

# *Gammaproteobacteria* as a Possible Source of Eicosapentaenoic Acid in Anoxic Intertidal Sediments

Elke Freese · Heike Rütters · Jürgen Köster ·  
Jürgen Rullkötter · Henrik Sass

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**Abstract** Eicosapentaenoic acid (EPA;  $n$ -20:5 $\omega$ 3) was found to be a constituent of phospholipids in three mesophilic strains of *Gammaproteobacteria*, which were isolated from anoxic most probable number series prepared with sediments from an intertidal flat of the German North Sea coast. Their partial 16S rRNA gene sequences identified the isolates as close relatives of *Shewanella colwelliana*, *Vibrio splendidus*, and *Photobacterium lipolyticum*. So far, eicosapentaenoic acid has mainly been reported to occur in eukaryotes and some piezophilic or psychrophilic bacteria. With decreasing temperature, relative contents of EPA (up to 14% of total fatty acids) increased in all strains. Additionally, *Shewanella* and *Vibrio* spp. showed a significant increase in monounsaturated fatty acids with lower growth temperature. Analysis of the phospholipid compositions revealed that EPA was present in all three major phospholipid types, namely, phosphatidyl glycerol (PG), cardiolipin and phosphatidyl ethanolamine (PE). However, EPA was enriched in PG and cardiolipin relative to PE. In the tidal flat sediments from which the isolates were obtained, substantial amounts of EPA-containing PG were detected, whereas other typical micro-eukaryotic phospholipids—being also a possible source of EPA—were abundant at the sediment surface but were

present in clearly lower amounts in the anoxic layers beneath 5 cm depth. Therefore, the EPA-containing PG species in the deeper layers in these sediments may indicate the presence of *Gammaproteobacteria* closely related to the isolates. These bacteria appear to be an important source of EPA in buried, anoxic sediments beneath the layers harboring significant populations of benthic eukaryotes.

## Introduction

In bacteria, membranes do not solely act as a cell envelope; they also play a central role in many physiological processes like respiration, chemotaxis, and substrate uptake [23]. These processes rely on a narrow range of membrane phase behavior and organization that may be disturbed by changing environmental conditions [15, 23]. Bacteria are, however, able to alter the composition of their phospholipid fatty-acid side-chains in order to maintain appropriate membrane organization and function [58]. At low temperatures or high pressures, the content of unsaturated fatty acids often increases with a concomitant decline in saturated fatty acids [1, 23]. Polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA;  $n$ -20:5 $\omega$ 3) and docosahexaenoic acid ( $n$ -22:6 $\omega$ 3), are known to be particularly effective in the adjustment of membrane fluidity due to their low melting points [23, 37]. For a long time, these PUFAs were considered membrane components exclusively of eukaryotes, e.g., unicellular photosynthetic microeukaryotes [60, 61, 66] and fish ([6] and references therein). Accordingly, the presence of PUFAs in sediments was related to eukaryotic organisms [5, 48, 62].

Since the first detection of EPA in marine bacteria by Oliver and Colwell [45] and Johns and Perry [29] in the

E. Freese · H. Rütters · J. Köster · J. Rullkötter · H. Sass  
Institute of Chemistry and Biology of the Marine Environment (ICBM),  
Carl von Ossietzky University of Oldenburg,  
P.O. Box 2503, 26111, Oldenburg, Germany

H. Sass (✉)  
School of Earth, Ocean and Planetary Sciences,  
Cardiff University,  
Park Place,  
Cardiff CF10 3YE, Wales, UK  
e-mail: sassh@earth.cf.ac.uk

early to mid-1970s, several EPA-producing bacteria have been isolated. These isolates were generally psychro- or piezophiles from polar regions and the deep sea [10, 13, 20, 40, 44]. However, the recent isolation of mesophilic EPA-producing *Shewanella* species from a temperate estuary [55] and from shallow seawater samples [19, 27, 28] indicated that EPA may not be restricted to psychrophiles and piezophiles.

All so far known EPA-producing prokaryotes affiliate with only a few genera within two bacterial phyla: The *Gammaproteobacteria* (e.g., genera *Shewanella*, *Moritella*, *Colwellia*, *Alteromonas*, and *Photobacterium*) and the *Bacteroidetes* (e.g. *Flexibacter* and *Psychroserpens*). Therefore, EPA may be a potentially useful marker for these genera in environmental microbial communities if a eukaryotic origin can be excluded. In fact, EPA was detected among the microbial lipids in various polar or deep-sea sedimentary environments (e.g. [9, 12, 39]). However, substantial amounts of polar lipid-bound EPA were also found in temperate regions, e.g., in tidal flat sediments of the German North Sea coast—not only in the surface layers, but down to a depth of 35 cm [51], and more recently down to a depth of a few meters [18]. EPA in these ‘subsurface’ sediment layers was originally assumed to be derived from buried photosynthetic microeukaryotes or protozoan cysts [51].

In the present study, we analyzed three mesophilic bacterial isolates that contain significant amounts of EPA and that were isolated from the aforementioned [51] tidal flat. Bacteria related to these isolates may be a significant source of phospholipid-bound EPA in deeper, anoxic sediments beneath the layers dominated by benthic microeukaryotes. To reveal the potential as source organisms, the intact phospholipids of the isolated bacteria were analyzed and compared to those found in the original sediments. We also analyzed the physiological capacities of the isolates to find out whether they can grow under in situ conditions. To study the role of EPA in adaptation to in situ temperatures, the temperature dependence of fatty- acid patterns of these strains was investigated in detail.

## Materials and Methods

### Source and Cultivation of Organisms

Pure cultures of marine strains of *Gammaproteobacteria* were isolated from the highest positive dilutions of most probable number (MPN) dilution series prepared with anoxic media and different carbon substrates (Table 1, Rütters et al., unpublished results). The MPN series were

**Table 1** Morphological and physiological properties of the EPA-containing isolates, their isolation substrates, and respective MPN counts

	<i>Shewanella</i> sp. NB72	<i>Vibrio</i> sp. NB73	<i>Photobacterium</i> sp. SAMA2
Shape	Rod	Oval-rod	Oval-rod
Size (µm)	1.2–3.0×0.5–0.9	1.5–2.5×1.2–1.5	1.2–2.5×1.0–1.5
Temperate range (°C)	4–35	4–35	4–37
Isolation substrate	Laminarin	Chitin	Amino acids
MPN count (g <sup>-1</sup> )	1.1×10 <sup>6</sup>	2.4×10 <sup>8</sup>	8.0×10 <sup>5</sup>
MPN count (% of total cell count)	0.29	6.3	0.02
Substrates utilized			
Peptone	(+)	+	+
Cellulose	–	–	+
Chitin	–	+	+
Laminarin	+	+	+
Monosaccharides	+	+	+
Fatty acids	+	+	+
Alcohols	–	+	+
Mono- and dicarboxylic acids	+	+	+
Amino acids	(+)	+	+
Fermentation of			
Carbohydrates	+	+	+
Amino acids	(+)	+	+
Electron acceptors			
Nitrate	+	+	+
Nitrite	–	–	–
MnO <sub>2</sub>	+	–	–
Fe(III)	–	–	–
Thiosulfate	+	–	(+)

Electron donors were tested aerobically at 20°C.  
(+) Growth very weak or present only in part of the experiments

prepared with bicarbonate-buffered and sulfide-reduced artificial seawater as described previously [35, 56]. MPN series were inoculated with sediments from an intertidal flat near the village of Neuharlingsiel (53°42.5' N, 7°42.05' E, East Frisian Wadden Sea, NW Germany; [51]) and incubated at 20°C for 6 weeks. Strain SAMA2 was isolated in October 1998 from the upper 0.5 cm of the sediment ( $T_{in situ}=12^{\circ}\text{C}$ ) with amino acids (alanine 2.0 mmol·l<sup>-1</sup>, asparagine 1.0 mmol·l<sup>-1</sup>, arginine 1.0 mmol·l<sup>-1</sup>, and cysteine 1.0 mmol·l<sup>-1</sup>) as substrates. Strains NB72 and NB73 were both obtained from a depth of 5 cm in January 2000 ( $T_{in situ}=3^{\circ}\text{C}$ ) with laminarin (0.1%) and chitin (0.1%) as substrates, respectively.

Although the strains were isolated under anoxic conditions, they turned out to be facultative aerobes and, for further investigations, were routinely grown under oxic conditions to achieve higher growth yields. It is expected that this does not affect the production of EPA, as in *Bacteria*, PUFAs can be synthesized also under anoxic conditions [38, 41] and the fatty-acid patterns between aerobically and anaerobically grown cells are very similar [59]. For fatty-acid analysis, cells were grown in oxic artificial seawater buffered with 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate [56] using sodium lactate (10 mmol·l<sup>-1</sup>) as substrate at 10, 20, or 30°C. Close to the extremes of their temperature range for growth, some strains formed only very little biomass, and under the microscope, cells appeared unhealthy. These 'weak' cultures were not analyzed for their FA patterns. Since the analysis of intact phospholipids requires a relatively large amount of biomass, several individual cultures (four to eight depending on cell density) were combined before harvest. Parallels were not analyzed because parallel cultures generally show only little variation in their lipid patterns [34]. Cells were harvested at the end of their exponential or during the early stationary growth phase, washed with saline phosphate buffer (9 g l<sup>-1</sup> NaCl, 7.5 mmol l<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub>, 7.5 mmol l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2), freeze-dried and stored at -20°C. Intact phospholipids were analyzed from cells grown at 20°C.

Physiological experiments were performed to test the metabolic capacities of the isolates, their ability to grow under anoxic conditions, and the temperature range for growth. Electron donor tests and temperature series were prepared in 22-ml screw-cap tubes filled with 5 ml medium and with air as headspace. Anaerobic electron acceptor utilization and fermentation were tested in completely filled screw cap tubes and analyzed as described previously [35]. For temperature and electron acceptor tests, sodium lactate (10 mmol·l<sup>-1</sup>) served as electron donor.

#### 16S rDNA Sequencing and Phylogenetic Analysis

The phylogenetic affiliation of the three strains was determined by analysis of partial 16S rRNA genes. Nucleic

acids were extracted, and the bacterial 16S rDNA was amplified, using the *Bacteria*-specific primers 8f and 1492r [36] as described previously [53]. Polymerase chain reaction (PCR) products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequence analysis was performed by Amodia Biosystems (Braunschweig, Germany). For phylogenetic analysis, about 550-bp-long fragments were used, as very high similarities (99.3% to 100%) to well described species were obtained, and it was shown that phylogenetic assignments from partial and full-length sequences are very similar [54]. In order to find highest similarities to other bacteria, the novel 16S rDNA sequences of strains SAMA2, NB72, and NB73 were compared using the FASTA3 tool of EMBL-EBI (<http://www.ebi.ac.uk>). The partial 16S rRNA gene sequences of strains NB72, NB73, and SAMA2 are available at EMBL-EBI under the accession numbers AJ866937 to AJ866939.

#### Analysis of Whole-Cell Fatty-Acid Patterns

Freeze-dried cells (approximately 20 mg) were saponified under reflux overnight with 5 ml of 5% KOH solution in methanol/water 4:1 v/v under a nitrogen atmosphere. Afterwards, the alkaline solution was decanted into a separation funnel, and the solution was acidified with hydrochloric acid (2 mol·l<sup>-1</sup>). Non-saponifiable, solid residues were extracted three times with 10 ml dichloromethane each. These dichloromethane extracts were added to the acidified methanol/water phase in the separation funnel. After phase separation, the organic phase was removed, and the aqueous phase was re-extracted three times with 10 ml of dichloromethane each. Combined organic phases were dried over anhydrous sodium sulfate and evaporated to dryness.

#### Derivatization of Fatty Acids and Analysis by Gas Chromatography

An aliquot of the extracted total fatty acids was transformed into their trimethylsilyl (TMS) esters using *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. The TMS derivatives of fatty acids were analyzed on a gas chromatograph (Hewlett Packard HP 5890 series II, Hewlett Packard, Waldbronn, Germany) equipped with a KAS3 cold injection system (Gerstel, Mühlheim a. d. Ruhr, Germany) and a flame ionization detector on a DB-5HT column (30 m×0.25 mm, 0.1 µm film thickness, J&W, Folsom, CA, USA). For gas chromatography-mass spectrometry (GC-MS), a Finnigan MAT SSQ 710B mass spectrometer (Finnigan-Thermoquest, San Jose, CA, USA) was used. Positions of double bonds were determined using dimethyldisulfide as described by Dunkelblum et al. [16] for selected samples.

## Phospholipid and PLFA Analysis

Phospholipid analysis was performed as described in Rütters et al. [52]. In brief, the freeze-dried sediment samples were extracted using a modified Bligh and Dyer procedure [8]. The resulting extracts were separated by liquid chromatography on a silica gel column into various fractions of neutral and polar lipids. The most polar fraction (eluted with dichloromethane/methanol) was analyzed on an high-performance liquid chromatography instrument (Thermo Separation Products, San Jose, CA, USA) coupled to a Finnigan LCQ ion trap mass spectrometer equipped with an electrospray source (Thermoquest-Finnigan, San Jose, CA, USA) and to an evaporative light-scattering detector (Alltech ELSD 500, Deerfield, IL, USA) used for qualitative and quantitative analysis, respectively. Quantification was done after external calibration of ELSD signals with phospholipid standards representing major phospholipid types. Fatty-acid side-chains were assigned according to MS/MS experiments (cf. [51, 52]). Only in the case of cardiolipin the fatty-acid side-chains were only tentatively assigned because not enough information was obtained from MS/MS experiments to rigorously identify all four fatty-acid side-chains. For analysis of polar lipid fatty-acid (PLFA) patterns, an aliquot of the most polar fraction was transesterified by mild alkaline hydrolysis as described by White et al. [64]. The methyl esters obtained were identified by GC-MS (for details see Rütters et al. [52]).

## Results and Discussion

### Physiology and Phylogeny

The three bacterial strains were isolated under anoxic conditions and grew well by fermentation of amino acids or carbohydrates. Aerobically grown, they utilized a wide range of carbon sources, including biopolymers, carbohydrates, and fatty and amino acids (Table 1). All strains grew with nitrate as electron acceptor. Strain NB72 also reduced manganese oxide and thiosulfate. The temperature range for growth was similar for all strains ranging from 4°C to 35°C, defining them as psychrotolerant mesophiles. With respect to their nutritional capacities and their temperature range for growth, the strains can be seen as well adapted to life in oxic and anoxic sediment layers.

Analysis of partial 16S rRNA genes revealed that the three strains belonged to the *Gammaproteobacteria* and were closely related to already described species. Strains SAMA2 and NB73 affiliated with the *Vibrionaceae*, strain SAMA2 being most closely related to *Photobacterium lipolyticum*<sup>T</sup> (99.5% sequence similarity, 588 bp) and strain

NB73 to *Vibrio splendidus*<sup>T</sup> (99.3% sequence similarity, 588 bp). Strain NB72 turned out to be a member of the genus *Shewanella* within the *Alteromonadaceae*, with a 100% sequence similarity to *Shewanella colwelliana*<sup>T</sup> (587 bp).

### Fatty-Acid Patterns

Generally, the fatty-acid patterns of the three strains agree well with those of their closest relatives [13, 21, 33, 44, 50, 55], with *n*-16:0 and *n*-16:1 $\omega$ 7 being the dominant fatty acids (Table 2). All strains contained relatively high amounts of EPA (up to 14%). The fatty-acid patterns of *Photobacterium* sp. SAMA2 and *Vibrio* sp. NB73 were very similar to each other but differed from that of *Shewanella* sp. NB72 by containing higher amounts of *n*-16:1 $\omega$ 7 and *n*-18:1 $\omega$ 7 fatty acids. *Shewanella* sp. NB72, in turn, possessed much higher amounts of branched-chain fatty acids (10–24%), with *i*-15:0 being the most prominent one. *Trans*-fatty acids (mainly *trans*-16:1 $\omega$ 7) were found in *Vibrio* sp. NB73, with the highest relative abundance of about 10% in the culture grown at 10°C (Table 2).

### Temperature Dependence of Whole-Cell Fatty-Acid Patterns

All three strains changed their fatty-acid patterns with changing temperatures (Table 2, Fig. 1). In *Shewanella* sp. NB72 and *Vibrio* sp. NB73, the proportion of monounsaturated fatty acids (MUFAs) increased at lower temperatures (Table 2, Fig. 1), with the most pronounced change occurring between cells grown at 30°C or 20°C. In contrast, in *Photobacterium* sp. SAMA2, only relatively minor changes in saturated straight-chain fatty acids (SCFAs) and MUFAs were observed, and the ratio of MUFAs to SCFAs remained almost constant (Table 2).

Relative abundances of EPA increased with decreasing growth temperature in all strains. In *Shewanella* sp. NB72, the relative increase in EPA was highest between cells grown at 30°C to 20°C (max. 5% of total fatty acids). In *Vibrio* sp. NB73 (maximum, 6.4% of total fatty acids) and *Photobacterium* sp. SAMA2 (maximum, 13.9% of total fatty acids), largest differences in relative abundances of EPA were observed between cultures grown at 20°C and 10°C. These findings agree with data for other *Vibrio* [21, 30, 49] and *Shewanella* species [55, 63]. As in *Photobacterium* sp. SAMA2, the relative abundances of MUFAs in cells grown at 10°C, 20°C, or 30°C vary only slightly; the relative increase of EPA appears to be the main mechanism of adaptation to low temperatures. Likewise, a mutant strain of *Photobacterium profundum* produced little MUFAs but elevated levels of EPA and was neither low-temperature nor high-pressure sensitive [1]. Data summarized

**Table 2** Cellular fatty acid composition (%) after whole-cell hydrolysis of the isolates

Fatty acid	<i>Shewanella</i> sp. NB72			<i>Vibrio</i> sp. NB73			<i>Photobacterium</i> sp. SAMA2		
	10°C	20°C	30°C	10°C	20°C	30°C	10°C	20°C	30°C
Saturated straight-chain fatty acids (SCFAs)									
<i>n</i> -12:0	3.2	5.1	4.2	3.1	4.6	4.3	0.5	1.3	0.7
<i>n</i> -13:0	0.2	2.7	0.2	0.5	–	0.2	0.2	–	0.1
<i>n</i> -14:0	5.5	6.4	4.4	4.0	4.7	8.4	2.0	4.0	3.4
<i>n</i> -15:0	1.1	1.0	1.0	0.6	0.4	1.8	0.5	2.3	0.8
<i>n</i> -16:0	20.6	23.9	28.4	23.4	30.2	34.3	27.6	24.9	28.6
<i>n</i> -17:0	0.2	0.3	0.5	0.3	0.2	0.5	0.1	0.5	0.1
<i>n</i> -18:0	1.8	1.9	4.7	4.0	1.7	13.6	1.3	3.4	1.8
<i>n</i> -20:0	–	–	–	–	–	0.4	–	–	–
Branched-chain fatty acids (BCFAs)									
<i>i</i> -13:0	3.5	–	5.2	0.1	–	0.2	–	–	–
<i>i</i> -14:0	0.3	0.5	0.4	–	–	–	0.1	–	–
<i>i</i> -15:0	13.8	9.3	16.2	0.3	–	0.6	0.1	0.6	0.2
<i>ai</i> -15:0	0.5	–	0.6	–	–	0.1	0.1	0.7	0.1
<i>i</i> -16:0	0.5	–	0.3	0.1	–	0.2	0.1	–	0.1
<i>i</i> -17:0	0.4	0.4	1.0	–	–	–	–	–	0.1
<i>i</i> -18:0	0.2	–	0.1	0.1	–	0.4	0.2	–	0.2
Mono- and polyunsaturated fatty acids (MUFAs, PUFAs)									
<i>n</i> -14:1 $\omega$ 5	0.4	–	0.4	0.2	0.3	0.3	0.6	1.0	0.5
<i>n</i> -15:1 $\omega$ 6	–	–	–	–	–	–	1.1	–	1.1
<i>n</i> -16:1 $\omega$ 9	1.8	2.1	1.0	<0.1	<0.1	0.9	1.0	1.4	0.7
<i>n</i> -16:1 $\omega$ 7c	31.5	32.5	24.6	27.9	44.6	22.6	38.0	41.5	45.1
<i>n</i> -16:1 $\omega$ 7t	–	–	–	9.9	–	1.5	–	–	–
<i>i</i> -17:1 $\omega$ 7	0.1	–	0.3	–	–	–	0.1	–	–
<i>n</i> -17:1 $\omega$ 8	1.0	0.7	0.8	0.2	–	0.2	0.1	–	–
<i>n</i> -17:1 $\omega$ 6	–	–	–	–	–	–	0.3	–	0.4
<i>n</i> -18:1 $\omega$ 9	2.8	3.5	2.2	0.5	0.5	2.8	0.5	1.9	0.6
<i>n</i> -18:1 $\omega$ 7c	5.5	4.6	3.3	17.2	11.3	5.4	11.3	14.3	14.4
<i>n</i> -18:1 $\omega$ 7t	–	–	–	0.6	–	0.2	–	–	–
11Me-18:1	–	0.4	–	–	0.3	–	–	–	–
<i>n</i> -20:1 $\omega$ 7	1.0	–	–	0.9	–	0.3	0.4	–	–
<i>n</i> -20:5 $\omega$ 3	4.0	5.0	0.3	6.4	1.3	0.9	13.9	2.5	1.2
$\Sigma$ SCFAs	32.6	41.3	43.4	35.9	41.8	63.5	32.2	36.4	35.5
$\Sigma$ BCFAs	19.3	10.2	24.1	0.6	–	1.5	0.7	1.3	0.7
$\Sigma$ MUFAs	44.1	43.8	32.6	57.4	57.0	33.9	53.4	60.1	62.8
UFAs/SFAs	0.9	0.9	0.5	1.7	1.4	0.5	2.1	1.7	1.8

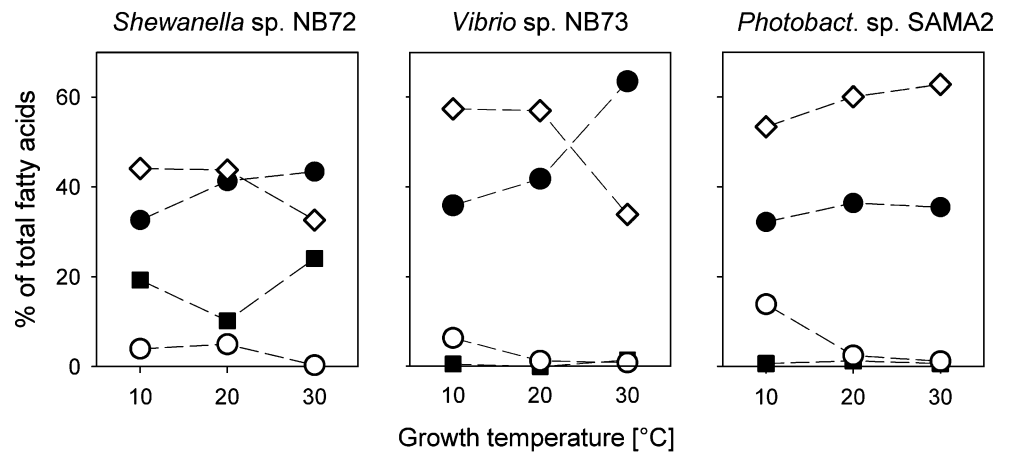
Fatty acids are denoted as total number of carbon atoms, followed by the number of double bonds and the position of the double bonds counted from the aliphatic end of the molecule. Structural isomers are denoted by prefixes: *i* iso and *ai* anteiso.

(–) Not detectable, *UFAs* unsaturated fatty acids (MUFAs+PUFAs), *SFAs* saturated fatty acids (SCFAs+BCFAs)

by Hirota et al. [26] suggested that EPA production of species related to *Shewanella pneumatophori* is limited to a maximum growth temperature of approximately 30°C, probably indicating that temperature-sensitive enzymes are involved in biosynthesis. This may explain the low values found in *Shewanella* sp. NB72 grown at 30°C. From the literature, it appears that the amount of EPA produced exclusively depends on temperature but is independent of other environmental parameters like salinity [24], anoxia [38, 41], or variations in nutrient composition and availability, although these were shown to influence the composition of other fatty acids [30].

Branched fatty acids (BCFAs) have repeatedly been reported to promote cold adaptation [4, 11, 58]. However, the results obtained with *Shewanella* sp. NB72 are ambiguous. Although the relative contribution of BCFAs is higher at 10°C than at 20°C, highest values were observed in cells grown at 30°C. In the latter case, however, it appears that BCFAs compensate for a strong decrease in MUFAs. Similarly, increasing contribution of BCFAs at lower and higher temperatures were observed for *Desulfobacterium autotrophicum* [46]. For other bacteria, no clear changes in BCFAs with varying growth temperature were observed [25, 43].

**Figure 1** Relative abundances of saturated straight-chain fatty acids (SCFAs, filled circles), branched-chain fatty acids (BCFAs, filled squares), mono-unsaturated fatty acids (MUFAs, open diamonds) and eicosapentaenoic acid (EPA, open circles) as percentage of total fatty acids in the isolates investigated



Presence of EPA in Bacterial Phospholipids

Major phospholipid types of all three strains were phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), and cardiolipin (CL). PE was the most abundant compound in *Shewanella* sp. NB72 and *Vibrio* sp. NB73, accounting for 60% and 77% of total phospholipids. These values agree well with literature data [e.g. 14, 19, 45]. In contrast to literature data for other *Photobacterium* spp. [17, 31, 57], which show a clear dominance of PE, in *Photobacterium* sp. SAMA2, PE and PG were present in equal amounts accounting for 38% of total phospholipids each.

The most abundant molecular species of all three major phospholipids types possessed combinations of 16:0 and 16:1 fatty-acid side-chains (Table 3). EPA as a side-chain

was detected in all three phospholipid types. EPA-containing species were most abundant in PG (17–30% of all PG molecular species) and CL (15–30% of all CL molecular species), in which EPA occurred in combination with saturated and monounsaturated C<sub>16</sub> and C<sub>18</sub> fatty-acid substituents (Table 3). PE species containing EPA were less abundant (3–10% of all PE molecular species). Furthermore, in PE, EPA was exclusively found in combination with saturated and monounsaturated C<sub>16</sub> fatty acids. Even greater differences in the combination of EPA with other fatty acids between PG and PE were reported for *Shewanella gelidimarina* [42] and a “*Vibrio*” sp. [24, 25]. Whereas EPA is preferentially combined with BCFAs in PE in these organisms, it is associated with straight-chain saturated or monounsaturated fatty acids in PG.

**Table 3** Most abundant phospholipid species in bacterial isolates at 20°C

	<i>m/z</i>	Fatty acid side-chains	<i>Shewanella</i> sp. NB72	<i>Vibrio</i> sp. NB 73	<i>Photobacterium</i> sp. SAMA2
<b>PG</b>	717	16:1/16:1	28	33	34
	719	16:1/16:0	100	100	100
	745	16:1/18:1	42	59	68
	747	16:0/18:1	38	17	17
	765	<b>16:1/20:5</b>	<b>25</b>	<b>16</b>	<b>12</b>
	767	<b>16:0/20:5</b>	<b>54</b>	<b>17</b>	<b>30</b>
	793	<b>18:1/20:5</b>	<b>20</b>	<b>10</b>	<b>24</b>
<b>CL</b>	1346	16:0/3x 16:1	72	63	86
	1348	2x 16:0/2x 16:1	100	100	100
	1374	16:0/16:1/2x18:1	70	84	54
	1376	16:0/16:1/18:1/18:0	25	28	23
	1394	<b>16:0/2x 16:1/20:5</b>	<b>67</b>	<b>20</b>	<b>52</b>
	1396	<b>2x 16:0/16:1/20:5</b>	<b>78</b>	<b>19</b>	<b>47</b>
	1422	<b>16:0/16:1/18:1/20:5</b>	<b>45</b>	<b>13</b>	<b>30</b>
<b>PE</b>	686	16:1/16:1	20	38	60
	688	16:1/16:0	100	100	100
	714	16:1/18:1	27	39	72
	736	<b>16:0/20:5</b>	<b>16</b>	<b>n.d.</b>	<b>7</b>

Abundances are given relative to most abundant phospholipid molecular species (in %). EPA-containing species are printed in bold.

### Presence of EPA in Temperate Tidal Flat Sediments

To reveal a potential contribution of the isolates and closely related bacteria to the sediment EPA pool particularly in the anoxic layers, the original sediments were analyzed for their intact phospholipids contents. In the October 1998 core from which *Photobacterium* sp. SAMA2 was isolated, EPA accounted for up to 15% of the PLFAs. Although absolute amounts of polar-lipid-bound EPA contents generally decreased with depth, even between 10 and 20 cm, i.e., in the anoxic part of the core, up to 4% of the PLFAs were still found to be EPA (data not shown). However, the upper 5 cm and the deeper layers (e.g., 11–15 cm) differed with respect to the EPA-containing intact phospholipid types (Tables 4 and 5): In the uppermost 5 cm of the sediment, EPA was one of the major substituents in phosphatidyl choline (PC), phosphatidyl serine (PS), and phosphatidyl inositol (PI). In addition, PC and PS species with 22:5 and/or 22:6 fatty acids were detected in the uppermost 5 cm. The presence of these typical eukaryotic lipids in the upper anoxic layers (1–5 cm depth) may be explained by the vertical migration of photosynthetic microeukaryotes like diatoms or the presence of other microeukaryotes like ciliates [2, 3, 61, 66]. In contrast, at 11–15 cm depth, phosphatidyl glycerol was found to be the major phospholipid type containing EPA. At this depth, PC was mainly substituted with saturated and monounsaturated C<sub>18</sub> fatty acids. C<sub>22</sub> PUFAs like those found in microeukaryotes [2, 3, 61, 66] were not detected in any phospholipid type at that depth.

In the January 2000 core, from which *Shewanella* sp. NB72 and *Vibrio* sp. NB73 were isolated, 8% and 3% of the PLFAs were found to be EPA at the surface and at a depth of 2–6 cm, respectively. As in the October 1998 core,

EPA was one of the major fatty-acid side-chains in PC and PI at the sediment surface, with smaller portions being present in PG and PE. However, already at a sediment depth of 2–6 cm, the detected phospholipid species indicate that the proportion of eukaryotic biomass is low. The most abundant molecular species among PC contained two 18:1 fatty-acid substituents, while relative abundances of EPA-containing phospholipid species remained nearly constant among PG (Tables 4 and 5).

### *Gammaproteobacteria* as a Source of EPA in Anoxic Sediment Layers

Since PUFA-producing bacteria were so far known only from polar regions or deep-sea habitats, it was assumed that buried algal biomass was the source of EPA detected in ‘subsurface’ sediment layers (cf. [5, 7]). However, in the phospholipids of photosynthetic microeukaryotes, PUFAs with 16, 18, 20, and 22 carbon atoms occur in addition to EPA (e.g., [2, 3, 32]). In our sediments, PUFAs other than EPA were only detected among the phospholipids in the uppermost centimeters of sediment, but not in deeper layers (Table 5). Furthermore, in deeper sediment layers, the major EPA-containing phospholipid type was phosphatidyl glycerol, a common bacterial phospholipid, whereas contents of PC and PI that are major microeukaryotic lipids [2, 3, 32] decreased rapidly with depth in both sediment cores (Table 4). Therefore, photosynthetic microeukaryotes such as diatoms are the most likely source of EPA-containing phospholipids in the surface sediments, whereas EPA bound to PG detected in deeper sediment layers indicates a bacterial origin. *Gammaproteobacteria* related to the genera *Photobacterium*, *Vibrio*, and *Shewanella* such as our isolates appear to be promising candidates as source

**Table 4** Contents of major phospholipid types in tidal flat sediments (μg/g sediment dry weight)

Sampling date	October <sup>a</sup>			January	
	0–1 cm	1–5 cm	11–15 cm	0–1 cm	2–6 cm
Phosphatidyl glycerol (containing ether bonds)	3.0	2.1	0.3	1.3	0.7
Phosphatidyl glycerol	<b>4.8</b>	<b>3.1</b>	<b>1.2</b>	<b>6.5</b>	<b>3.2</b>
Cardiolipin	2.4	2.2	1.3	n.q.	4.5
Phosphatidyl ethanolamines	<b>1.2</b>	<b>0.9</b>	n.q.	<b>2.9</b>	<b>1.4</b>
Phosphatidyl cholines (containing ether bonds)	<b>3.2</b>	<b>1.3</b>	<b>0.2</b>	<b>3.0</b>	<b>0.6</b>
Phosphatidyl cholines	<b>5.1</b>	<b>2.9</b>	<b>0.7</b>	<b>4.7</b>	<b>1.5</b>
Phosphatidyl serines	<b>1.6</b>	<b>1.0</b>	n.d.	n.d.	n.d.
Phosphatidyl inositols	<b>3.7</b>	<b>2.0</b>	n.d.	<b>2.0</b>	<b>1.0</b>
Σ Unknowns	3.7	2.7	0.5	2.8	1.5

EPA-containing phospholipid types are printed in bold.

n.q. Not quantifiable, n.d. not detected

<sup>a</sup>Data from Rütters et al. [51]

**Table 5** Major phospholipid molecular species in tidal flat sediments

	<i>m/z</i>	Fatty acid side-chains	Sampling date				
			October			January	
			Sediment depth: 0–1 cm	Sediment depth: 1–5 cm	Sediment depth: 11–15 cm	Sediment depth: 0–1 cm	Sediment depth: 2–6 cm
<b>PG</b>	707	15:0/16:0	17	29	46	21	22
	717	16:1/16:1	18	15	<10	39	23
	719	16:1/16:0	100	100	100	100	100
	721	16:0/16:0	22	33	62	22	22
	733	16:1/17:0	27	33	41	31	35
	745	16:1/18:1	47	41	28	88	88
	747	16:0/18:1	44	62	66	53	58
	765	16:1/20:5	52	48	18	44	30
	767	16:0/20:5	26	22	10	17	17
	773	18:1/18:1	55	47	54	54	46
<b>PE</b>	674	15:0/16:1	26	19	n.q.	27	21
	686	16:1/16:1	31	22	n.q.	59	52
	688	16:1/16:0	100	100	n.q.	69	70
	700	16:1/17:1	25	17	n.q.	30	22
	702	16:1/17:0	39	43	n.q.	36	32
	714	16:1/18:1	98	80	n.q.	100	100
	716	16:1/18:0	33	27	n.q.	33	28
	728	17:1/18:1	22	12	n.q.	38	33
	734	16:1/20:5	n.d.	n.d.	n.q.	15	n.d.
	736	16:0/20:5	27	11	n.q.	13	<10
<b>PCM</b>	742	18:1/18:1	27	21	n.q.	34	23
	762	18:1/20:5	40	12	n.q.	20	11
	764	18:0/20:5	42	15	n.q.	29	12
	762	O-16:0/16:1	14	40	33	23	34
	776	O-16:0/17:1	13	50	74	13	61
	790	O-16:0/18:1	23	57	100	18	54
	810	O-16:0/20:5	100	100	79	100	100
	812	O-16:0/20:4	24	38	28	44	49
	836	O-16:0/22:6	62	62	17	45	68
	838	O-16:0/22:5	48	42	25	47	37
<b>PC</b>	862	O-18:1/22:6	13	19	n.d.	n.d.	32
	864	O-18:1/22:5	16	27	n.d.	23	n.d.
	776	16:0/16:1	33	36	33	41	43
	802	16:1/18:1	19	25	17	28	37
	804	16:0/18:1	35	45	54	35	43
	824	16:0/20:5	100	100	36	100	98
	830	18:1/18:1	24	38	100	61	100
	850	18:1/20:5 or 16:0/22:6	75	52	14	93	89
	852	18:0/20:5 or 16:0/22:5	45	42	10	57	78
	878	20:1/20:5	27	16	<10	32	39
<b>PS</b>	808	18:0/20:5	100	100	n.d.	n.d.	n.d.
	834	18:0/22:6	26	63	n.d.	n.d.	n.d.
<b>PI</b>	807	16:0/16:1	88	100	n.q.	18	n.q.
	883	20:5/18:0	100	33	n.q.	100	n.q.
	885	20:4/18:0	37	25	n.q.	93	n.q.
	909	20:5/20:1	42	22	n.q.	39	n.q.
	911	20:4/20:1	42	33	n.q.	26	n.q.

Abundances are given relative to most abundant phospholipid molecular species (in %). PUFA-containing species are printed in bold. *n.q.* Not quantifiable, *n.d.* not detected



organisms because PG is one of their major EPA-containing phospholipid types. However, contents of PE, the most abundant phospholipid type in our isolates, were surprisingly low in deeper sediments. This may be explained by the contribution of other EPA-producing bacterial genera possessing PG as their major phospholipid type. Molecular surveys of sediments from the area have revealed a large diversity of so far uncultured *Bacteria* including *Gammaproteobacteria* only remotely related to any known genera [65]. However, the assumption that bacteria closely related to the three isolates investigated in this study are abundant in the tidal flat sediments, and hence, a likely source of EPA is supported by the repeated isolation from highest dilutions of MPN series ([35], Sass and Rütters, unpublished results) but also by molecular investigations. Hardwick et al. [22] showed that members of the genera *Vibrio* (up to 28%), *Shewanella* (up to 6%), and related *Gammaproteobacteria* can represent a significant fraction of the microbial communities in coastal sediments.

Using the conversion factors presented by Rajendran et al. [47], which are 100  $\mu\text{mol}$  PLFAs per-gram bacterial dry weight and  $5.9 \times 10^{12}$  cells per-gram dry weight, MPN counts given in Table 1 can be converted into PLFA contents. The estimated potential contribution of EPA by our isolates ranges from 0.0006 (*Photobacterium* sp. SAMA2, October 1998) to 0.078  $\mu\text{g}$  EPA  $\text{g}^{-1}$  sediment (*Vibrio* sp. NB73, January 2000). These values are close to those found in situ. In the October 1998, core EPA contents in the PLFA fraction were 0.54  $\mu\text{g}$  EPA  $\text{g}^{-1}$  sediment in the 1- to 5-cm-depth interval [51] in which a significant contribution of eukaryotes was found as indicated by the high contents of PC and PI (Table 4). In contrast, in the 11- to 15-cm-depth interval, which was dominated by bacterial lipids, only 0.09  $\mu\text{g}$  EPA  $\text{g}^{-1}$  sediment was found [51]. This indicates that bacteria may indeed be the main source of EPA in the layers underneath the zone harboring photosynthetic microeukaryotes. Since all three strains were isolated under anoxic conditions and grew well by fermentation and were capable of using alternative electron acceptors, it can be expected that they are actively growing and present in numbers also at greater depths.

## Conclusions

The presence of eicosapentaenoic acid in anoxic sediment layers has previously been attributed to buried algal cells, microeukaryotes, or burrowing zoobenthos. The presence of EPA in bacterial lipids was rather seen as a physiological curiosity restricted to psychrophiles from polar regions or the deep sea. However, the occurrence of EPA in our investigated strains of *Shewanella*, *Vibrio*, and *Photobacterium* spp. suggests that the ability to produce PUFAs

is more widespread among mesophilic *Gammaproteobacteria* than previously known. As these strains were isolated from anoxic layers and grew well anaerobically, our results suggest that *Gammaproteobacteria* are the likely source of EPA present in deeper, anoxic sediments, particularly, as it has been shown that various bacteria can synthesize PUFAs under strictly anoxic conditions (e.g., [38, 41]). Analysis of the intact phospholipids shows that, at the sediment surface, EPA is present in lipids typically found in photosynthetic microeukaryotes, whereas in the anoxic layers, EPA is mostly present in PG, as in our bacterial isolates. These results indicate that the interpretation of the presence of algal cells in sediment based on the occurrence of EPA has to be done with caution. Analysis of intact phospholipids can provide supporting information to allow a better distinction between eukaryotic and bacterial microbial community members.

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