes (Osram L 36W/830 Lumilux, Germany). The incident light intensity was 150 m^2s^{-1} of photosynthetically active radiation (PAR). The light intensity was measured using a PU 550 digital lux-meter (Metra, Czech Republic) equipped with a PAR sensor calibrated according to the LI-185 quantum sensor (LI-COR, USA). Cultures were grown in a modified Galdieria-nutrient medium [9] and aerated by a mixture of air and CO_2 (2%, v/v). Temperature was 39 °C ± 0.5 °C. The volume of the algal suspension in each cultivation photobioreactor was 150 mL. Four different pH values were used: pH 1, 2, 3 and 4 [10]. The cultures were cultivated for 10 days and were controlled by measuring the optical density. The biomass was harvested by centrifugation and lyophilized.

2.3. Isolation of lipids

Isolation and pretreatment of lipids were carried out according to Bligh and Dyer [11], with modifications as described previously [12]. For full description of the experiments see the Supplements.

2.4. FAMEs analysis

Analysis of fatty acids in the form of methyl esters (FAME) was described previously [13,14]. A complete description of all conditions of FAME analysis by GC-MS is in the Supplements.

2.5. 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.

2.6. Statistics

All experiments were repeated at least thrice. Two-way ANOVA analyses were performed to determine significant differences ($p < 0.05$) between cultivation at different pH. In the case of significant effects, the Student–Newman–Keuls post-hoc test was applied. Three replicates ($n = 3$) of each cultivation (pH 1, 2, 3, and 4) were used for each sampling time. The software Statistica for Windows (version 12.0, Dell) was used for analyses. Data were presented as means ± SD.

3. Results and discussion

3.1. Identification and production of fatty acids

Table 1 gives the contents of total fatty acids and FA in triacylglycerols and polar lipids in the alga *G. sulphuraria* cultured autotrophically at different pH. It shows that the content of individual acids, as dependent on the pH of the culture, is not much different. Guschina and Harwood [15] assumed that algae respond to a decrease in cultivation pH by increasing the production of saturated FAs. *Euglena gracilis* [11] cultured at pH 2 and pH 5 showed basically no difference between the proportions of FA (content of saturated FA at pH 2 was 27.6%, whereas at pH 5 it was 27.9%) [16] while for *Chlamydomonas* sp. the total content of saturated FA at pH 1 was 37.2%, while at pH 6 it was 43.0% [17], i.e. it decreased with decreasing pH at variance with the theory of Guschina and Harwood [15]. Still, the saturated/unsaturated FA ratio in another *Chlamydomonas* sp. cultured at pH 2.7 and pH 7.0 was 1.38:1.00 [18], i.e. the content of saturated FA complied with the theory. The results are thus largely contradictory and satisfactory explanation for this phenomenon has not yet been proposed.

As stated above (Table 1), the theory of Guschina and Harwood [15] on the influence of pH on the unsaturation of FA appears to be only partially tenable. We therefore analyzed the lipids using two approaches, i.e. analysis of polar lipids using LC-MS (see below) and also analysis of molecular species of phospho- and glycolipids, including betaine lipids and their regioisomers, see also below.

3.2. Separation of polar lipids

The commonly used methods of analysis of lipids include TLC and HPLC and the preferred connection of HPLC with mass spectrometry (LC-MS). Separation can be carried out on both normal and reverse phases. Normal phase silica methods exploit amine or diol stationary phase, and lipids are essentially separated according to the polarity of the head groups. Reverse phase separates lipids according to the non-

Table 1
Fatty acid composition in *Galdieria sulphuraria* cultivated at different pH.

FA	pH 1	pH 2	pH 3	pH 4
Total lipids				
M	1.8 ± 0.2[a]	2.0 ± 0.3	2.1 ± 0.2	1.9 ± 0.2
P	23.1 ± 1.3	26.3 ± 1.5	27.2 ± 1.5	27.4 ± 1.4
Po	3.8 ± 0.4	3.6 ± 0.4	3.1 ± 0.3	3.7 ± 0.5
S	4.2 ± 0.4	4.4 ± 0.5	4.1 ± 0.4	4.6 ± 0.3
O	17.3 ± 0.8	16.8 ± 1.2	17.5 ± 1.5	17.1 ± 1.2
L	36.2 ± 1.2	34.8 ± 1.8	35.1 ± 2.2	33.8 ± 2.3
Ln	9.6 ± 0.7	9.0 ± 0.8	8.9 ± 0.7	9.3 ± 0.9
Unkn + tr[b]	4.0 ± 0.6	3.1 ± 0.3	2.0 ± 0.3	2.2 ± 0.3
Triacylglycerols				
M	3.4 ± 0.3	4.2 ± 0.4	4.4 ± 0.4	4.3 ± 0.4
P	25.2 ± 1.8	25.9 ± 2.3	26.4 ± 1.5	26.0 ± 2.4
Po	4.9 ± 0.4	5.7 ± 0.4	5.4 ± 0.4	5.1 ± 0.5
S	6.3 ± 0.5	7.1 ± 0.6	7.7 ± 0.6	6.9 ± 0.7
O	21.4 ± 1.9	19.8 ± 1.1	19.7 ± 1.2	22.5 ± 1.9
L	29.8 ± 2.1	31.5 ± 1.5	32.2 ± 2.3	30.6 ± 2.5
Ln	5.4 ± 0.5	2.8 ± 0.3	2.2 ± 0.3	1.3 ± 0.2
Unkn + tr	3.6 ± 0.4	3.0 ± 0.3	2.0 ± 0.2	3.3 ± 0.3
Polar lipids				
M	2.1 ± 0.2	2.3 ± 0.3	2.0 ± 0.2	2.4 ± 0.2
P	26.1 ± 2.4	27.6 ± 2.5	30.2 ± 2.6	29.9 ± 2.5
Po	4.2 ± 0.4	4.7 ± 0.4	5.1 ± 0.4	4.8 ± 0.4
S	3.7 ± 0.3	3.9 ± 0.3	4.0 ± 0.4	3.8 ± 0.3
O	19.4 ± 1.4	22.3 ± 2.3	21.4 ± 2.1	21.0 ± 1.3
L	29.1 ± 2.3	23.2 ± 2.1	22.5 ± 2.3	21.8 ± 1.5
Ln	13.6 ± 1.0	14.2 ± 0.8	13.1 ± 1.0	13.7 ± 0.9
Unkn + tr	1.8 ± 0.2	1.8 ± 0.2	1.7 ± 0.2	2.6 ± 0.3

[a] Arithmetic means from three analyses ± standard deviations.
[b] Unknown fatty acids and those present in traces.