Chapter 6

Influence of Culture Conditions on the Fatty Acids Composition of Green and Purple Photosynthetic Sulphur Bacteria

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Additional information is available at the end of the chapter

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1. Introduction

As was published in [1], based on 16S rRNA sequences, prokaryotes are divided into two primary groups: Archaebacteria (Archaea) and Eubacteria (Bacteria). With the exception of Cyanobacteria, all the main lineages of photosynthetic organisms belong to the Eubacterial Phyla and the unrelated halobacterial species.

Within Eubacteria, photosynthetic organisms are found according to the following divisions: (i) gram-positive bacteria belonging to the family Heliobacteriaceae, (ii) green non-sulphur bacteria (also known as filamentous green bacteria) such as *Chloroflexus aurantiacus*, (iii) green sulphur bacteria such as *Chlorobium tepidum*, (iv) cyanobacteria, (v) and several genera of proteobacteria (or purple bacteria) [1].

Anoxygenic green and purple photosynthetic sulphur bacteria (GPSB/PPSB) are anaerobes. They do not release O_2 by photosynthesis, and as electron donors for this process, they use sulphur and its derivatives. These microorganisms have bacteriochlorophyll (Bchl) and accessory carotene pigments that include spirilloxanthin, okenone and chlorobactene pigment series.

Photosynthetic pigments in green photosynthetic sulphur bacteria (GPSB) include Bchl c, d and e, as well as chlorobactene and isorenieratene. PPSB may contain Bchl *a* or *b*. In general, these photosynthetic microorganisms present one kind of Bchl, except in GPSB - which could have small quantities of Bchl *a* - they belong to the Chlorobiaceae family. PPSB comprise the families Chromatiaceae (e.g., *Chromatium or Halochromatium)* and Ectothiorhodospiraceae.

Morphological patterns are limited in bacteria, and phenotypical together with molecular properties have been used for the identification of these microorganisms. Chemotaxonomy takes into account fingerprint methods using proteins, nucleic acids, sugars isoprenoids and fatty acids, among other molecules. For example, an average of fatty acids can be easily obtained by gas chromatography (or by gas-liquid chromatography). This average is called a 'fatty acid profile' and a part of this profile is a pattern [2]. The pattern is generated by cleaning and adapting the fatty acid profile. Both are highly reproducible when growth conditions, the physiological age of the cells and analysis are well standardized. Subsequently, the fatty acid patterns can be used for the identification of bacteria, library generation, taxonomy, epidemiological and ecological studies [2], and obviously it is very useful for detecting bacteria in samples of different origins.

Photosynthetic prokaryotes have a great variety of fatty acids, and they are useful for distinguishing these microorganisms; actually, they are recommended for the description and identification of new species ([3, 4]). For example, *Chloroflexus aurantiacus*, a green photosynthetic non-sulphur bacterium (GPNSB), contains straight-chain, saturated and monounsaturated fatty acids as its main constituents, but its pattern is distinctly different from the pattern of GSPB [5].

Fatty acids from whole cells of *Chlorobium* are within the range of C12-C18, and the main ones are: n-tetradecanoic (C14:0), hexadecenoic (C16:1) and n-hexadecanoic (C16:0) [5-7].

Currently, studies have reported 12 fatty acids in complete cells from GPSB, and the fatty acid C14:0 accounts for 14% [6] to 27.10% [5]; the fatty acid C16:1 accounts for at least 37.3% in these bacteria [5].

Hexadecanoic is the third main fatty acid in GPSB [6] in quantities from 10% in *Cb limicola v. thiosulfatophilum* to 29% in *Cb. phaeovibroides*; finally, the fatty acid C18:1 could be the other main one in GPSB, where it has been detected in quantities between 2.9% and 10.33% in the genus *Chlorobium* [5-7].

Minor fatty acids have been reported in these microorganisms and they include: C12:0, 12-Me-C14:0, 5-Me-C16:0, 14-Me-C16:0, C15:0, 15-Me-C16:1, C17:0, C18:0 and a cyclic fatty acid [5-7]. GPNSB (like *Chloroflexus*) are characterized by the presence of C17 and C18-C20 as the main fatty acids, and these are not detected in GPSB [5].

In oxygenic photosynthetic cyanobacteria - for example, in *Nostoc* and *Anabaena* - it has been reported [8] the presence of 17 major fatty acids (≥0.9%) that included saturated even-carbon, straight-chain C16:0 and C18:0; the first one was higher for *Anabaena* (27.7-34.72%) and lower for *Nostoc* isolates (18.5 to 26.1%). 26 minor components (at least 0.03%) and 10 trace-level components (average ≤ 0.03%) were also recorded.

Fatty acids of the Chromatiaceae family have been more studied than those from GPSB, and analysed strains of this family include members of the genera *Amoebobacter, Halochromatium* (e.g. *Chromatium salexigens*), *Thiocapsa*, *Allochromatium* (*Allochromatium vinosum* = *Chromatium vinosum*) and *Thiocystis.* As gram-negative bacteria, they have as main fatty acids: C18:1ω7>C16:1>C16:0. The proportions of these compounds are variable among

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species of these genera; however, C18:1 may be present with more than 50% (e.g., *Chromatium salexigens* DSM4395). The C16:1 contributes at least with 16 % (e. g., *Chromatium tepidum*, DSM 3771) and 36% as maximum (e. g., *Thiocystis gelatinosa*) [cited in 7], and the C16:0 fatty acid has been reported in quantities of 10.4% in *Chromatium* sp. (strain T7s9) to 25.8% in *Chromatium tepidum* (DSM 3771); it is important to note that in *Chr. tepidum*, the main fatty acids C18:1, C16:0 and C16:1 contribute with 46.38%, 25.86% and 16.17%, respectively, so the pattern for this strain is C18:1>C16:0>C16:1. Other fatty acids detected in minor quantities in members of the Chromatiaceae family include: C12:0, C13:0, C14:0, aC17:0, C18:1w9. C17:0, C18:0 and C20:1 [7-9].

The other family of PPSB, the Ectothiorhodospiraceae, has C18:1 as the main fatty acid, which contributes with 74.7% of the total extracted (96.7%) in *Ectothiorhodospira shaposhnikovii* [cited in 7]. The fatty acid C16:0 is the next in abundance, and C16:1 or C18:0 might be the third. The relative abundance of these major fatty acids as well as the less abundant are very different among the strains of this family (e.g., *Ec. halophila, Ec. halochloris, Ec. abdelmaleki, Ec. shaposhnikovii, Ec. vacuolata, Ec. salini, Ec. magna*, *Halorhodospira halophila* and *Thiorhodospira sibirica*) [cited in 7]. These minor fatty acids are: C12:0 [10] in *Ec. shaposhnikovii*; C19:0d8,9 (a phospholipid detected in *Halorhodospira halophila*) and 19:0Cy (detected in different strains of *Ectothiorhodospira halophila*, *Ec. Mobilis*, *Ec. salini* and *Ec. Abdelmaleki*). Also, C21:0 has been detected in the phospholipids of *Ec. shaposhnikovii* and *Halorhodospira halophila* DSM 244, while the eicosanoic (C20:0) fatty acid was found in *Halorhodospira halophila* BN9626 [cited in 7].

This chapter describes the influence of $MgSO_4$ and NaCl on three cultures of GPSB (*Chlorobium* genus) as well as the effect of culture age (15-30 days) in two strains of *Chromatium* (*Halochromatium*). Some considerations about the uses of fatty acids other than chemotaxonomy as well as new applications of fatty acid analyses like lipidomics by mass spectrometry are also included.

2. Fatty acids analysis

The analysis of fatty acids as methyl esters has been used to identify bacteria species. Their composition and the ratios of these compounds are useful in identifying microorganisms. They are conserved if bacteria are grown in similar conditions [11]. Also, it has been pointed out that information such as phylogenetic data and similarities derived from the fatty acid analysis are in broad agreement with 16S rDNA results, and appear to accurately distinguish between most species [12].

The number of bacterial species evaluated is a limitation in comparing the information available about the fatty acid composition from bacteria [12]. GPSB and PPSB are also not an exception compared to the fatty acid studies on chemoheterotrophic bacteria. There are very few investigations about the influence on fatty acid compositions of the culture medium for bacterial growth (e.g., salinity), the age of the cells or the physical factors (e.g., temperature) used to culture the microorganisms.

Fatty acid analysis may also depend on the extraction technique; in some studies, different procedures have been applied to investigate the fatty acid compositions of *Pseudomonas diminuta* and *P. maltophilia*, and the results have revealed that the qualitative and quantitative features of these compounds are different depending on the techniques used. Nevertheless, there are no changes in their profiles. As such, the main as well as the minor fatty acids are very useful in distinguishing among these strains [13].

Different techniques and methods for fatty acid analysis have been developed since Abel et al. (1963) [14] proposed quantitative and qualitative analyses for characterizing microorganisms, where it was indicated that gas chromatography (GC) has the necessary sensitivity, rapidity and selectivity for such analyses. They considered that this is an extremely selective method, such that a single normal-sized colony of microorganisms is sufficient. This method requires a short time between preparation to examination, and organic and inorganic nutrients do not interfere. The fatty acid methyl esters (FAMEs) from cell membranes have been analysed using GC. These fatty acids were extracted from cell hydrolysates and derivatized to volatile methyl esters for further detection by GC [14].

Other detection methods for fatty acid analysis have also been applied. These include gas-liquid chromatography and mass spectrometry (MS), which is a rapid, automated method for analysing mixed populations, and the various configurations of MS offer combined advantages of speed, sensitivity and selectivity. The method also requires that molecules be vaporized into the gas phase before they can be ionized and analysed.

Gas chromatographic or high pressure liquid chromatographic separation increases the capability for analysing complex mixtures, and they together provide two complementary kinds of characterization. Such chromatographic separation could even distinguish chirality, which is usually invisible to MS [15]. Fatty acids analysis and quantification by GC is carried out by the extraction of lipids from microorganisms, followed by hydrolysis and methylation. The resulting FAME profiles are used for the identification and differentiation of these microorganisms [16]. Actually, there are standardized and automated techniques to asses these compounds; using the Microbial Identification System (MIS) it is a possibility, in a short time, for acquiring precise information about bacteria fatty acid compositions. This system is applied most frequently in the clinical market and it is limited in the number or environmental species they can identify. Nevertheless, microbial fatty acid profiles are unique from one species to another [2].

The MIS system – also known as the MIDI Sherlock Holmes System – which identifies microorganisms using FAMEs and GC, consists of large microbial libraries; the procedure for FAME analysis [17] may be summarized by the following steps: 1) harvesting (the removal cells from the culture media), 2) saponification (the lysis of the cells to liberate fatty acids), 3) derivatization (methylation, the formation of FAMEs), 4) extraction (the transfer of the FAMEs from the aqueous phase to the organic phase by the use of an organic solvent), 5) base wash (an aqueous wash or the organic extract prior to chromatographic analysis).

Alternative procedures have also been proposed for the extraction and separation of FAMEs without the previously described steps, such as by the direct injection of the culture broth,

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allowing the separation and quantification of alcohols and volatile and non-volatile fatty acids. This method simplifies the procedure for fatty acid identification and quantification [18].

Pyrolysis-MS is another technique that allows the direct analysis of bacteria (depending on the organism). With pyrolysis, the bacteria are broken (in an inert atmosphere) using heat to produce volatile, low molecular weight substances that are detected and quantified. This technique involves the analysis of the total cellular composition of the bacteria, and a single bacterial colony is sufficient for analysis [19].

This technique can be used to provide GC data from non-volatile/solid materials. A GC with an ion trap mass spectrometer (IT-MS) could be used for this kind of analysis [20].

2.1. Fatty acid definition

Lipids are defined as natural products that may be isolated from biological materials by extraction with organic solvents and which are usually insoluble in water. This definition includes sterols, terpenoids, isoprenoids, waxes and pigments, many of which are not present in bacteria [21], and the simplest are the fatty acids, as in Table 1. These are the fundamental building blocks of complex lipids, like fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides [22].

Microbial fatty acids are typically 12-24 carbons long and the common membrane fatty acids are 14-20 carbons long. These compounds are either ester- or ether-linked. The latter is rare, and has been found in the phospholipids of Archaea [23]. In general, bacteria contain fatty acids between C10 and C20 in length, but there are some eubacteria species that have poly-unsaturated fatty acids, like *Shewanella gelidimarina*, *Shewanella hanedai*, *Colwellia psychrerythraea* [24], *Shewanella donghaensis* [25], cyanobacteria and mycobacteria.

Fatty acids are essential to cells and their metabolic functions. These compounds are typically associated with energy storage and the structural fluidity of membranes; they form compounds that play important roles in cell signalling processes [26]. In addition, they function as metabolic intermediates and form part of the higher molecular weight lipids [27]. These carbon-based 'backbone' chains, may be elongated, shortened or altered by the activity of the elongase or desaturase enzymic activity, respectively, and longer fatty acids are results of lipogenesis. Moreover, the size of the fatty acid structure depends upon the addition or removal of double hydrogen bonds and catabolic activity [26].

Structurally, fatty acids are long chain monocarboxylic acids of the general formula R.COOH. Usually, the R-group has a long chain - commonly unbranched - consisting of an even number between eight and 24 carbon atoms [27]. The chain has a hydrophobic aliphatic tail that is either saturated or unsaturated [22], depending on the presence of double bounds in the R-group. Acids with one double bound are known as 'mono-enoic', those with two as 'di-enoic', and so on [27].

Fatty acids are structurally diverse and contain distinct classes that are defined at the molecular level, based on the degree of branching, the number and position of double bonds, the chain length, the *cis-trans* isomer conformations, and the presence of functional groups [22].

The major groups of fatty acids are: a) straight chain, b) branched chain, c) unsaturated, and d) cyclopropane. Straight chain fatty acids are found elsewhere in nature (e.g., C12, C14, C16, C18 and C20). However, the corresponding β− hydroxymyristic acid 3-OH and C12 are not normally found in higher organisms, but are common components of the boundary of lipids present in the walls of gram-negative bacteria. Branched fatty acids comprise two types: the iso-form (the methyl group is located on the penultimate carbon atom) and the anteiso (the methyl group is located in the antepenultimate carbon atom). The unsaturated acids found in bacteria are monounsaturated, whereby the double bound is most frequently found between carbon 11 and 12. The cyclopropane fatty acids - rarely found in higher organisms - are frequently encountered in bacteria. The biosynthetic precursors of these acids are the corresponding monounsaturated fatty acids; as a consequence, the major example is the cis-11-12 methylene hexadecanoid acid [21]. Table 1 contains some examples of fatty acids commonly found in bacteria.

Fatty acid	Simplified formula	Common name	Systematic name
SATURATED			
$C_{10}H_{20}O_2$	C16:0	Capric	Decanoic
$C_{12}H_{24}O_2$	C12:0	Lauric	Dodecanoic
$C_{14}H_{28}O_2$	C14:0	Myristic	Tetradecanoic
$C_{16}H_{32}O_2$	C16:0	Palmitic	Hexadecanoic
$C_{17}H_{34}O_2$	C17:0	Margaric	Heptadecanoic
$C_{18}H_{36}O_2$	C18:0	Stearic	Octadecanoic
$C_{20}H_{40}O_2$	C20:0	Arachidic	Eicosadecanoic
UNSATURATED			
$C_{16}H_{30}O_2$	C16:1ω9	Palmitoleic	Hexadec-9-enoic
$C_{18}H_{34}O_2$	C18:1ω9	Oleic	Octadec-9-enoic

Table 1. Examples of fatty acids found in bacteria: formula, common and systematic names [modified from 27]

2.2. Nomenclature of fatty acids

Fatty acids are designated by the total number of carbon atoms followed by the total number of double bonds (e.g., a 16 carbon alkanoic acid is 16:0), beginning with the position of the double bound closest to the methyl-end (*ω*) of the molecule. The double bond position is indicated by the number closest to the carboxyl-end of the fatty acid molecule with the geometry of either c (*cis*) or t (*trans*). For example, 16:1ω9*c* is a phospholipid fatty acid with a total of 16 carbons and with one double bond located between nine and 10 carbons from the methyl-end of the molecule in the *cis* configuration [45].

2.3. Analysis of fatty acids from photosynthetic bacteria

Numerous papers have been published about the influence of the culture media in growing bacterial cells as well as the salinity in the bacteria fatty acid compositions. These have been done especially in heterotrophic bacteria, and it has been observed that when *Escherichia coli*

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was grown in a limited concentration of ammonium salts, these bacteria produced high quantities of cyclopropane fatty acids [28].

The analysis of salinity effects on the fatty acid composition of photosynthetic bacteria has been done in PPSB, such as *Chromatium purpuratum* (or *Halochromatium purpuratum*), *Ectothiorhodospira mobilis* and *Ec. halophila* [29], using different salt concentrations; the results showed only quantitative alterations, but their fatty acid pattern did not witness changes.

The age of the cells is another factor that may change the fatty acid composition of bacteria as well as the cells of all living organisms. In many techniques, it has been suggested that it is very important to use bacterial cells of the same age for fatty acid analysis, especially for chemotaxonomical purposes.

In order to study the influence of $MgSO_4$ and NaCl and the age of the cells in the fatty acid composition of GPSB and PPSB, respectively, the bacterial strains included in Table 2 were used. This table contains the origin, the phenotypical characteristics and optimal culture conditions (NaCl) of the microorganisms.

	Strain	Origin	Main pigments	Shape	NaCl(%)*
Chromatiaceae					
T9s642	[a]*Chromatium* sp.	TL, Mexico	Bchl *a, spx*	Rods	2
DSMZ4395	[a]*Chr. salexigens*	GSC, France	Bchl *a*, *spx*, *ly*, *rh*	Rods	7
Chlorobiaceae					
T11s	[a]*Chlorobium* sp.	TL, Mexico	Bchl *c, cb*	Rods	2
DSMZ249	*Cb. limicola f.***	THS, USA	Bchl *c, cb*	Rods	0
DSMZ266	*Cb. phaeobacteroides*	LB, Norway	Bchl *e, irt*	Rods	0

DSMZ (=DSM) = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; GSC = Giraud Saltens Camargue; TL = Tampamachoco Lagoon (Veracruz); THS = Tassajara Hot Spring; LB = Lake Blankvann; *Bchl* bacteriochlorophyll, *spx* spirilloxanthin, *ly* lycopene, *cb* chlorobactene, *rh* rhodopinal; *NaCl (%) = optimal salinity requirement.

[a]Marine origin strains. The genus *Chromatium* was reclassified into *Halochromatium*. ***Chlorobium limicola f. thiosulfatophilum*=actually *Chlorobaculum thiosulfatifilum*

Table 2. List of strains used in this work: their origins and optimal salinity

For all the experiment, two wild strains were used: a PPSB strain T9s642 (*Chromatium* sp.) and a GPSB strain T11s (*Chlorobium* sp.). These photosynthetic bacteria were isolated from superficial water samples collected in Tampamachoco, a coastal lagoon located in Tuxpan (Veracruz, Mexico) [7].

For culturing these bacteria, a basal medium enriched with water samples was used. This was also used for the isolation and growth of these photosynthetic bacteria and contained the following: distilled water 950 ml, KH_2PO_4 1.0 g, NH_4Cl 0.50 g, $MgSO_4 \cdot 7H_2O$ 0.40 g, $CaCl_2 \cdot 2H_2O$ 0.50 g, and NaCl 2.0% (only for marine strains). It was sterilized by autoclave and, once cooled, was supplemented with a 1.0 mL SL10 solution (GPSB) and SL12 (PPSB), Vitamin B12 1.0 mL

(2.0 mg/100 mL distilled water), and 30 mL of sodium bicarbonate solution 5% [30]. 6.0 mL and 10.0 mL of $Na_2S{\cdot}9H_2O$ 5% (v/v distilled water) were added as an electron donor for *Chromatium* and *Chlorobium*, respectively. The pH was adjusted to 7.5 for the culture medium of *Chromatium* and 6.5 for *Chlorobium.*

2.3.1. Influence of $MgSO_4$ and NaCl on cultures of GPSB

The influence of $MgSO_4$ and NaCl on cultures of GPSB (T11s) were observed by growing the bacteria in the basal medium, as described before, and modified as follows: condition T11S = NaCl (2.0%) and $MgSO_4{\cdot}7H_2O$ 0.3% g; condition C11S = NaCl (0.0%), $MgSO_4{\cdot}7H_2O$ 0.04% g; and condition MG11S = NaCl (0.0%) and $MgSO_4{\cdot}7H_2O$ 0.3% g, respectively. Conditions T11S and C11S are optimal for marine and freshwater GPSB, respectively. In all cases, the pH was adjusted to 6.5 using a diluted H_2S sterilized solution. Strains of GPSB, DSMZ249 and DSMZ 266 were used as references, and they were cultured under the condition C11S (optimal for freshwater strains).

2.3.2. Influence of cell age on the fatty acid composition of PPSB

In order to investigate the influence of bacterial cell age on the fatty acid composition of PPSB, a wild strain of *Chromatium* sp. (*Halochromatium* sp.), isolated from Tampamachoco lagoon, and a collection strain of *Chromatium salexigens* DSMZ4395, were used. The fatty acids of the cultures of these bacteria were analysed after 15, 20 and 30 days of growth, and they were incubated under the standard conditions as described previously.

All the bacteria cultures (green and purple) were grown in 1.5 L glass flasks, the temperature of incubation was 23ºC and, as the light source, bulbs of incandescent (for *Chromatium* strains) and fluorescent light (*Chlorobium* strains) were used. In all cases, the light intensity was 2000 Lux.

The mass cultures of GPSB and PPSB were obtained by adding a previously sterilized and neutralized solution of $Na_2S{\cdot}9H_2O$. It was added to the cultures when the sulphur source (electron donor) was depleted, which was detected using an acetate paper pH tester. Cultures were grown for 15 days (with the exception of *Chromatium* cultures, as indicated before) under the conditions described. Once the absence of sulphur in the bacterial cultures was detected, they were centrifuged (5,000-1000 rpm) and afterwards freeze-dried for further methylation and fatty acid analysis.

2.3.3. Fatty acid extraction and analysis by GC and GC-MS

The procedure for total fatty acid analysis has been described previously in [7], and the lyophilized cells of GPSB and PPSB (250 mg and 500 mg, respectively) were used for fatty acid extraction by saponification followed by diazomethane derivation. The resulting FAMEs were identified using a standard methyl ester mixture and the final identification was confirmed using GC and MS (GC-MS). Cell lipids from each sample were saponified by adding 2.0 mL benzene and 8.0 mL KOH solution (5.0 g/100 mL methanol), containing 5% (w/v) under a

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temperature of 80°C (using a cover bath), over four hours. Once the sample was cooled to room temperature, it was acidified to pH = 1 with a H_2SO_4 solution (20% v/v).

FAMEs were prepared by adding to each sample 2.0 g N-Nitroso-N-methylurea (Sigma) dissolved in a pre-cooled solution of 30.0 mL diethyl ether, 2.0 g KOH and 6.0 mL distilled water. This mixture was stirred for 5-10 minutes and the supernatant was removed and placed in a new tube containing KOH pellets cooled on ice. Finally, diazomethane was added and the dried lipids were achieved within 10-20 min and the content was evaporated at 40°C in a water bath.

The FAMEs were analysed by injecting 2.0 μL of the sample, previously dissolved with n-hexane (0.04-1.0 mL), in a HP-5890A gas chromatograph equipped with a flame ionization detector and a silica capillary column (15 m x 0.25 mm I.D.) with cross-linked methyl silicone (HP-1, Hewlett-Packard). The column was programmed from 175°C to 300°C within 15 minutes. The injector and detector temperature were 275°C and 300°C, respectively. Helium was used as a carrier gas with a flow rate of approximately 1.0 mL/min, and the split ratio was approximately 1:50. Finally, a HP3396 integrator was used for the chromatogram integration and the identification of the FAMEs was done by comparing the retention time of each fatty acid, using a standard mixture of FAMEs.

For the GC-MS analysis, an HP-5890 gas chromatograph attached to a HP5989X quadripole mass spectrometer was used with a methyl polysiloxane column TRB1 (30 m). The injector and detector temperatures were both 225°C, and two ramps were used: one from the initial temperature 10°C/min to 240°C and the other with 40°C/min to 270°C. The injection mode was splitless.

The standard deviations and means were calculated based on the integration areas of the fatty acid peaks.

3. Results

3.1. Influence of the $MgSO_4$ and NaCl cultures on GPSB

Strain T11S was isolated from superficial water samples from Tampamahoco lagoon (Veracruz, México). One of the characteristics of this bacterium is that it is able to tolerate physical and chemical environmental changes. For this reason, it was selected to investigate the changes in fatty acid composition due to salinity (NaCl and $MgSO_4$). However, no changes of the fatty acid profile have been observed as a response to NaCl and $MgSO{\cdot}7H_2O$ changes. In all the strains analysed, 12 fatty acids comprising saturated, unsaturated, branched and cyclic acids were detected, as has been reported previously [7].

All the fatty acids detected in the GPSB had chains with no more than 18 carbons (Table 2). In the *Chlorobium* collection strains (DSMZ266, DSMZ249) and in T11S, the same fatty acids were detected. The standard deviations for almost all the fatty acids identified were very small, with the exception for the C18:0 fatty acid.

Wild and reference strains of *Chlorobium* have C14:0 (6.14-21.3%), C16:0 (11.9-22.9%) and C16:1 (26.9-31%) as the main fatty acids, a profile that was reported for the same strains, cultured in similar conditions [7]. These results confirm the use of FAMEs to characterize bacteria, because they are conserved in composition and proportion if they are grown in similar conditions [11].

All the strains were incubated in similar physical (temperature and illumination) conditions, but specific culture conditions were used for each one. The culture medium for DSMZ266 and DSMZ249 was the same as with C11S (this is without NaCl and 0.04 g $MgSO_4 \cdot 7H_2O$), but it is evident that the condition C11S conserves the same fatty acid pattern as T11S and MG11S.

The collection strains have less saturated fatty acids than T11S, while MG11S and C11S have similar quantities of these fatty acids; inversely, the collection strains have more unsaturated fatty acids than the experimental cultures (T11S, MG11S and C11S). It is very important to note that the branched fatty acids recorded were similar to all the strains cultured in the same conditions (DSMZ266, DSMZ249 and C11S); it is evident that the bacterium of marine origin (T11S) cultured in optimal conditions produced more branched fatty acids than the strains cultured in freshwater conditions as in DSMZ266 and DSMZ249. It has been pointed out that the presence of branched chain fatty acids confers to the membrane a greater degree of flexibility [31].

The cyclic fatty acid was present in greater quantities in DSMZ249 than in DSMZ266 and T11S. When there was an increase in NaCl or Mg salts, the production of C17Cy fatty acid decreased too. It is evident that in optimal conditions for T11S, there was less production of C17Cy fatty acid, so the presence of this fatty acid is as follows: MG11S>C11S>T11S. Cyclopropane acid production in cells is a mechanism for preserving membrane integrity, preventing the formation of abnormal structures [32].

The values of the standard deviation for each fatty acid of T11S, MG11S and C11S were between 0.009 and 1.828, which is evidence that the changes in the fatty acid compositions of *Chlorobium* sp. due to salinity (NaCl and $MgSO_4$) were not so large to loss its identity (Table 3).

3.2. Influence of the culture age (20 and 30 days of growth) on the fatty acids composition of PPSB

It has been proposed that cell age is among the factors that affect the fatty acid compositions of living organisms. The influence of this factor in the fatty acid composition of bacterial cells has been studied in heterotrophic bacteria, and cultures of 24 and 48 hours incubation have been proposed as the optimal age (Microbial Identification System) for the fatty acid analysis of these bacteria. However, the exponential phase has been proposed for fatty acid analysis in photosynthetic bacteria [33]. In addition, stationary or low stationary phases, after three and six days of growth, have been indicated too [34]. Nevertheless, and frequently, the age of the cells that they used for fatty acid analysis was not mentioned.

In the present experiment, the cells of two strains of PPSB from marine origin were used. For fatty acid analysis, the cells of these bacteria were collected after 15, 20 and 30 days of growth in optimal conditions according to their origins. They were also incubated under the same light and temperature conditions. The results of the fatty acid analysis of *Chromatium salexigens*

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Fatty acids	DSMZ249 (3)		DSMZ266 (2)		T11S (4)		MG11S (4)		C11S (3)	
SATURATED	X	SD	X	SD	X	SD	X	SD	X	SD
C12:0	1.489	0.342	0.970	0.414	1.943	0.156	1.881	0.910	2.384	0.264
C14:0	6.114	2.353	13.626	0.406	20.606	1.001	17.366	1.371	21.305	0.860
C15:0	0.757	0.076	1.119	0.532	0.191	0.093	0.441	0.096	0.278	0.162
C16:0	11.932	0.621	11.960	0.622	22.973	1.828	21.435	0.492	19.949	0.696
C17:0	0.230	0.158	0.246	0.050	0.202	0.035	0.429	0.009	0.224	0.015
C18:0	0.838	0.066	0.663	0.252	0.660	0.255	0.334	0.364	0.325	0.018
Sum (%)	21.360		28.584		46.574		41.888		44.465	
UNSATURATED										
C16:1(9)	31.034	0.293	36.009	0.009	26.912	1.172	26.912	0.922	27.215	1.524
C18:1	10.298	0.055	2.689	2.147	2.972	0.188	3.255	0.107	4.643	1.754
Sum (%)	41.332		38.697		29.884		30.167		31.858	
BRANCHED										
12-Me-C14:0	0.516	0.016	0.421	0.070	0.458	0.296	0.973	0.070	0.272	0.013
5-Me-C16:0	0.632	0.078	0.538	0.100	1.163	0.041	1.477	0.335	1.326	0.076
aC17:0	0.862	0.097	0.608	0.011	1.383	0.053	0.770	0.042	0.595	1.123
15-Me-C16:1	0.620	0.176	0.461	0.061	ND	-	ND	-	0.239*	-
Sum (%)	2.629		2.029		3.004		3.220		2.432	
CYCLIC										
17Cy	3.336	0.146	1.780	0.271	0.827	0.038	2.014	1.897	1.426	0.051
TFAE (%)	68.657		71.090		81.289		77.288		80.181	

TFAE = Total fatty acid extracted; X = mean (%); SD = Standard deviation; Experimental culture (number of independent cultures analysed) ND = not detected.

*It was detected only in one of the three cultures of the same analysed strain.

Table 3. Influence of NaCl and $MgSO_4 7\ H_2O$ on the fatty acid composition of a wild strain of GPSB (T11s)

DSM4395 (*Halochromatium salexigens*) and *Chromatium* sp. T9s642 are shown in Table 4 and Figures 1 and 2, respectively.

The fatty acid analysis of these bacteria revealed the presence of nine of these compounds. All these fatty acids have been reported before for members of the Chromatiaceae family [7, 9]. The major fatty acids were C18:1, C16:1 and C16:0 and less quantities of: C12:0, C14:0 and C17:0, C18:0 and C20:1. These conformed between 92% and 93% of the total compounds extracted from the cells of two *Chromatium* strains (DSMZ4395 and T9s642). In this analysis, fatty acids less than 0.1% were also taken into account because minor fatty acids are very important in identifying microorganisms, as reported by Núñez-Cardona [7, 8]. For some strains of Cyanobacteria,it has been reported trace quantities of fatty acids (less than 0.03%) [8], and it has been indicated that minor fatty acids could be useful as biomarkers.

There were no qualitative changes in the fatty acid compositions of DSMZ4395 and T9s642 due to cell ages, and the quantitative changes were minimal: both strains conserved the fatty acid pattern that it is characteristic for members of the Chromatiaceae family, but strain DSMZ4395 produced almost the same quantities of C16:0 (16.2-16.8%) and C16:1 (17.5-18.2%), which is characteristic of halophilic microorganisms like *Halochromatium purpuratum* [29] and

	DSMZ4395-30		DSMZ4395-15		T9s642-20		T9s642-15	
Fatty acid	X	SD	X	SD	X	SD	X	SD
C12:0	0.680	0.015	0.718	0.129	1.049	0.253	0.705	0.057
C14:0	0.873	0.064	0.837	0.054	0.201	0.010	0.237	0.008
C16:1	18.283	0.387	17.520	0.527	27.714	0.102	27.414	0.336
C16:0	16.195	1.249	16.810	0.200	10.498	0.327	11.605	0.887
C17:0	0.428	0.085	0.330	0.069	0.289	0.132	0.413	0.064
C18:1(9)	48.911	1.193	50.526	2.405	44.674	0.141	45.893	0.219
C18:1(11)	5.005	0.223	4.773	0.085	5.757	0.023	5.622	0.296
C18:0	2.784	0.260	2.696	0.272	2.168	0.444	1.928	0.145
C20:1	0.624	0.030	0.724	0.084	0.634	0.004	0.627	0.011
TFAE (%)	93.783		94.934		92.983		94.445	

Table 4. Average (%) and standard deviation (SD) of each fatty acid identified in whole cells of *Chromatium* strains, concentrated at 15, 20 and 30 days of growth

Thiocapsa halophila [7]. Nevertheless, in *Thiohalospira halophila*, a chemolithoautotrophic, halophilic, sulphur-oxidizing gammaproteobacteria member of *Ectothiorhodospiraceae*, fatty acids such as 10MeC16:0, C16:0 and C18:0 are predominant (more than 80%). Furthermore, it has been pointed out that C16:0 is often the main fatty acid in halophilic bacteria [35]. However, this was not applicable for DSMZ4395. In this strain, the major fatty acids are the unsaturated acids (C16:1 and C18:1). This property was characteristic of other halophilic chemolithoautotrophic bacteria, like *Thiohalomonas denitrificans* (DSM15841), where C18:1 is less than 18.58% (Σ:C18:1ω9, 18:1ω7, C18:1ω5) [36]. In contrast, in the *Chromatium salexigens* strain (DSMZ4395) this is the main fatty acid (48.9-50.5%).

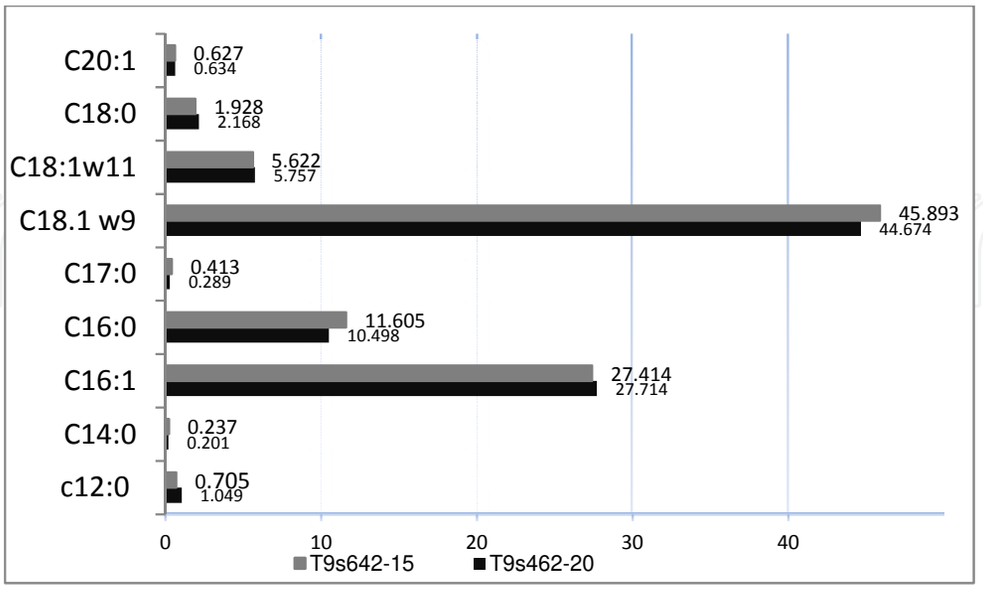

Figure 1. Fatty acids (%) of the wild culture T9s642-15 and T9s642-20 from cells centrifuged after 15 and 20 days of growth, respectively.

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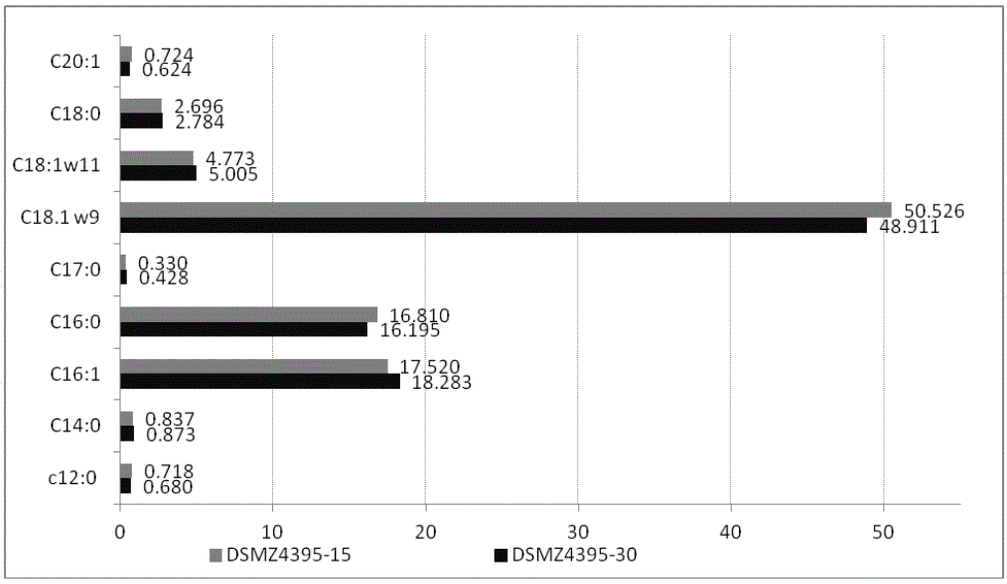

Figure 2. Fatty acids (%) of DSMZ4395-15 and DSMZ4395-30, obtained in cells centrifuged after 15 and 30 days of growth under standard conditions, respectively.

On the other hand, in the wild *Chromatium* sp. strain T9s642, as in some mesophilic eubacteria, the fatty acid C16:1 was present in greater quantities than C16:0 and *Chromatium salexigens* (DSMZ4395) produced more saturated fatty acids (Figures 1 and 2). Nevertheless, it is evident that for the fatty acid compositions in both bacteria, the age factor causes a increase of C18:1 and C16:0 in younger cells, and also a small increase of C16:1, in the oldest.

The influence of age on the fatty acid compositions of bacteria has also been studied in microorganisms like *Serratia marcescens* and *Escherichia coli*, which have more C16:0, C16:1 and C18:1 as well as more cyclic fatty acids. When these cultures are in stationary phases (24-48 hours), monounsaturated fatty acids (e.g., C16:1 and C18:1) are transformed to cyclic forms (17Cy and 19Cy) and C16:0 increases too [37].

The changes in branched fatty acids, such as iC15:0 and C15:0 (e. g , increases and decreases, respectively), are the result of aged cells in a strain of *Bacillus* [38]. This microorganism, as in many other gram-positive bacteria, is characterized by high quantities of branched fatty acids, which are responsible to maintain the fluidity and the membrane structure.

3.3. Uses of fatty acids analysis (other than chemotaxonomy)

Two goals of chemotaxonomy are to measure the number of changed molecules as a descriptor of the chemical relevance of two bacteria and also to provide a list of changed molecules to discover biomarkers [39].

Bacteria contain lipids in concentrations of 0.2-50% of dry weight, and for fatty acid analysis lipid compositions are important. These are mainly: phospholipids (structural elements of the cell membrane), glycolipids (elements of cell membrane structures but less common than phospholipids; abundant in Actinomycetes), lipid A (present in the outer membrane of gram-

negative bacteria; part of the lipopolysaccharides) and lipoteichoic acids (present in gram-positive bacteria) [2].

Profiles of fatty acids have been used to distinguish pathogenic bacteria species for fish, like the genus *Tenacibaculum* (agents of flexibacteriosis in marine fish) whose cells are characterized by the presence of large amounts of branched (36.1–40.2%) and hydroxylated (29.6–31.7%) fatty acids [40].

The presence of bacteria in sponges has been detected because they produce large quantities of iso-, anteiso-cyclopropyl- and monomethyl-branched fatty acids. Using fatty acid analysis, it is possible to distinguish different symbionts (photosynthetic and non-photosynthetic) present in marine sponges and provide estimates of bacterial symbiont abundances [41].

Special fatty acids from phospholipids have been used for characterizing bacteria and photosynthetic life forms. Fatty acids linked to these molecules in the members of Chromatiaceae and Ectothiorhodospiraceae were analysed, and it is evident that the fatty acid pattern does not change. As has been indicated before, these compounds have great value in determining bacterial phylogeny, providing a useful set of features for characterizing strains, and they give important information about microbial communities in environmental samples.

Phospholipid fatty acids could be used as biomarkers because they degrade rapidly after cell death and are not present in storage lipids or in anthropogenic contaminants. Bacteria contain phospholipids as relatively constant proportions in their biomass [41], and with these it is possible to estimate the microbial biomass in environmental samples where the bacteria of subsets in microbial communities contain specific signature fatty acids in their phospholipids [42]. Using phospholipid fatty acids (as methyl esters) analysis, it is possible to measure attributes or microbial communities, such viable biomass and microbial structures as well as nutritional and physiological status [41, 43]; these compounds, as well as bacterial fatty acid ratios, have been used to disclose bacterial connections in the marine food web and their importance in supplying material and energy to the higher trophic levels [44].

In addition, lipids have been used to establish possible links between modern and ancient microbial communities and to add to the understanding of the evolutionary histories of the organisms in the various environments. The analysis of phospholipids provides information about the viable biomass and biological diversity, and on the other hand information about the glycolipids of the activity of photosynthetic microorganisms in the environment. In addition, sterols and hopanoids - which are better preserved in the geological records - offer insights into the presence of microorganisms in paleoenvironments [45]. In bacterial membranes, the fatty acids of phospholipids are linked by ester bonds to glycerol and form a phospholipid bilayer, while archaeal membranes have phospholipids containing two polar heads linked by isoprenoid chains and ether linkages to glycerol [44]. As such, these linkages are markers for detecting archaeal bacteria.

3.4. Lipidomics studies: some examples

Lipidomics has been applied in physiological studies, such as the change in the lipidomic profiling of *Chlamydomonas nivalis* as a response to nutritional stress [46]. In *Nitzschia closterium*

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f. minutissima, lipid metabolomic changes in the different growth phases of these microorganisms have been studied. Some biomarkers have been selected and identified, including free fatty acids and lipids such as harderoporphyrin, phosphatidylglycerol, 1,2-diacylglycerol-3-O-40-(N,N-trimethyl)-homoserine, triacylglycerol, cholesterol, sulfoquinovosyldiacylglycerol, lyso-sulfoquinovosyldiacylglycerol, monogalactosyldiacylglycerol, digalactosyldiacylglycerol and lyso-digalactosyldiacylglycerol [47].

Lipidomics has revealed that under cold stress, *Stephanodiscus* sp. produces the following compounds as biomarkers: triacylglycerol, phosphatidylcholine, phosphatidylglycerol, sulfoquinovosyldiacylglycerol, monogalactosyldiacylglycerol, lyso-phosphatidylglycerol, lyso-phosphatidylcholine, lyso-monogalactosyldiacylglycerol and lyso-sulfoquinovosyldiacylglycerol [48].

In addition, lipidomics have been applied to the understanding of the lipogenesis of free fatty acids from the symbiosis between cnidarian-dinoflagellate [26] as well as the study of the lipidomics of human pathogenic mycobacteria. It is possible to get the lipidomics datasets to assess lipid changes during infection or else among clinical strains of mycobacteria tuberculosis [39].

4. Conclusions

As was mentioned before, the influence of NaCl in the culture medium increases the concentration of the saturated fatty acids C16:0 and decreases the production of the unsaturated C16:1 and C18:1. The presence in the culture medium of both $MgSO_4$ and NaCl reduces the production of the saturated fatty acids C14:0 and C18:0 and increases the production of the unsaturated C16:1 and C18:1. The change of the relative abundances of the fatty acids due to the changes of $MgSO_4$ and NaCl in the culture media of *Chlorobium* is not sufficient to alter the fatty acid pattern of the *Chlorobium* strains analysed. Minimal quantitative alterations were observed by the cell age, the pattern and profile of the fatty acids in PPSB and GPSB are very conservative in these microorganisms could be used for chemotaxonomic approaches. Nevertheless it is very important to describe the culture conditions as well as the techniques for fatty acid analysis.

Although advances in the technologies for fatty acid analysis have been reported, the isolation and culturing of novel strains as well as the fatty acid analysis of photosynthetic prokaryotes remains scarce, and it is very important to encourage the investigation of these microorganisms. With new sources of genetic materials from photosynthetic bacteria and the application of new technologies - for example lipidomics - which are more precise, it will be possible to discover new fatty acid structures and to evaluate the sources of these compounds of biotechnological interest and novel chemotaxonomic biomarkers. In addition, the physiological role of fatty acids in the cell membranes of photosynthetic bacteria and their role in environmental trophic chains could be explored.

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