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Polar lipid biomarkers of free-living bacteria from oligotrophic marine waters

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Abstract. Free-living marine bacteria isolated from oligotrophic Mediterranean waters were enriched in culture to characterize their phospholipid fatty acids (PLFAs). Odd chain *iso*- and *anteiso*-FAMEs and *n*−16:0 were the predominant structural PLFAs, together with a homologous series identified as mid-chain methoxy FAMEs. The dominant methoxy fatty acids identified were 9-CH_3O-15:0, 9-CH_3-16:0 and 11-CH_3O-17:0, occuring as pairs of stereoimers. Methoxy fatty acids accounted for up to 37% of PLFAs of free-living bacteria, which sets them as promising new biomarkers for bacteria of oligotrophic waters. Although similar homologues have already been characterized in a variety of eukaryotes and prokaryotes, methoxy fatty acids are identified here for the first time in marine bacteria. Analytical difficulties that may hinder the characterization of these biomarkers are presented, and structural elucidation keys by gas chromotography coupled to mass spectrometry are discussed. Whilst bacterial branched fatty acids were transferred to storage lipids of bacterivorous flagellates methoxy acids were not transferred to higher trophic levels in the studied conditions.

Introduction

In marine oligotrophic areas, carbon and nitrogen are exchanged from dissolved organic pools to a microbial food web linking free-living bacteria, flagellates and ciliates through food-web relationships (Azam et al. 1983; Rassoulzadegan and Sheldon 1986; Sherr and Sherr 1988; Caron et al. 1991). This microbial loop, also referred to as the microbial food web, allows the transfer of part of the bacterial production to higher trophic levels. It also recycles part of this production, for instance 10–16% of ingested bacterial amino acids are excreted by bacteriophages (Ferrier-Pagès et al. 1998). In marine oligotrophic settings, the bacterioplankton contributes to a significant portion of total biomass (Fuhrman et al. 1989). The occurrence of a microbial food web, comprising bacteria and bacteriophages, has also been pointed out in the aphotic layer (Tanaka and Rassoulzadegan 2002). Microorganism communities of the microbial food web are most commonly characterized by cell counting. However most marine bacteria are non-culturable and their

morphology does no allow to discriminate species. Molecular techniques using the diversity of DNA or rRNA provide valuable information on prokaryotic diversity and on the occurrence of some clones in natural populations (Pace et al. 1986; Giovanni et al. 1990; Schmidt et al. 1991; Field et al. 1997).

Phospholipids are important components of bacterial cell membranes, their composition regulates membrane fluidity and permeability. Phospholipid ester-linked fatty acids are an excellent estimate of the viable microbial biomass in marine sediments (White et al. 1979). Furthermore phospholipid fatty acid (PLFA) composition also characterizes the structure of the microbial consortia, and this approach has been extensively used to describe microorganism community structure as well as biomass abundance (White et al. 1979; Balkwill et al. 1988; Zelles 1997; Ibekwe and Kennedy 1998; Rütters et al. 2002). When compared to our knowledge of sedimentary microorganisms, very little attention has been paid so far to the lipids of free-living bacteria and bacterivorous nanoflagellates of the microbial loop (Ederington et al. 1995; Harvey and Macko 1997; Zhukova & Kharlamento 1999), most likely because of difficulties in isolating these small heterotrophs.

In order to enlarge the application of PLFAs to the microbial food web of oligotrophic areas, the present contribution describes for the first time phospholipid composition of pelagic bacteria and heterotrophic nanoflagellates from surface Northwestern Mediterranean waters. PLFA characteristics of bacteria are documented with emphasis on a novel series of compounds proposed as biomarkers for free-living bacteria. Next, the fate of these novel biomarkers was tested by analyzing lipids of bacterivorous nanoflagellates, and determining whether they were transferred to higher trophic levels.

Bacteria and bacterivorous flagellates were isolated from Northwestern surface Mediterranean waters and grown in culture of large volume so as to yield a cell number consistent with gas chromatography detection limits of lipids.

Experimental

Isolation of pico and nanoplankton and cultures

Cultures of bacteria and of bacterivorous nanoflagellates were developed to mimic the marine oligotrophic conditions. Seawater from 50 cm below the surface was collected from oligotrophic Northwestern Mediterranean Sea off Villefranche Bay. The water was successively filtered on sterile 0.8 and 0.2 μm Nuclepore filters. The latter filtrates were used to inoculate free-living bacteria into a culture medium. This medium consisted of boiled wheat grains placed into 5 l of seawater filtered on GF/F and 0.22 μm Nuclepore membranes, and was sterilized in an autoclave before inoculation. Cultures were incubated in the dark at 12 °C and bacterial cells were monitored until they reached a minimum abundance of 10^7 cells ml^{-1}. For monitoring, subsamples of 200 μl

of the bacterial suspensions were preserved in borax-buffered formaldehyde (0.3% vol/vol final concentration), stained with DAPI and counted under UV light using a Zeiss Axiophoth epifluorescence microscope (Ferrier-Pagès et al. 1998). The bacterial culture selected for harvest contained small coccoid forms of about 0.2 μm in length, not epiphytic nor agglomerated. Four liters of this culture were filtered on 0.2 μm Durapore pre-extracted membrane, under low vacuum, and this sample will be named B in the text.

Bacterivorous nanoflagellates *Pseudobodo* sp. were isolated from Northwestern Mediterranean Sea by successive filtrations on 4.0 and 0.8 μm Nuclepore filters. Half a liter of this filtrate was inoculated into bacterial cultures that contained at least 10^5 bacteria ml^{-1}. Flagellate and bacteria abundances were monitored by epiflurescence after DAPI staining. After a lag of a few days, flagellate numbers rose up to 10^4–10^5 flagellates ml^{-1} while bacterial counts dropped. The flagellate peak lasted less than 24 h and was followed by a sudden cell drop, a well-known scenario in such experiments (Alonso et al. 2000). When flagellate number decreased, bacteria shaped as filamentous rods rapidly developed in the culture. Such rapid changes in community structure caused difficulties in harvesting flagellates at the pertinent time. The culture where the flagellate number remained the highest during harvest was selected for analysis. This culture of 1.4 l was first concentrated on 8 μm Nuclepore membrane by gravity and then filtered on 0.22 μm Soxhlet-cleaned Durapore filters under low vacuum. This sample will be named B & F in the text. Subsamples for bacteria and flagellate counts were taken before and in the course of the sampling procedure.

Lipid extraction and separation

Lipids were extracted from particles retained on the filter by extraction in a monophasic solvent mixture of CH_3OH:CH_2Cl_2:saline water (2:1:0.8). The particle filter was mixed with 60 ml of this mixture and extracted by ultrasonication for 20 min and let in contact under Argon on an ice bath for 4 h. The supernatant was collected and the filter was rinsed twice with the solvent mixture. Joined extracts were placed into a separatory funnel, into which CH_2Cl_2 and saline water was added until a ratio of 2:2:0.8 was reached, and phase partition was let to occur for 30 min. The lower organic phase was collected and the aqueous-methanolic phase was rinsed by 40 ml of CH_2Cl_2. The organic phases were vacuum concentrated down to 10 ml and were dried overnight on Soxhlet-cleaned $MgSO_4$. After filtration on cleaned wool, the dried lipid extract was reduced down to 500 μl. Lipid classes were separated by Thin Layer Chromatography using an elution sequence described elsewhere (Méjanelle et al. 2002). Silica bands on which lipid classes of interest were adsorbed were scrapped off from the plate, and lipids were eluted by CH_2Cl_2:CH_3OH (2:1). A known amount of the internal standard, deuterated 23:0 methyl ester, was added to the selected lipid classes: Phospholipid fatty

acids (PLFA), Free Fatty Acids (FFA) triacylglycerol esters (TAG) and alkyl esters, generally designed as wax esters (WE). PLFAs were transmethylated and FFAs were methylated by 250 μl of boron-trifluoride in methanol (14%) for 30 min at 65 °C, and the other lipid classes for 60 min. Reaction times were kept as short as possible for better preservation of intrachain cyclopropane rings. After the reaction, the methyl esters were extracted by 3×2 ml of diethyl ether–hexane (1/1). The combined extracts were neutralized by rinsing three times with milliQ water (Waters) and dried overnight on $MgSO_4$. The FAME extracts were filtered and evaporated to 0.5 ml under a pure nitrogen stream.

GC and GC-MS analyses

The methyl esters of various lipid classes were analyzed and quantified by gas chromatography and flame ionization detection (GC-FID). Peaks were attributed according to retention time comparison with authentic standard mixtures (PUFA 2, Matreya Inc., PA, USA and Bacterial Mixture, Supelco, Bellefonte, PA, USA) and by mass spectrometry.

Samples were dissolved in hexane and analyzed by GC-FID using both a polar and a non polar column for FAME separation. A Silar 5CP column (Chrompack, France) (25 m $\times$ 0.25 mm internal diameter, 0.25 μm film thickness) was mounted on a Girdel 3000 chromatogram (Girdel, France) equipped with a Ross injector heated up at 250 °C and with a split flow of 20 ml min^{-1}. The oven temperature was raised from 100–195 °C at 2°/min and then held at 195 °C for 70 min. Helium was used as carrier gas at a flow of 50 cm s^{-1}.

A 30 m DB5 column (0.25 mm internal diameter, 0.25 μm film thickness, Chromoptic, France) was used on a DI200 chromatograph (Delsi, France), using helium as the carrier gas at a flow of 50 cm s^{-1}. The sample was injected into a Ross injector heated up at 250 °C and with an split flow of 20 ml min^{-1}. The oven temperature was raised from 100–300 °C at 2 °C/min and then held at 300 °C for 20 min. Detector and injector temperatures were 330 and 280 °C, respectively.

Both chromatographs were equipped with flame ionization detectors, fed with air at 300 ml min^{-1} and with hydrogen at 30 ml min^{-1}, and are maintained at 330 °C. Helium was used as make up gas (30 ml min^{-1}). The FID detector response was digitized by a Nelson 900 interface and processed with a Nelson 2600 software package (Perkin Elmer). Relative concentrations of FAMEs were calculated from their GC-FID response areas, relatively to that of the internal standard, deuterated C23:0 FAME.

Analyses by GC-mass spectrometry (GC-MS) were performed with a Girdel 32 chromatograph coupled to a quadrupole mass analyzer Nermag R10-10C. Samples were injected with a Ross type injector into a DB5 capillary column similar to that used for GC analyses and the temperature of the oven was

identically programmed. Fragmentation was obtained in electron impact mode at 70 eV and ion source and transfer line were kept at 310 °C. Data were recorded by the PDP 11/23 + Sidar 111 software by scanning from mass 31–600 amu/s.

The nomenclature used for fatty acids in this paper is the *n*-designation.

Results

Microorganisms in presence and lipid class profiles

The concentration of small coccoid bacteria in the B culture was $5.20 \pm 0.74 \times 10^6$ cells ml^{-1}. In the B & F culture, the concentration of filamentous bacteria and nanoflagellates were $8.60 \pm 1.62 \times 10^7$ and $9.92 \pm 0.36 \times 10^5$ cells ml^{-1}. The volume of the B & F culture filtered comprised $3.57 \pm 0.15 \times 10^9$ nanoflagellates and $8.60 \pm 0.89 \times 10^7$ filamentous bacteria, some of them up to 0.5 μm in length. The closeness in size of bacteria and nanoflagellates made it impossible to separate the bacteria from the flagellates by filtration. In the B & F sample the flagellate lipids are thus expected to dilute lipid bacterial fingerprint.

In the B culture, 47.6% of the fatty acids methyl esters (FAMEs) were esterified to PLFA, and 52.4% occurred as free fatty acids. The PLFA represented 0.29 fg $cell^{-1}$, a value that compares well with the literature (White et al 1979). In the B & F culture, 54.7% of the FAMEs occurred as phospholipid esters, 43.4% occurred as free fatty acids, 0.7% occurred esterified to TAG and 1.1% esterified to alkyl esters. Phospholipids generally constitute the dominant lipid class in marine bacteria, where they account for up to 96% of total lipids (Gérin and Goutx 1993). In the B and B & F samples, a significant proportion of FFAs was also present. FFAs are produced by hydrolysis of other lipid classes and are usually thought to stem from non-living decaying detritus. FFAs in the B and B & F cultures may originate from a pool of dead cells. Another relevant source for these FFAs is an artifactual hydrolysis of phospholipids in the course of cell harvest and extraction. During repeated extractions, the membranes are broken up by ultrasonic extraction and membrane enzymes, such as phospholipases, are brought into the extraction solution. Phospholipases may then hydrolyze phospholipids into FFAs, a bias clearly affecting lipid class profile in the course of extraction of diatoms (Bergé et al. 1995). About an hour was necessary for harvesting the B and B & F cultures, and the extraction method comprised a step of ultrasound extraction and a step where the cells were let in contact with the solvent for 4 h under an inert gas; hydrolysis might have occurred during those steps. The FFAs analyzed in B and B & F cultures may thus, at least partly, originate from phospholipids.

In the B & F sample, other lipid classes were present in low abundance: reserve lipids (TAG, WE) and sterols. These lipid classes are not synthesized

by bacteria and are probably contributed by the nanoflagellates present in the B & F culture.

Phospholipid fatty acid patterns of free-living bacteria and nanoflagellates

The B culture exclusively comprised pelagic free-living bacteria, therefore the phospho-ester linked fatty acids (PLFAs) of this sample depicts the imprint of pelagic bacteria from oligotrophic areas. They were dominated by 16:0 and branched acids (40% of the sum of PLFAs), often used to fingerprint bacterial occurrence and relative abundance from marine particles or sediments (Perry et al. 1979; Volkman et al. 1980; Kaneda, 1991). The *anteiso* isomers were more abundant than their *iso* homologues for 15:0- and 17:0-PLFAs, a feature observed in other bacteria (Ibekwe and Kennedy 1998; Rütters et al. 2002) and methyl branched 15:0 and 17:0 dominated over the normal isomers (Figure 1). *Iso*-16:0 occurred in bacterial PLFAs, whereas the *anteiso* homologue could not be detected. Besides these well documented bacterial

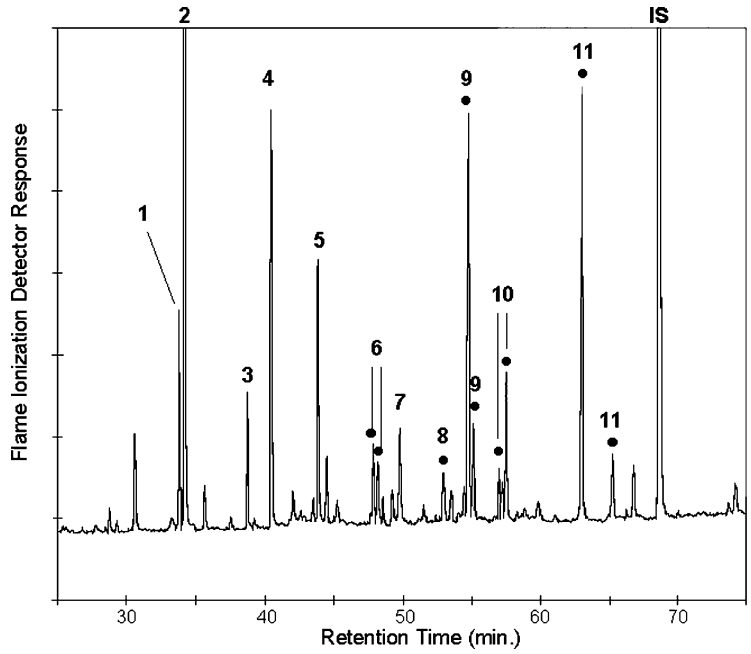

Figure 1. Partial GC chromatogram of PLFAs extracted from the bacterial culture, and analyzed as FAMEs on an apolar DB5 column. Peak labeled by a full circle are mid-chain methoxy FAME, and IS represents the internal standard (deuterated 23:0) peak. Peak numbers correspond to the following FAME structures: 1, *iso*-15:0; 2, *anteiso*-15:0; 3, *iso*-16:0; 4, 16:0; 5, *anteiso*-17:0; 6, two isomers of 4-CH_3O-14:0; 7, 18:0; 8, 6-CH_3O-15:0 and 5-CH_3O-15:0; 9, two isomers of 9-CH_3O-15:0; 10, two isomers of 9-CH_3O-16:0; 11, two isomers of 11-CH_3O-17:0.

biomarkers, other branched acids were detected in the bacterial culture: isomers of *iso*- and *anteiso*-15:0 and 17:0 in trace amounts and branched monounsaturated 15:1 and 17:1 accounting for less than 1% of total FAMEs. Cultures of bacteria have shown highly variable proportions of branched and monounsaturated fatty acids, some comprising more branched than monounsaturated acids (Perry et al. 1979; Findlay et al. 1990; Rajendran et al. 1992; Nichols et al. 1993).

No monounsaturated PLFA were identified in B. 16:1($n-7$), 18:1($n-9$) and 18:1($n-7$) are common constituents of some marine bacteria (Zhuckova and Kharlamento 1999), but not all bacteria comprise monounsaturated FAMEs. The B & F sample showed a different PLFA composition, with less branched compounds and, on the contrary, a dominance of monounsaturated PLFA. Those differences more probably reveal the flagellate imprint.

Table 1. Comparison of retention times of mid-chain methoxy FAMEs and other FAMEs relatively to the normal saturated 16:0, on apolar and polar capillary columns

Elution on a DB5 apolar column		Elution on a Silar polar column	
Compound	Relative retention time	Compound	Relative retention time
16:0	1.000	16:0	1.000
4-CH_3O-14:0	1.182	4-CH_3O-14:0	1.224
4-CH_3O-14:0	1.189	4-CH_3O-14:0	1.254
18:0	1.233 ± 0.003	18:0	1.288 ± 0.007
6-CH_3O-15:0 and 5-CH_3O-15:0[a]	1.345		
9-CH_3O-15:0	1.352	9-CH_3O-15:0	1.539
9-CH_3O-15:0	1.361	9-CH_3O-15:0	1.572
9-CH_3O-16:0	1.413	20:0	1.722 ± 0.025
20:1($n-9$)	1.417 ± 0.002	C20:4($n-3$)	1.737 ± 0.004
{9-CH_3O-16:0	**1.420**	C20:5($n-3$)	1.844 ± 0.025
20:1($n-7$)}	**1.420 ± 0.009**	9-CH_3O-16:0	1.914
20:0	1.451 ± 0.004	22:1($n-9$)	1.933
20:4($n-3$)	1.370 ± 0.001	9-CH_3O-16:0	1.940
20:5($n-3$)	1.377 ± 0.001	Deuterated 23:0	1.975 ± 0.017
22:5($n-3$)	1.563 ± 0.000	22:5($n-3$)	2.291
{11-CH_3O-17:0	**1.566 ± 0.015**	22:6($n-3$)	2.418 ± 0.035
C22:6($n-3$)}	**1.571 ± 0.001**	11-CH_3O-17:0	2.493 ± 0.017
{11-CH_3O-17:0	**1.622 ± 0.017**	11-CH_3O-17:0	2.580 ± 0.014
22:1($n-3$)}	**1.625**		
22:0	1.655 ± 0.004		
Deuterated 23:0	1.715 ± 0.010		

When compounds were identified in several samples, the average and standard deviation are given. Chances of co-elutions are indicated by relative retention times underlined in bold and brackets. The deuterated 23:0 is the internal standard used for quantitation. [a]Composite peak of two structures, as shown by mass spectrometry.

Three prominent peaks eluted in the second half of the chromatograms of the B and of the B & F PLFAs. The relative retention times of those peaks matched those of 20:5($n-3$) and 22:6($n-3$) and 22:1($n-9$) on the DB5 column

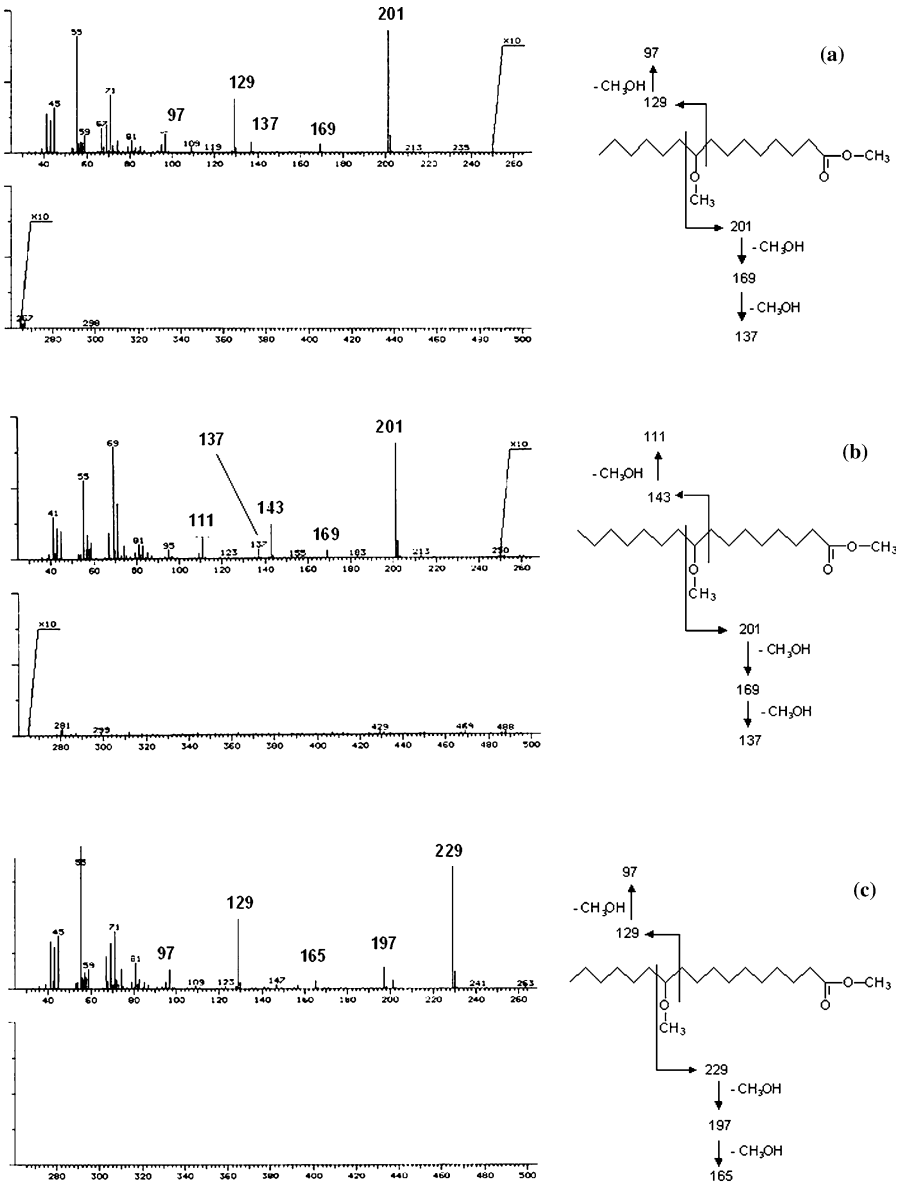

Figure 2. Mass spectra of the three dominant methoxy FAMEs, obtained on the electron impact mode, and fragmentation schemes of the elucidated structures: (a) 9-CH_3O-15:0; (b) 9-CH_3O-16:0 and (c) 11-CH_3O-17:0.

and they were close to retention times of 20:1($n-7$), of 20:5($n-3$) and 22:6($n-3$) on the polar Silar column (Table 1). Most bacterial strains are devoid of polyunsaturated acids (Bobbie and White 1980; Vestal and White 1989) and the occurrence of polyunsaturated acids in marine bacteria is restricted to psychrophilic and barophilic strains (DeLong and Yayanos 1985, Fang et al. 2002). Therefore the attribution of these peaks to polyunsaturated acids in surface bacteria was highly questionable and GC-MS analyses were performed to confirm the structures of these three compounds. In the B & F sample the spectra obtained under the electron impact mode matched polyunsaturated FAMEs, even though the peak of 22:6($n-3$) showed traces of a coeluting compound in minor amount. In the extract of the B sample, mass spectra of the three prominent peaks showed characteristics that clearly deny their attribution to the polyunsaturated FAMEs matching their retention times (Figure 2).

Structural elucidation of methoxy FAMEs

Prevailing fragmentation yielded intense fragments in the m/z range from 100 to 250 and the three large peaks corresponded to structures resolved chromatographically. Homologues of these series were detected at other retention times on the chromatogram of PLFA of the B culture. The following discussion will focus on the structural elucidation of these compounds.

The mass fragments in the m/z range from 100 to 250 occurred as two suites of fragments separated by 32 m/z unit gap, corresponding to gradual loss of CH_3OH groups (Figure 2). A first suite encompasses three fragments of 32 mass unit apart, and the second suite is constituted by a couple of fragments. Structures in agreement with such peak suites are mid-chain methoxy-FAMEs (Barnathan et al. 1998). Fragmentation in α of either side of the ether branch produces two fragments, the one comprising the methyl side of the FAME bears only one –O–CH_3 group (a), while the fragment comprising the carboxyl end of the FAME bears both the mid-chain and the terminal –O–CH_3groups (b). Diagnostic fragment suites yielded by methoxy-FAMEs of different alkyl chain lengths are computed in Table 2. The structure of the three prominent bacterial FAMEs eluting at the retention times of C20:5($n-3$), C22:1($n-9$) and C22:6($n-3$) are 9-CH_3O-15:0, 9-CH_3O-16:0 and 11-CH_3O-17:0, respectively. Methoxy FAMEs occurred as pairs of isomers chromatographically well resolved (Figure 1). The carbon bearing the methoxy also bears an hydrogen atom and two distinct alkyl chains (the carboxyl and the methyl end of the fatty acid). Therefore this carbon is a chiral center and chromatographically resolved isomers more probably correspond to stereoisomers. On the basis of retention times, the dominant series members can easily be mistaken for mono and polyunsaturated fatty acids commonly found in marine algae, crustacean and psychrophyllic bacteria (Table 1). Structure elucidation by GC-MS is therefore a requisite for analyzing marine FAMEs, to

Table 2. Diagnostic series of fragments calculated for methoxy FAMEs of different alkyl chain lengths and methoxy locations

Fragment A			Fragment B			
Location of the –OCH_3 from the methyl end	*m/z* values		Location of the –OCH_3 from the carboxyl end	*m/z* values		
	A	$[A - OCH_3]^+$		B	$[B - OCH_3]^+$	$[B - 2\times(OCH3)]^+$
3	73	41	3	117	85	53
4	87	55	4	131	99	67
5	101	69	5	145	113	81
6	115	83	6	159	127	95
7	**129**	**97**	7	173	141	109
8	**143**	**111**	8	187	155	123
9	157	125	9	**201**	**169**	**137**
10	171	139	10	215	183	151
11	185	153	11	**229**	**197**	**165**
12	199	167	12	243	211	179
13	213	181	13	257	225	193

Fragment A comprises both the mid-chain O–CH_3 group and the methyl end of the FAMEs, and fragment B comprises the mid-chain methoxy group and the carboxyl end of FAME. The *m/z* values in bold correspond to the fragments of the dominant isomers in bacterial phospholipids, whose spectra and fragmentation are presented in Figure 1.

ascertain whether they are common unsaturated FAMEs or methoxylated ones.

In order to definitively confirm the mass spectra identification, co-injection of the samples with authentic standards would be required. Synthesis of various methoxy acids has been reviewed by Carballeira (2002), and recently that of 9-methoxypentadecanoic acid has been reported (Carballeira and Miranda 2003). This homologue was identified in the B sample and co-injection with synthetic homologues would unambiguously confirm the mass spectra interpretation and allows further identification of the stereochemistry of the asymmetric carbon.

Molecular patterns of flagellate fatty acids

In the B & F culture, PLFA and FFA may come from filamentous bacteria and from flagellates. In these lipid classes, percentages of branched FAMEs were lower than in the B culture and higher than values reported for nanoflagellate FAMEs (Zhukova and Kharlamento 1999). PLFA and FFA of the B & F culture comprise monounsaturated and polyunsaturated FAMEs that were absent in B. They were dominated by 18:2($n-6$) and also comprised 20:5($n-3$) and 22:6($n-3$). Polyunsaturated FAMEs accounted for a considerable portion of PLFA and FFA in B & F, which confirms that flagellates are the initial

Table 3. Comparison of methoxy FAME series identified in marine free bacteria to those previously reported in algae and bacteria

Compound	*Schizymenia dubyi*	*Lyngbya majuscula*	*Thiobacillus*	*Helicobacter pylori*	*Rhizobium leguminosarum*	*Escherichia coli*	Marine free-living bacteria
7-CH_3O-12:1($n-8$)		9					
4-CH_3O-14:0							Present work
7-CH_3O-14:0		5					
7-CH_3O-14:1($n-10$)		1, 5					
5-CH_3O-15:0 and 6-CH_3O-15:0							Present work
9-CH_3O-15:0	8						Present work
9-CH_3O-16:0							Present work
9-CH_3O-17:0	8					7	
9-CH_3O,10-CH_3-16:0						7	
9-CH_3,10-CH_3O-16:0						7	
7-CH_3O,9-CH_3-16:1($n-12$)		2					
11-CH_3O-17:0				6		7	Present work
10-CH_3O-18:0			4				
11-CH_3O-18:1($n-7$)					10		
11-CH_3O-18:0			4		7		
12-CH_3O-18:0			4		7		
7-CH_3O,9-CH_3-18:2($n-10$)		2, 3					
11-OCH_3,12-CH_3-18:0					7	7	
11-CH_3O-19:0				6	7	7	
11-CH_3,12-CH_3O-18:0					7	7	
13-CH_3O-19:0					7	7	
13-CH_3O-20:0			4				
13-CH_3O-21:0	8						
15-CH_3O-23:0	8						

Occurrence of a given methoxy FAMEs in an organism is indicated by a number referring to the following literature. 1, Cardellina II et al. (1978); 2, Mynderse and Moore (1978); 3, Loui and Moore (1979); 4, Kerger et al. (1986); 5, Gerwick et al. (1987); 6, Orgambide et al. (1993); 7, Inamoto et al. (1995); 8, Barnathan et al. 1998; 9, Mesguishe et al. 1999; 10, Drouin et al. 2000.

Table 4. Molecular composition of the lipid classes of marine free-living bacteria (culture B) and of bacteria and nanoflagellates (Culture B & F)

	Culture B		Culture B & F			
FAME	PLFA	FFA	PLFA	FFA	TAG	WE
Saturates						
iso-14:0	0.55	0.37	0.30	0.10	nd	nd
14:0	2.3	4.1	2.2	2.7	nd	2.8
iso-15:0	5.4	6.7	6.6	1.9	0.38	0.8
anteiso-15:0	22.2	6.1	5.8	1.8	0.61	1.3
15:0	1.1	4.4	3.4	1.5	0.46	2.5
iso-16:0	3.2	2.6	1.9	1.2	0.53	nd
16:0	10.4	39.9	12.0	16.6	13.9	21.6
isomer of *iso*-17:0	bql	bql	nd	nd	nd	nd
isomer of *anteiso*-17:0	0.70	0.75	0.76	1.00	nd	nd
iso-17:0	0.46	0.72	0.62	1.1	3.8	1.1
anteiso-17:0	6.6	3.1	2.0	1.8	4.2	3.2
17:0	0.51	2.8	0.33	0.8	2.3	1.7
18:0	2.5	19.1	4.3	4.5	14.9	7.6
19:0	nd	nd	0.02	0.11	0.78	nd
20:0	nd	nd	0.08	0.33	4.2	0.80
21:0	nd	nd	0.01	0.07	nd	nd
22:0	nd	nd	0.07	0.36	2.3	0.66
Sum	56.0	90.6	40.2	35.8	48.5	44.1
Monounsaturates						
14:1	nd	nd	0.42	0.56	nd	0.59
Br 15:1	0.79	nd	nd	nd	nd	Nd
15:1	nd	nd	7.9	1.5	nd	nd
15:1	nd	nd	0.98	0.54	2.5	nd
16:1	nd	nd	0.91	0.37	2.2	nd
16:1($n-7$)	nd	nd	19.1	23.4	2.1	20.8
16:1	nd	nd	0.61	0.57	1.9	bql
br 17:1	nd	0.66	1.5	0.47	2.4	1.8
17:1 or Δ17:0[a]	1.4	2.3	1.2	0.72	nd	nd
18:1($n-9$)	0.25	nd	nd	6.1	17.9	11.0
18:1($n-7$)	nd	nd	nd	10.6	13.1	6.6
Br 19:1	nd	1.1	0.30	nd	nd	nd
19:1 or Δ19:0[a]	0.67	0.90	0.53	0.82	1.2	6.3
C20:1($n-9$)	nd	nd	nd	0.18	1.5	nd
C20:1($n-7$)	nd	nd	0.05	0.13	3.4	nd
C22:1($n-9$)	nd	nd	bql	nd	nd	6.1
Sum	3.2	5.0	33.5	45.9	48.1	53.1
Polyunsaturates						
16:2	nd	nd	0.24	nd	nd	nd
18:2($n-6$)	nd	nd	22.8	nd	nd	nd
18:3($n-3$)	nd	nd	nd	0.74	nd	nd
18:4($n-3$)	nd	nd	nd	1.9	0.92	1.1
18:2($n-3$)	nd	nd	0.95	2.9	2.5	1.7
20:4($n-3$)	nd	nd	0.18	1.2	nd	nd
20:5($n-3$)	nd	nd	0.52	4.9	nd	nd
20:5	nd	nd	nd	0.17	nd	nd

Table 4. Continued.

FAME	Culture B		Culture BF			
	PLFA	FFA	PLFA	FFA	TAG	WE
20:4($n-6$)	nd	nd	nd	0.40	nd	nd
22:5($n-3$)	nd	nd	0.69	1.03	nd	nd
22:6($n-3$)	nd	nd	0.78[b]	4.1	nd	nd
22:5	nd	nd	0.05	0.24	nd	nd
22:4	nd	nd	0.07	0.54	nd	nd
Sum	0	0	26.3	21.2	3.4	2.8
Methoxy fatty acids						
CH_3O-FAME [a]	0.19	nd	nd	nd	nd	nd
4-CH_3O-14:0	2.1	nd	nd	nd	nd	nd
4-CH_3O-14:0	1.7	nd	nd	nd	nd	nd
6-CH_3O-15:0 and 5-CH_3O-15:0[c]	0.88	nd	nd	nd	nd	nd
9-CH_3O-15:0	10.3	nd	nd	nd	nd	nd
9-CH_3O-15:0	2.6	nd	nd	nd	nd	nd
9-CH_3O-16:0	0.94	nd	nd	nd	nd	nd
9-CH_3O-16:0	3.9	nd	nd	nd		nd
11-CH_3O-17:0	14.0	2.1	bql[b]	nd	nd	nd
11-CH_3O-17:0	1.6	nd	nd	nd	nd	nd
Sum	37.3	2.1	bql	0	0	0
Others						
Lactone 85	0.97	nd	nd	nd	nd	nd
Unknown	1.6	1.8	nd	nd	nd	nd
Unknown	nd	0.55	nd	nd	nd	nd
Sum	2.6	2.3	0	0	0	0

PLFA, phospholipid fatty acids; FFA, free fatty acids; TAG, triacylglycerol ester-linked fatty acids; WE, fatty acids esterified to alkyl esters. FAME abundances are given in percentage of total FAMEs of the corresponding lipid class. Double bond location is indicated when the compound's retention time matched that of authentic standards. bql, Below quantitation limits; nd, non-detected.

[a]Monounsaturated and cyclopropyl FAMEs cannot be distinguished on the basis of mass fragmentation and retention times.

[b]GC-MS analysis indicated a composite peak of 22:6($n-3$) and 11-CH_3O-17:0, the latter in minor amount.

[c]Tentative structural elucidation of a composite peak.

source of essential FAMEs in the microbial loop (Zhukova and Kharlamento 1999).

Nanoflagellates of the B & F culture are bacteriophages, thus bacterial fingerprints observed in B can be expected to be transferred to FAMEs of reserved lipids of B & F (Harvey et al. 1997). Some characteristics of bacterial FAMEs were indeed observed in TAG- and WE-FAMEs of B & F culture, for instance the occurrence of *iso*- and *anteiso*- 15:0 and 17:0 isomers. On the contrary, methoxy FAMEs identified in B were not transferred to the reserve lipids of flagellates whereas these compounds were more abundant than *iso*- and *anteiso*- acids in the B culture. Reserve lipids in B & F constituted only 3% of total lipids.

Discussion

Origin of mid-chain methoxy FAMEs: analytical artifact or biological source

The occurrence and the biological activity of FAMEs bearing a methoxy group at various locations of the alkyl chain have been reviewed (Carballeira 2002). Amongst this family of compounds, mid-chain locations of the methoxyl group characterized malyngamides (*N*-substituted amides) of cyanobacteria and total fatty acids or polar lipids of bacteria (Table 3). A series of mid-chain methoxylated FAMEs was also identified in the total lipids of a red algae, and the authors proposed that they might arise from symbiotic bacteria associated to the algae (Barnathan et al. 1998).

Methoxy FAMEs can be generated from alteration of cyclopropane FAMEs during acid catalyzed transesterification of phospholipids of bacteria (Orgambide et al. 1993). Cyclopropane fatty acids can be important constituents of bacterial acids (Guckert et al. 1986). Accordingly, the methoxy FAMEs evidenced in the present study might be suspected to be artifactual products of cyclopropane-containing biomarkers. The occurrence of cyclopropane FAMEs could not be ascertained by GC-MS in the cultures under study because of their low amounts and of their coelution with monounsaturated acids. In B, a structure matched cyclopropane-17:0 and/or monounsaturated 17:1 and another one could be cyclopropane-19:0 and/or 19:1 (Table 4). These compounds were present in low percentages in PLFA as well as in FFA of the B culture, whereas methoxy acids occurred essentially in PLFAs. This distinct repartition rules out that methoxy acids could derive from cyclopropane acids. In addition, temperature and duration of the transmethylation procedure were optimized for the recovery of polyunsaturated and cyclopropanoic acids in commercial mixtures of FAMEs. One hour transmethylation at 80 °C resulted in a decrease of polyunsaturated FAMEs and the total loss of cyclopropanoic ones. When the reaction was realized at 65 °C for 1 h, polyunsaturated FAMEs were quantitatively recovered and some loss of cyclopropanoic FAMEs occurred. Finally, when the transmethylation was done at 65 °C for 30 min, both polyunsaturated fatty acids and cyclopropanoic FAMEs were quantitatively recovered. We selected the latter conditions for the transmethylation of phospholipids and FFA of B and B & F, to limit the artifacts observed when higher temperature was used, alike in the procedure used by Orgambide et al. (1993).

Another artifactual source of methoxylated FAMEs was identified in freshwater sediments, where 3-methoxy-FAMEs were formed from 3-hydroxy-fatty acids in the course of saponification, accounting for about a tenth of their hydroxylated precursors (Stefanova and Disnar 2000). In the present study this artifact could not have produced mid-chain methoxyl FAMEs from corresponding hydroxyl derivatives because no mid-chain hydroxy-FAMEs could be detected and because the analytical procedure included no saponification.

The aforementioned clues point to a genuine origin of mid-chain methoxylated FAMEs in the structural lipids of free-living marine bacteria. Mid-chain methoxylated acids have already been reported in non-marine bacteria (Inamoto et al. 1995). The series of methoxylated FAMES reported in a Sicilian red algae showed similarities with the series identified in the present work, and the authors also confided to a genuine bacterial origin of methoxylated FAMEs (Barnathan et al. 1998). Finally, the biosynthetic pathway and the gene responsible for the addition of a mid-chain methoxy group were clearly established in some mycobacteria (Yuan and Barry 1996).

Unfortunately, no information on the types of bacteria occurring in this sample are available. However, free-living bacteria has been studied by amplified ribosomal DNA in surface (5 m depth) open Mediterranean and showed that they were composed by a diverse assemblage lacking representatives of the Archaeal domain, and that the assemblage structure was stable at the time scale of a few days and at the space scale of a few kilometers (Acinas et al. 1997). Further characterization by cloning and sequencing 16S ribosomial DNA of free-living bacteria from 5m showed that most clones were related to α Proteobacteria, and to a lesser extend to γ Proteobacteria (Acinas et al. 1999). We can only speculate that bacteria producing methoxy FAMEs might be affiliated to these domains. Further work in collaboration with molecular biologists is required and is on progress.

The series of methoxy FAMEs occurring in bacteria from oligotrophic waters was dominated by homologues with an alkyl chain length from 15 to 17 carbon atoms and the ether branch located at an odd carbon from C_9 to C_{11}. The most abundant series member, 11-CH_3O-17:0, has been reported in other bacteria, and in a sample of red algae, possibly produced by the bacteria associated with the algae (Table 3). Two homologues, 9-CH_3O-15:0 and 11-CH_3O-17:0 may be biosynthetically related by a chain elongation pathway similar to that proposed by Barnathan et al. (1998). The occurrence of even homologues in low amounts, 4-CH_3O-14:0, 5-CH_3O-16:0 and 6-CH_3O-16:0, nonetheless suggests that other pathways exist, or that the methoxyl group may be added on monoenoic acids (Yuan and Barry 1996).

The occurrence of these biomarkers, essentially esterified to phospholipids and almost absent as FFA in oligotrophic bacteria (Table 4) suggests that they have a biological role on the membrane properties. Similarly, most methoxy FAMEs were identified in polar lipids in other organisms than bacteria (Carballeira 2002), and ω-methoxy-FAMEs esterified to phospholipids showed higher biological activity than the corresponding free acids (Pidgeon et al. 1993). While antifungal, antibacterial and antitumor activities of 2-methoxy-acids were demonstrated and while ω-methoxy-phospholipids display antiviral activities, the only demonstrated biological activity of saturated mid-chain methoxylated PLFAs is an increase in membrane fluidity (Carballeira 2002).

The identification of mid-chain methoxy-FAME in the B culture provides the first report of these biomarkers in marine free-living bacteria. The sum of methoxy FAMEs represented about 28% of the PLFAs and 2% of the FAMEs

of the FFA of the B culture and their abundance set them as promising bacterial biomarkers (Table 4). In the B & F sample, only the dominant isomer could be detected and was coeluting with 22:6($n-3$). Most bacteria present in this sample were filamentous bacteria that develop in cultures after flagellate bloom and they are not typical of oligotrophic area. Although methoxy FAMEs are constituents of small coccoid bacteria typical of oligotrophic waters, they do not occur in all bacteria living in marine waters.

Transfer of bacterial methoxylated FAMEs to higher trophic levels?

The B & F sample comprises both heterotrophic nanoflagellates and their prey, free-living bacteria, so that lipid profile of this sample is a surimposition of both fingerprints. Monounsaturated FAMEs may originate from both flagellates and bacteria, while polyunsaturates, not observed in the B sample, were probably contributed by flagellates, in agreement with previous report on flagellate fatty acid composition (Zhukova and Kharlamento 1999). Reserve lipids of zooplankton usually comprise FAMEs originating from their diet (Waldock and Nacimiento 1979; Ederington et al. 1995). Nanoflagellates of the B & F culture are bacterivores grazing on small coccoid bacteria alike bacteria of the B culture. Bacteria did not comprise significant amounts of reserve lipids, so that wax esters and triacylglycerol esters of the B & F culture were most probably contributed by the flagellates only. The fatty acid composition of reserve lipids of flagellates showed the incorporation of the bacterial *iso*- and *anteiso*- 15:0 and 17:0 FAMEs, a feature already observed in total FAMEs of bacterivorous ciliates (Harvey et al. 1997). In contrast, methoxylated FAMEs were not incorporated to reserve lipids of flagellates. Owing to the low level of reserve lipids in the B & F culture, storage lipid synthesis and transfer of diet acids to storage lipids may not be triggered in the studied oligotrophic system. The lower levels of methoxy FAMEs in B & F, relatively to levels observed in the small coccoid bacteria of the B culture indicate that methoxy FAMEs may be restricted to small coccoid bacteria and may be less abundant in filamentous ones.

Conclusions

This study is the first report of phospholipid-ester linked fatty acids of free-living bacteria typical of oligotrophic waters. The occurrence of odd chain *iso*- and *anteiso*-FAMEs, together with mid-chain methoxy FAMEs characterizes the PLFAs of these bacteria. FAMEs bearing a methoxy group at various locations have been identified in a variety of organisms, including marine organisms and five methoxy FAMEs were identified in the phospholipids of coccoid bacteria typical of oligotrophic conditions. In contrast, filamentous bacteria developing in cultures after flagellate bloom were almost devoid of methoxy FAMEs The occurrence of these biomarkers in free-living

bacteria is of promising geochemical significance. The dominant methoxy homologues coelute with polyunsaturated fatty acids commonly found in algae, crustacean and psychrophyllic bacteria, which counsels systematic GC-MS confirmation of FAMEs from marine samples.

Unlike free-living bacteria of oligotrophic surface waters, bacterivorous nanoflagellates comprise polyunsaturated FAMEs, and thus are a source of essential lipids for higher trophic levels of the microbial loop. Establishing the fate of bacterial compounds in the microbial loop by using bacterial fingerprinting FAMEs is limited by the low levels of reserve lipids in nanoflagellates.

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