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Polyunsaturated fatty acids in Antarctic bacteria

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Abstract: Thirty eight strains of Antarctic bacteria were screened for the ability to produce polyunsaturated fatty acids (PUFA). Five strains contained eicosapentaenoic acid (20:5ω3) in the range of trace to 3.3% of total fatty acids, and up to 1.4 mg g^{-1} dry weight. Thirteen strains produced polyunsaturates including 18:2ω6, 18:3ω3, 18:4ω3 and 20:4ω6 in the range of trace to 7.0% of total fatty acids. Although the data set is currently small, the proportion of Antarctic strains found to produce PUFA's is higher than that found for temperate marine bacteria (and is similar to that recorded for barophilic bacteria). This suggests that the Antarctic environment has naturally selected for bacterial strains capable of maintaining membrane lipid fluidity by the production of PUFA. These results highlight the potential of Antarctic bacteria for possible consideration in the industrial production of PUFA. The fatty acid composition of *Flectobacillus glomeratus* is reported and discussed in relation to other closely related Antarctic flavobacteria. Fatty acid composition is also shown to represent an important chemotaxonomic tool to aid with the identification of Antarctic bacteria.

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Key words: polyunsaturated fatty acids, bacteria, chemotaxonomy, Antarctica

Introduction

The production of polyunsaturated fatty acids (PUFA) represents a significant biotechnological resource due to their unique characteristics. In humans, PUFA such as eicosapentaenoic acid [20:5ω3; (EPA)] exhibit a wide range of beneficial physiological effects including lowering of plasma cholesterol and triacylglycerols, prevention of certain cardiovascular diseases (atherosclerosis and thrombosis) and reducing the risk of breast, colon and pancreatic cancers (Kelly 1991). PUFA also have high nutritional value in the diet of many economically important mariculture species, increasing the overall health and growth rate of oysters, scallops and salmonoid fish (Langdon & Waldock 1981, Bell *et al.* 1991). A cheap and reliable source of PUFA is therefore of great interest in many industrial and health fields.

At present, only certain fish oils and algal species are utilized as industrial sources of PUFA. Bacterial sources of PUFA, however, possess two distinct advantages over these:

1) The majority of algal species generally require strictly controlled growth conditions in terms of nutrients, light quantity and quality, oxygenation and carbon dioxide levels; these factors can result in considerable expense. In contrast, most bacteria are not fastidious, and can often be grown on the waste products of other agricultural or industrial processes.

2) Bacteria, because of their ability to be cultured and maintained almost indefinitely, can be considered as a more easily renewable resource for the production of PUFA than fish oils extracted from rapidly diminishing fish stocks.

Historically, it was considered that non-photosynthetic bacteria did not contain long chain PUFA (Goldfine 1972). However, since 1977, numerous reports have appeared confirming the presence of PUFA in a range of eubacterial species, mostly isolated from temperate marine environments (Johns & Perry 1977, DeLong & Yayanos 1986, Wirsen *et al.* 1987, Jacq *et al.* 1989, Yazawa *et al.* 1988a&b, Akimoto *et al.* 1990, Matsui *et al.* 1991, Temara *et al.* 1991, Intriago 1992).

Survival in the extreme conditions of the Antarctic environment may lead to the synthesis of novel lipids by microorganisms. Lipid composition is central to this survival as the membrane bilayer must remain in a fluid state to enable normal cellular processes to continue (Singer & Nicolson 1972, Langworthy 1982). Microorganisms regulate membrane fluidity in response to low growth temperatures by altering the lipid composition of the membrane to reduce the melting point of its constituent phospholipids (Melchior 1982). This reduction in phospholipid melting point can be achieved by increasing the degree of fatty acid unsaturation, which may include the synthesis of PUFA (Russell 1990). Indeed, an increase in fatty acid unsaturation with decreasing growth temperature has been observed for a number of Antarctic microorganisms (Fukunaga & Russell 1990, Finegold *et al.* 1990). Antarctic bacteria were therefore examined for the ability to produce PUFA as an adaptation to low temperature environments.

Materials and methods

Twenty three bacterial strains were obtained from the Australian Collection of Antarctic Microorganisms (ACAM), University of Tasmania. Isolates were chosen on the basis of potential

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Table I. Brief characteristics of ACAM strains

ACAM Strain	Identification*	Isolated from*	Depth (m)*	Gram reaction	Oxidase	Catalse	O/F	Growth at 25°C
113	*Pseudomonas* sp.	Burton Lake	10	(-) Rod	(+)	(+)	O	(-)
123	Unidentified	Burton Lake	10.3	(-) Pl. Rod	(+)	weak (+)	O	(-)
146	*Pseudomonas* sp.	Burton Lake	11.2	(-) Rod	(-)	(-)	N/G	(-)
149	Unidentified	Burton Lake	12	(-) Seg. Fil	(+)	(-)	N/G	(-)
150	*Pseudomonas* sp.	Burton Lake	12	(-) Rod	(+)	(+)	O	(+)
151	*Pseudomonas* sp.	Burton Lake	12.3	(-) Rod	(+)	weak (+)	O	(-)
162	*Pseudomonas* sp.	Burton Lake	11.8	(-) Pl. Rod	(+)	(-)	O	(-)
166	*Pseudomonas* sp.	Burton Lake	11.5	(-) Rod	(+)	(+)	O	(-)
167	*Flectobacillus glomeratus*	Burton Lake	10.7	(-) Rod	(+)	(-)	N/G	(-)
171	Unidentified	Burton Lake	11	(-) Pl. Rod	(+)	(-)	G/A	(-)
179	Unidentified	Burton Lake	10.5	(-) Pl. Rod	(+)	(-)	G/A	(-)
181	Unidentified	Burton Lake	11.6	(-) Rod	(-)	(+)	N/G	(-)
188	*Pseudomonas* sp.	Burton Lake	10.4	(-) Rod	(+)	weak (+)	N/G	(-)
282	Unidentified	Penguin guano	Surface	(-) Cocci	(+)	(+)	A	(+)
283	Unidentified	Penguin guano	Surface	(-) Cocci	(+)	(+)	A	(+)
284	Unidentified	Penguin guano	Surface	(-) Cocci	(+)	(+)	A	(+)
285	Unidentified	Penguin guano	Surface	(-) Cocci	(+)	(+)	A	(+)
286	Unidentified	Penguin guano	Surface	(-) Cocci	(+)	(+)	A	(+)
298	Unidentified	Penguin guano	Surface	(-) Coccibacilli	(+)	(+)	A	(-)
299	Unidentified	Penguin guano	Surface	(-) Rod	(+)	(+)	A	(+)
301	Unidentified	Penguin guano	Surface	(-) Coccibacilli	(+)	(+)	A	(+)
302	Unidentified	Penguin guano	Surface	(-) Coccibacilli	(+)	(+)	A	(-)
304	Unidentified	Penguin guano	Surface	(-) Cocci	(-)	(+)	O	(-)

Legend: N/G = No visible growth; O = Oxidative; O/F = Oxidative/Fermatative growth
F = Fermentative; Pl. = Pleomorphic; A = Alkaline reaction
Seg. Fil = Segmented filaments; G/A = Growth no acid; *Mancuso *et al.* (1991)

psychrophilic or psychrotolerant growth. These are referred to by their three digit numeric codes (Table I). In addition, fifteen isolates were collected from Antarctic marine and lake sites during the 1990/91 summer season, and are denoted by a three letter code (Table II). A further two isolates were isolated from Antarctic marine sediment from Marchant's Landing near Davis Station, and are denoted by a two letter and single numeric code (Table II). Selected biological and biochemical characteristics of the strains were examined as described by Smibert & Krieg (1981).

For biomass production, strains were inoculated into 10 ml of Zobell's medium (Zobell 1946) and incubated at 15°C until turbidity was apparent. The 10 ml cultures were then used to inoculate 90 ml volumes of Zobell's medium contained in 500 ml conical flasks pre-rinsed in chloroform. Flasks were incubated at 15°C with agitation provided by a magnetic stirrer or orbital shaker (100 rpm) for 24–48 h until sufficient mass of estimated late-log phase cells were present for harvest.

Broth cultures (100 ml) were transferred to chloroform-rinsed 250 ml teflon centrifuge bottles containing 100 ml of 0.45 μm filtered natural seawater. Following centrifugation at 4600*g* for 25 min, the supernatant was removed and the cell pellet resuspended in a further 200 ml of 0.45 μm filtered natural seawater. Samples were again centrifuged for 35 min at 4600*g* and the supernatant decanted. The remaining cell pellet was collected using one of two procedures depending on the sample involved. For the majority of isolates, the cell pellets were resuspended in *c.* 3 ml of 0.45 μm filtered seawater and transferred to chloroform rinsed McCartney bottles and stored at -70°C until analysis. The extensive extracellular slime of strains JOO1 and JOO2 necessitated modification of harvest procedures due to difficulties experienced in obtaining a sufficiently compacted cell pellet. Cells were suspended in a small portion of seawater before transfer to chloroform-rinsed McCartney bottles and then stored as described above. For quantitative analysis, cell suspensions were transferred to a chloroform-rinsed, 30 ml test tube of known mass before freeze drying for 48 h. The amount of water lost was then determined by reweighing, and assuming a seawater salt concentration of 3.5% and density of 1.04 g l^{-1}, the mass of salt remaining in the sample was calculated and subtracted from the difference between initial and final test tube weights, to yield the dry mass of sample.

Lipids were extracted using the modified one-phase chloroform-methanol Bligh & Dyer extraction (White *et al.* 1979). A portion of the total lipid extract recovered from the lower chloroform phase was then saponified by reaction at 80°C for three hours using 3 ml 5% KOH in 80% MeOH (w/v) solution. After the addition of water (1 ml) this was further extracted with hexane/chloroform (4:1, v/v) to yield a nonsaponifiable neutral lipid fraction contained in the upper organic layer and free fatty acids in the lower aqueous layer. The

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Table II. Brief characteristics of recent Anarctic isolates.

Strain	Gram reaction	Oxidase	Catalase	O/F	Growth at 25°C
AS1	(-) Rod	(+)	(+)	O	(+)
AS2	(-) Rod	(+)	(+)	O	(+)
AOY	(-) Coccobacilli	(-)	(+)	F	(+)
BAB	(-) *	(+)	(+)	N/G	(-)
BOY	(-) Rod	(+)	(+)	G/A	(+)
JAO	(-) Rod	(-)	(+)	N/G	(-)
JAW	(-) Rod	(+)	(+)	G/A	(+)
JCW	(-) Coccobacilli	(+)	(+)	G/A	(+)
JCO	(-) Rod	w(+)	(-)	N/G	(+)
JOO1	(-) Rod	(-)	(+)	N/G	(+)
JOO2	(-) Rod	(-)	w(+)	N/G	(+)
PBO	(+) Cocci	(-)	(+)	O	(+)
PBP	(-) Rod	(-)	(+)	N/G	(+)
PBY1	(-) *	(+)	(+)	F	(-)
PBY2	(-) *	(+)	(+)	N/G	(-)

Legend: N/G = No Visible Growth O = Oxidative
O/F = Oxidative/Fermentative Growth F = Fermentative
G/A = Growth, no acid"
* Weak cell wall lead to lysis on staining

neutral fraction was removed. Fatty acids were collected by acidification of the remaining aqueous layer with concentrated HCl and extraction with hexane/chloroform (4:1, v/v). Subsequent methylation was achieved by reaction of the fatty acids for one hour at 80°C with a MeOH/HCl/$CHCl_3$ (10:1:1, 3 ml) solution. The resultant fatty acid methyl esters (FAME) were extracted with hexane/chloroform (4:1, v/v). Samples were then diluted for gas chromatographic analysis with chloroform containing nonadecanoic acid methyl ester (19:0 Me ester) internal standard. Samples containing hydroxy fatty acids were further derivatized by reaction with bis(trimethylsilyl)trifluoroacetamide (BSTFA) to form trimethylsilyl ethers for GC analysis.

Analyses of the FAME were performed with a Hewlett Packard 5890 GC equipped with a 50 m x 0.32 mm internal diameter cross-linked methyl silicone fused-silica HP1 capillary column and flame ionization detector. Samples were injected at 50°C in the splitless mode with a 2 min venting time. After 1 min the oven was temperature-programmed from 50–150°C at a rate of 30°C min^{-1}, then at 2°C min^{-1} to 250°C and 5°C min^{-1} to a final temperature of 300°C which was maintained for 15 minutes. Hydrogen was used as the carrier gas, and the injector and detector were maintained at 290 and 310°C respectively. Peak areas were quantified using chromatography software (DAPA Scientific Software, Kalamunda, Western Australia).

Selected FAME samples were also analysed on the same GC equipped with a 60 m x 0.32 mm internal diameter Supelcowax polar column. Samples were injected on-column at 45°C. After 1 min the oven was temperature-programmed from 45–120°C at a rate of 30°C min^{-1}, then at a rate of 3°C min^{-1} to 250°C. Hydrogen was used as the carrier gas, and the detector temperature was maintained at 280°C. Peak areas were quantified using a Shimadzu C-R2AX plotter/integrator.

GC-mass spectrometric (GC-MS) analyses of samples were performed on a Hewlett Packard 5890 GC and 5970 Mass Selective Detector (MSD) fitted with a direct capillary inlet. The column, injector and chromatography conditions were similar to those described above, with helium as the carrier gas. Mass spectra were acquired and processed using a Hewlett Packard 59970C Workstation operated in scan acquisition mode. Typical MSD operating conditions were: electron multiplier 2000–2200 volts; transfer line 300°C; autotune file DFTPP normalized; electron impact energy 70eV; scan threshold 1500; 0.8 scans / second mass range 40–600 amu; solvent delay 7 min.

Compounds were identified by comparison of relative retention data from both non-polar and polar columns, and mass spectra with those previously reported. The geometry and position of double bonds in monounsaturated FAME were determined by GC-MS analysis of the dimethyl disulphide (DMDS) adducts produced as described in Nichols *et al.* (1986a).

Fatty acids are denoted as number of carbon atoms: number of double bonds followed by the position of the first double bond from the methyl (ω) end of the molecule. Subsequent double bonds in PUFA are methylene interrupted. The prefixes i, a and br indicate *iso, anteiso* and branching respectively. The suffixes c and t indicate *cis* and *trans* double bond geometry respectively. The prefixes 2-OH and 3-OH indicate substitution of a hydroxyl group at the position indicated.

Results and discussion

PUFA production

Five of the 38 Antarctic strains analysed (PBP, ACAM 171, ACAM282, AS1, AS2) produced the long-chain PUFA 20:5ω3 in the range of trace to 3.3% of the total fatty acids (TFA) (Tables III, IV & V). A representative gas chromatogram is shown in Fig. 1. In addition to EPA, these organisms also contained varying levels of 18:2ω6, 18:3ω6, 18:4ω3, 20:4ω3 and 20:4ω6 (Table VI). A further seven isolates (Table VII) contained at least one other PUFA component. Thus, of the Antarctic strains examined, 8% contained EPA and 28% of strains produced PUFA. The Antarctic strains analysed also demonstrated a widely distributed production of the diunsaturated fatty acid 18:2ω6, which was found in 75% of strains. Although, as with the PUFA, this diunsaturated fatty acid is generally considered rare amongst bacteria (Harwood & Russell 1984) a similar occurrence of 18:2ω6 from marine bacterial isolates has been reported by Oliver & Colwell (1973), who also found 18:2ω6 in 75% of the strains they analysed. Of five EPA-producing strains, four were found to be psychrotolerant, while the majority of non-EPA PUFA producers (five of the seven) were psychrophilic (Tables I & II).

Of the multitude of reports concerning bacterial fatty acid composition in systematic and microbial ecological studies, few note the presence of PUFA (Johns & Perry 1977, DeLong & Yayanos 1986, Wirsen *et al.* 1987, Jacq *et al.* 1989, Yazawa

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Table III. Percentage fatty acid composition of Antarctic strains possessing a high abundance of monounsaturated fatty acids

	Strains									
	282	283	284	285	299	301	304	JAW	PBO	PBP
Fatty acid										
10:0	0.3	0.1	0.1	0.1	-	-	-	-	-	-
12:0	0.2	TR	0.1	0.1	TR	-	-	-	-	TR
13:0	-	-	-	-	-	0.7	-	-	-	-
14:0	0.3	0.1	0.2	0.2	0.2	0.1	0.2	0.2	4.7	0.9
15:0	0.3	0.4	0.5	0.1	TR	3.8	0.1	-	1.5	0.6
16:0	1.5	0.5	1.5	1.1	1.7	TR	3.1	4.7	17.0	9.8
17:0	0.2	0.1	0.5	0.1	TR	2.3	0.1	0.1	0.5	1.1
18:0	0.7	0.2	0.7	0.6	1.4	-	1.9	0.6	1.7	4.6
Σ saturates:	3.4	1.5	3.4	2.3	3.4	6.8	5.4	5.5	25.5	17.1
i13:0	TR	-	-	-	-	-	-	-	-	TR
i14:0	TR	-	-	-	-	TR	TR	-	TR	TR
i15:0	1.3	0.4	0.2	0.7	TR	TR	TR	-	0.3	0.2
a15:0	0.6	0.2	0.2	0.1	TR	TR	TR	-	0.3	0.1
i15:1*	-	-	-	-	-	-	-	-	TR	-
a15:1*	-	-	-	-	-	-	-	-	0.1	-
i16:0	-	-	-	-	TR	-	-	-	-	-
i17:0	9.5	4.5	2.9	-	-	-	0.1	0.1	TR	-
i17:1*	0.3	0.2	TR	0.3	-	-	0.1	-	-	-
a17:1*	-	0.1	0.1	0.1	-	-	-	-	-	-
i19:0	-	-	-	0.1	-	-	-	-	-	-
i19:1*	-	0.1	TR	0.1	-	-	-	-	-	-
a19:1*	-	TR	TR	0.1	-	-	-	-	-	-
Σ branched:	11.8	5.4	3.5	1.6	TR	TR	0.2	0.1	0.7	0.3
14:1ω8c	-	-	-	-	-	TR	-	-	-	-
14:1ω7c	TR	0.2	TR	TR	TR	TR	TR	TR	0.2	-
14:1ω5c	0.2	-	0.3	0.2	0.1	-	0.1	0.2	0.7	-
15:1ω6c	0.5	1.9	1.0	0.4	-	-	-	-	0.2	-
16:1ω9c	-	0.5	-	-	1.2	0.5	-	-	5.3	0.7
16:1ω7c	30.0	19.8	30.3	32.4	23.7	23.7	35.4	33.7	1.4	1.6
16:1ω7t	-	-	-	-	-	-	-	0.3	20.3	-
17:1ω8c	12.9	24.9	21.7	10.2	2.7	2.3	3.5	-	1.6	0.3
17:1ω6c	0.4	TR	0.3	10.6	-	-	TR	-	-	TR
18:1ω5c	-	20.4	-	-	-	-	-	-	-	-
18:1ω9c	40.6	24.1	35.8	0.2	66.8	64.9	55.2	TR	42.7	3.6
18:1ω7c	-	-	0.4	40.0	1.5	1.7	0.1	60.2	1.1	62.9
Σ monounsat.:	84.6	91.8	89.7	93.9	96.0	93.0	94.3	94.3	73.4	69.3
18:2*	-	-	-	-	-	-	-	-	-	8.6
18:2ω6	TR	TR	TR	-	0.5	0.1	0.1	-	0.4	3.6
18:3ω3	-	-	-	-	-	-	-	-	-	0.1
20:5ω3	-	-	-	-	-	-	-	-	-	0.9
Σ PUFA:	TR	TR	TR	-	0.5	0.1	0.1	-	0.4	13.3
Total:	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

* Double bond position not determined TR = < 0.05%

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Table IV. Percentage fatty acid composition of Antarctic strains possessing a range of fatty acids

	Strains								
	146	149	150	179	286	BOY	JAO	AS1	AS2
Fatty acid									
10:0	-	-	-	-	-	-	-	-	-
12:0	-	-	-	-	2.7	0.5	1.3	1.1	1.0
13:0	-	-	-	-	0.2	-	TR	0.7	0.8
14:0	3.4	1.5	5.1	0.5	4.5	1.9	6.2	5.3	5.2
15:0	13.3	14.9	1.0	3.1	2.1	4.9	2.9	2.7	3.0
16:0	7.8	13.1	7.7	21.4	27.1	6.1	16.5	11.1	9.6
17:0	TR	1.2	0.1	1.9	0.9	0.2	0.7	0.8	0.7
18:0	3.3	7.0	0.4	0.1	1.9	1.2	4.1	0.7	0.6
Σ saturates:	27.8	37.6	14.3	27.1	39.5	14.8	31.7	22.3	20.9
i11:0	-	-	-	-	-	-	-	-	-
i13:0	1.4	-	0.3	-	3.1	-	-	10.1	9.8
i14:0	TR	0.8	1.3	-	0.2	1.6	0.9	0.3	0.3
i15:0	39.7	23.8	29.6	0.1	11.0	25.1	2.5	9.5	9.0
a15:0	6.3	23.2	16.7	-	0.8	20.4	33.1	-	-
i15:1*	17.4	0.9	1.3	-	-	14.6	0.7	TR	TR
a15:1*	0.4	0.2	0.2	-	-	1.6	11.1	-	-
i16:0	0.6	11.7	1.7	0.1	0.2	-	-	-	-
i17:0	0.2	0.4	2.4	0.1	0.7	0.3	0.2	0.4	0.3
a17:0	-	-	-	-	-	-	-	-	-
i17:1*	1.6	-	5.1	-	TR	1.3	-	0.2	1.0
Σ branched:	67.7	60.9	58.6	0.3	15.9	64.8	48.3	20.6	20.4
14:1ω8c	-	-	-	-	-	-	-	0.1	0.1
14:1ω7c	-	-	0.1	1.3	0.4	0.4	-	1.1	1.2
14:1ω5c	-	TR	TR	-	0.2	-	0.1	0.1	0.1
15:1ω8c	-	-	-	5.0	-	TR	TR	0.8	0.9
15:1ω6c	-	-	-	-	3.5	TR	TR	0.5	0.7
16:1ω9c	-	-	5.0	7.4	2.5	-	-	2.5	2.9
16:1ω7c	4.4	-	15.9	21.1	23.3	4.6	7.1	37.9	37.7
16:1ω7t	-	-	-	-	-	-	-	0.1	0.1
16:1ω5c	-	-	-	28.8	-	6.2	-	0.1	0.6
17:1ω8c	0.1	1.4	1.0	3.6	3.0	1.4	1.6	3.3	4.3
17:1ω8t	-	-	-	3.1	-	-	-	-	-
17:1ω6c	-	-	-	-	0.4	-	-	0.3	0.4
18:1ω7t	-	-	-	0.5	-	0.2	TR	TR	TR
18:1ω9c	-	-	3.3	0.3	3.7	5.8	8.5	2.2	1.9
18:1ω7c	-	-	1.4	1.5	3.4	0.7	0.8	4.3	4.0
19:1*	-	-	-	-	-	-	-	0.1	0.1
20:1ω9c	-	-	-	-	-	-	-	TR	0.1
Σ monounsat.:	4.5	1.4	26.7	72.6	40.3	19.3	18.0	53.4	54.9
18:2ω6	-	-	0.4	-	3.2	0.9	1.7	TR	TR
18:3ω3	-	-	-	-	1.1	0.2	0.2	-	-
18:3ω6	-	-	-	-	-	-	-	TR	0.1
18:4ω3	-	-	-	-	-	-	-	0.1	0.2
20:4ω3	-	-	-	-	-	-	-	TR	0.1
20:5ω3	-	-	-	-	-	-	-	3.3	2.9
Σ PUFA:	-	-	0.4	-	4.3	1.1	1.9	3.4	3.3
Total:	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

* Double bond position not determined TR = < 0.05%

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Table V. Percentage fatty acid composition of Antarctic strains ACAM 171, JOO1 and JOO2 which contain hydroxy fatty acids

	Strains		
	JOO1	JOO2	171
Fatty acid			
12:0	-	-	0.1
13:0	0.1	-	0.1
14:0	0.3	1.0	1.0
15:0	0.3	0.3	3.4
16:0	2.5	0.4	1.9
17:0	0.1	TR	TR
18:0	0.4	0.4	0.6
Σ saturates:	3.6	2.2	7.0
i13:0	-	-	2.0
i14:0	3.1	3.2	4.3
i14:1ω9c	2.5	2.0	-
i15:0	5.2	4.7	48.7
a15:0	30.8	32.5	0.7
i15:1ω10c	3.5	4.8	14.0
a15:1ω10c	25.0	25.3	-
i16:0	2.5	3.8	-
i16:1ω6c	0.2	0.5	4.8
i17:0	TR	TR	TR
Other	0.8	0.9	-
Σ branched:	72.9	76.8	74.5
3-OH 14:0	0.8	0.9	0.4
2-OH i15:0	0.3	TR	-
3-OH i15:0	0.3	0.4	6.6
2-OH a15:0	0.7	TR	-
3-OH a15:0	1.5	2.3	-
3-OH 16:0	0.2	1.1	0.4
3-OH i16:0	11.8	8.3	-
3-OH i17:0	1.5	1.1	1.2
3-OH a17:0	4.3	4.7	-
Σ hydroxy:	21.4	18.8	8.6
14:1ω5c	-	-	0.2
15:1ω6c	-	-	5.4
16:1ω5c	-	-	0.6
16:1ω7c	0.2	0.2	2.9
17:1ω6c	-	-	TR
17:1ω8c	0.1	0.2	TR
18:1ω9c	0.5	0.6	0.5
Σ monounsat.:	0.8	0.9	9.6
18:2ω6	0.5	0.4	0.3
20:4ω6	-	-	TR
20:5ω3	-	-	TR
Σ PUFA:	0.5	0.4	0.3
Total:	100.0	100.0	100.0

TR = < 0.05%

Table VI. Percentage PUFA content of EPA-producing Antarctic strains

PUFA	Strains				
	AS1	AS2	PBP	171	282
18:2ω6	TR	TR	1.0	0.3	0.0
18:3ω6	TR	0.1	-	-	-
20:4ω6	-	-	-	TR	-
20:4ω3	TR	0.1	-	-	-
20:5ω3	3.3	2.9	0.9	TR	TR
mg/g 20:5ω3	1.1	1.4	ND	ND	ND

ND = Not Determined TR = < 0.05%

et al. 1988a&b, Akimoto *et al.* 1990, Matsui *et al.* 1991, Temara *et al.* 1991, Intriago 1992), with only three of these being screening studies. Yazawa *et al.* (1988a) found that only 1.5% of over 5000 temperate marine strains analysed demonstrated the ability to produce EPA. A further investigation of over 7000 temperate marine isolates, derived predominantly from the intestinal contents of marine fish, showed only 1.7% produced EPA (Yazawa *et al.* 1988b). DeLong & Yayanos (1986) analysed 11 barophilic strains isolated from depths between 1200 and 10476 m, and found that 27% produced EPA, with a further 55% containing the more highly unsaturated PUFA 22:6ω3. While the analysis of 38 Antarctic strains may not provide as significant a statistical base, it is difficult to envisage that the random selection of strains employed in this study would have fortuitously contained a higher proportion of PUFA producers. Thus, while the proportion of Antarctic EPA-producing strains does not appear as great as those from the deep-sea environment, there appears a significant increase in the proportion of Antarctic bacteria that possess the ability to produce EPA when compared with temperate marine isolates (Fig. 2).

Bacteria may possess two possible mechanisms for the synthesis of unsaturated fatty acids: an oxygen-independent (anaerobic) pathway catalysed by a fatty acid synthetase, or an oxygen-dependent (aerobic) pathway involving fatty acid desaturation analogous to that of eukaryotic organisms. Historically, these two mechanisms have been considered mutually exclusive within the one bacterium (Fulco 1983 & references therein). However, Wada *et al.* (1989, 1991), Intriago (1992) and Morita *et al.* (1992) have recently demonstrated the existence of both biosynthetic mechanisms in four strains of eubacteria; *Pseudomonas* sp. strain E-3, *Flexibacter* sp. strains Inp2 & Inp3 and *Vibrio* sp. strain ABE-1. The type of biosynthetic pathway utilized by a bacterium is important, as the methylene-interrupted double bond system characteristic of ω3 and ω6 PUFA cannot be produced by the anaerobic system. Under the anaerobic system, sequential β,γ-dehydration would lead to $\Delta^{1,3}$ conjugated

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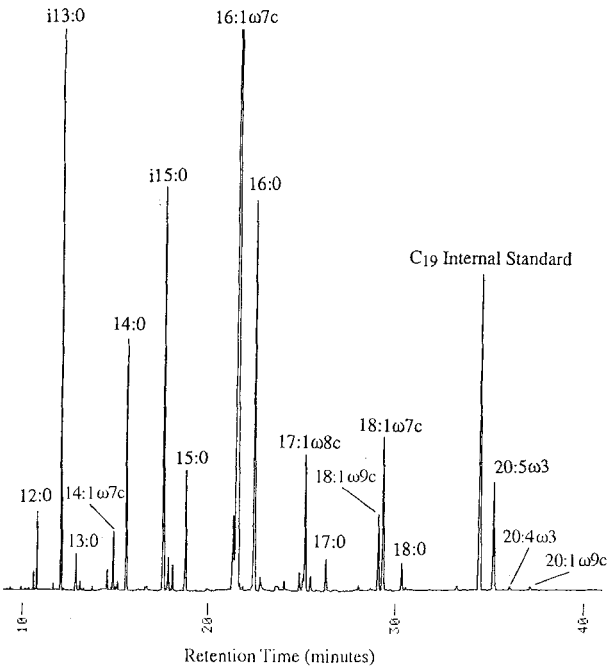


Fig. 1. Capillary gas chromatogram of fatty acids (as methyl esters) from Antarctic strain AS1. Unlabelled peaks are listed in Table VIII.

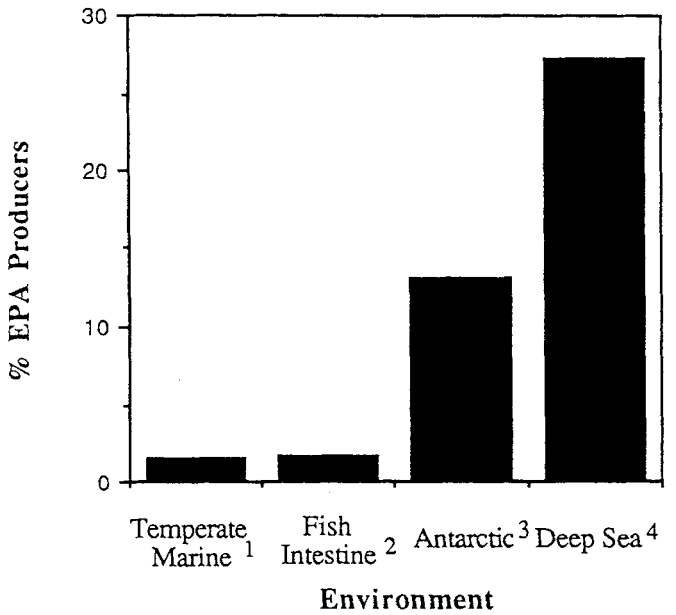


Fig. 2. The percentage of EPA-producing bacteria isolated from varying environments ([1] Yazawa *et al.* 1988a, [2] Yazawa *et al.* 1988b, [3] present study of Antarctic bacteria, [4] DeLong & Yayanos 1986).

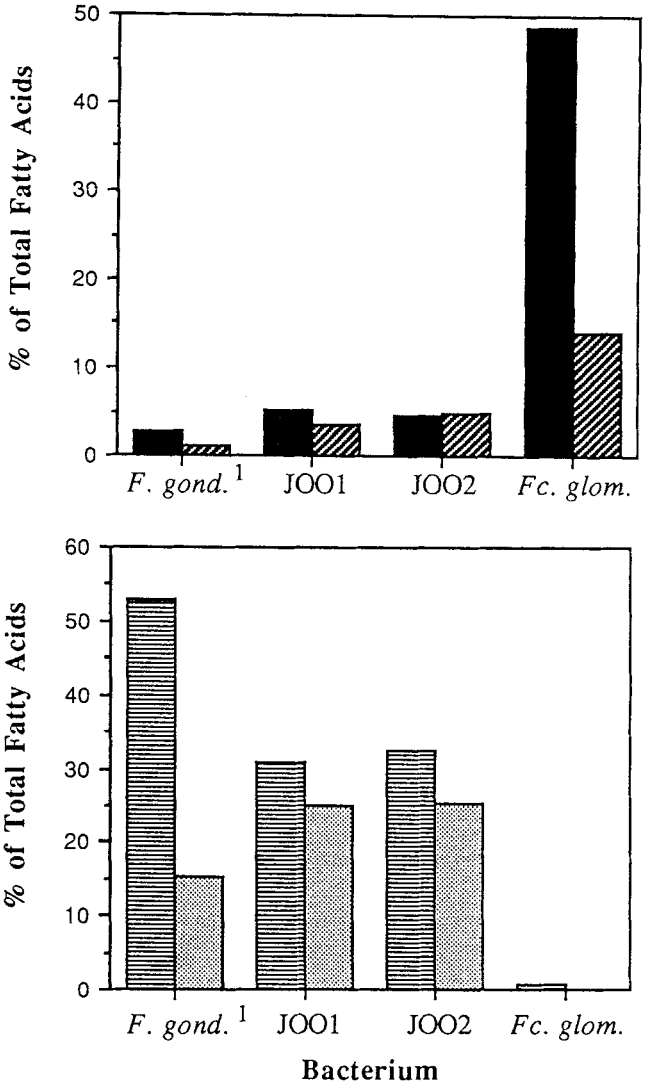


Fig. 3. Chemotaxonomic comparison of the major fatty acids from the related Antarctic species *Flavobacterium gondwanense* (*F. gond.*[1]), *Flectobacillus glomeratus* (*Fc. glom.*) and strains JOO1 and JOO2 ([1] Skerratt *et al.* 1991). Key: il5:0 il5:1ω10c al5:0 a15:ω10c

double bond systems which have not been found in bacteria (Fulco 1983). Alternatively, cycles of α,β and,β,γ-dehydration could yield 1,5 double bond systems. These do occur in some bacteria (*Bacillus licheniformis, Mycobacterium phlei*) although the anaerobic pathway has not been identified as the synthetic route in either case (Fulco 1969, Asselineau & Montrozier 1976). The Antarctic PUFA producers isolated must therefore be utilizing, at least in part, an oxygen-dependent fatty acid synthetic mechanism. From the fatty acid composition of strains AS1 and AS2 (Table IV) a possible PUFA biosynthetic pathway for these organisms may be suggested (Fig. 4), based on the presence of known eukaryotic PUFA intermediates 18:2ω6, 18:3ω3, 18:4ω3, 20:4ω3 and 20:5ω3 (Harwood & Russell 1984).

Fulco (1983) suggests that the apparent inability of most

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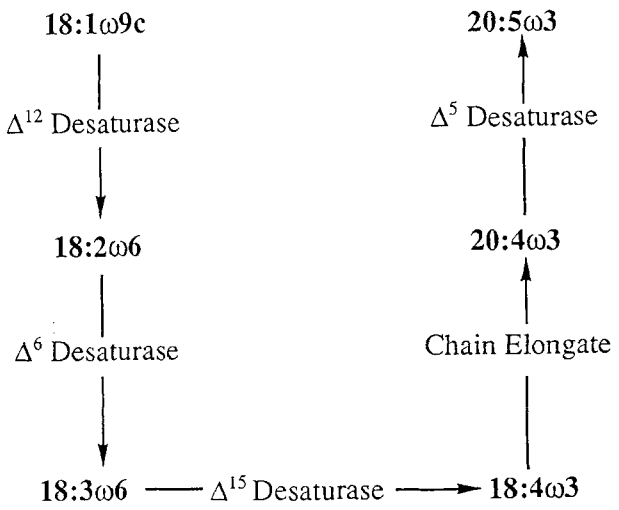


Fig. 4. Possible biosynthetic mechanism of PUFA production in Antarctic strains AS1 and AS2 based on the presence of known eukaryotic intermediates.

bacteria utilizing an oxygen-dependent system to produce PUFA does not stem from a basic mechanistic difference between bacterial and eukaryotic systems. Rather, he suggests that the great majority of bacteria contain only one desaturase system with a moderately high degree of positional specificity for double bond insertion; this is in contrast to the multi-enzyme desaturase systems required for PUFA synthesis exhibited by eukaryotes.

It would therefore appear that the Antarctic environment may have selected for organisms capable of PUFA production by providing a selective advantage to those organisms capable of modifying their desaturase action. Many bacteria adjust their fatty acid composition in response to lowered growth temperature by increasing the degree of fatty acid unsaturation to maintain a sufficient level of membrane fluidity (Melchior 1982). Such a transitory response is termed by Russell (1990) as a phenotypic adaptation, that is, one that occurs within the lifetime of the organism over a variable timescale, from minutes to seasonal or longer periods. Phenotypic adaptation is unlikely, however, to have lead to the ability to biosynthesize PUFA. The development of such a complex adaptive mechanism is only likely to have occurred from environmental pressure over an evolutionary timescale, where PUFA production would yield a particular survival advantage. The evolution of such an ability is termed by Russell (1990) as a genotypic adaptation. It has been proposed, therefore, that genotypic adaptation to a permanently cold environment may result in organisms more likely to produce PUFA. Both the deep oceans and Antarctica represent such permanently cold environments from which bacterial PUFA producers have now been isolated. The higher proportion of PUFA producers from deep-sea isolates observed by DeLong & Yayanos (1986) may be rationalized in terms of genotypic adaptation to high pressure as well as low temperature. It is well established that increasing hydrostatic pressure elicits a similar response to decreasing temperature upon membrane lipid fluidity and the adaptive responses employed by bacteria to maintain a suitable level of fluidity (Cossins & Sinensky 1984). Thus, deep-sea bacteria would have been expected to adapt to the additive effects of both temperature and pressure. Similarly, the results of this investigation support the proposition that the extreme temperature conditions of the Antarctic environment appear to have selectively enriched for bacteria able to maintain membrane lipid fluidity by the production of PUFA. We believe that barophilic bacteria may be a less attractive potential industrial source of PUFA, as the cost of necessary pressure facilities for growing such bacteria may be restrictive.

Trans-fatty acids

Seven strains contained 18:1ω7t and 16:1ω7t in the ranges trace to 0.5% and trace to 20.4% of TFA respectively. A further isolate also contained trace levels of 15:1ω6t. The occurrence of 20.4% 16:1ω7t within strain PBO (Table III) is believed to be the highest level of a *trans*-fatty acid found in any bacterium to date. As with PUFA biosynthesis, the *de novo* production and incorporation of *trans*-fatty acids into cellular lipids by bacteria appears to be a relatively limited ability (Makula 1978, Guckert *et al.* 1987, Nichols *et al.* 1986a&b, Intriago 1992). The high incidence of *trans*-fatty acid production observed may represent a further characteristic of Antarctic bacteria.

Chemotaxonomic aspects

The majority of ACAM and recently isolated Antarctic strains used in this study remain as yet unidentified. However, several ACAM isolates analysed have been tentatively identified on the basis of classical biochemical analysis (Table I). The fatty acid profiles of strains 113, 146, 150, 151, 162, 166 and 188, identified as *Pseudomonas* sp., are particularly unusual due to the predominance of branched-chain fatty acids. Only three species of this genus, *P. maltophilia*, *P. perlurida* and *P. pictorum* have been reported with such peculiar fatty acid compositions (Oyaizu & Komagata 1983). Based on the irregular occurrence of high levels of C_{15} branched-chain fatty acids (Tables V, VII & VIII) these strains may be closely related to one of the above species. It may also be suggested that based on this chemotaxonomic evidence, such strains should be transferred to a more representative genus.

P. maltophilia has i15:0, 16:0 and 16:1 as major fatty acids (Oyaizu & Komagata 1983). This is very similar to the fatty acid distribution displayed by strain 151 (Table VIII), but which also contains high levels of i15:1ω7c. The occurrence of i15:1ω7c may indicate a particular adaptation to the low temperature Antarctic environment. Such an unsaturated branched-chain fatty acid would be expected to cause a much greater disruption in acyl chain packing within the membrane bilayer, thereby increasing membrane fluidity in response to the lower environmental temperature. Strain 146 also exhibits this compositional pattern, but with slightly lower levels of 16:0 and

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Table VII. Percentage PUFA in non-EPA-producing Antarctic strains

PUFA	Strains						
	BAB	BOY1	JAO	181	188	286	302
18:3ω3	0.2	0.2	0.2	0.1	TR	0.8	TR
18:4ω3	0.7	-	-	TR	-	-	-

TR = < 0.05%

16:1ω7c. Similarly, strain 188 contains high levels of i15:0 and 16:1ω7c, but a higher proportion of a15:0 and a lesser amount of 16:0 than exhibited by the majority of *P. maltophilia* strains reported. Based on the similarity of their fatty acid compositions, however, these organisms may represent strains closely related to *P. maltophilia*. Similar to these three strains is ACAM 162 which possessed much higher levels of i15:0 and i15:1ω7c, but lower percentages of both 16:0 and 16:1ω7c. It appears that while related, a substantial amount of inter-strain variability is apparent.

Strain 113 is distinctive in possessing a15:0 as the major branched-C_{15} fatty acid, together with 16:0, 18:0 and i16:0 as major components. A predominance of a15:0 was found to be a marker solely for *P. perlurida* among *Pseudomonas* species (Wilkinson 1988, Fukunaga & Russell 1990), although Wilkinson (1988) did not observe such high levels of 16:0, 18:0 and i16:0.

Strain 166 (Table VIII) is considered to be a pseudomonad. However, its fatty acid composition is unusual in two respects. Firstly, there were almost equal levels of i15:0 and a15:0 as major fatty acids, while secondly, 16:1ω7c, a major component in *Pseudomonas* sp. is not present. Presently, there is no recognized *Pseudomonas* sp. with such a distribution of branched-chain and monounsaturated fatty acids (Wilkinson 1988). In contrast, strain 150 (Table IV) contains a15:0 and i15:0 as major fatty acids, but in an approximate 2:1 ratio, while retaining significant levels of 16:0 and 16:1ω7c. Such a distribution indicates a closer relation to strain 166 than any presently recognized *Pseudomonas* sp.

The fatty acid compositions of strains JOO1 and JOO2 were found to be very similar (Table V), with a range of hydroxy branched-fatty acids including br15:0 and 3-OHa17:0 characteristic of the *Flavobacterium–Cytophaga* group (Oyaizu & Komagata 1981). Particularly characteristic of both strains was the presence of high levels of a15:1ω10c and 3-OHi16:0, together with lesser levels of i15:1ω10c. To date, only one species of flavobacteria, *Flavobacterium gondwanense*, (isolated from Organic Lake, Vestfold Hills, Antarctica) has been reported to contain high levels of a15:1ω10c (Skerratt *et al.* 1991). Most other flavobacteria are characterized by high levels of i15:0 (~20–50% of TFA), 16:1ω7c (~5–30% of TFA) and usually substantial levels of 2-OHi15:0 (~5–30% of TFA) (Oyaizu & Komagata 1981). High levels of these components were not apparent in either *F. gondwanense* or strains JOO1 and JOO2.

Table VIII. Percentage fatty acid composition of Antarctic strains possessing a high abundance of branched fatty acids

	Strains					
	123	151	162	166	181	188
Fatty Acid						
14:0	2.4	2.8	2.9	1.1	0.8	4.9
15:0	9.4	3.2	9.8	14.4	9.5	0.7
16:0	2.9	16.9	3.9	7.5	5.7	4.4
17:0	TR	0.6	-	0.3	0.1	TR
18:0	0.6	9.9	1.4	1.8	1.9	0.2
Σ saturates:	15.2	33.5	17.9	25.1	18.0	10.1
i13:0	1.2	-	1.2	-	-	0.4
i14:0	0.1	-	0.3	1.4	0.5	1.6
i15:0	18.4	23.6	43.5	23.8	14.8	35.2
a15:0	4.6	3.4	5.5	26.0	19.4	19.6
i15:1*	18.4	10.4	23.2	9.6	15.0	1.3
a15:1*	0.5	-	0.5	3.9	8.4	-
i16:0	0.6	0.5	1.7	8.2	2.4	-
i16:1*	0.1	-	-	-	3.2	-
i17:0	0.5	TR	0.3	0.1	0.1	2.0
a17:0	-	-	-	-	-	TR
i17:1*	11.8	3.3	5.0	0.2	3.5	4.4
Σ branched:	56.1	41.3	81.2	73.3	67.5	64.4
14:1ω8c	-	-	TR	-	-	TR
14:1ω7c	0.1	-	TR	-	0.1	0.1
14:1ω5c	-	TR	-	-	-	TR
15:1ω6c	1.1	-	0.1	0.8	1.8	-
15:1ω6t	-	-	-	-	-	TR
15:1*	-	-	-	-	0.8	-
16:1ω9c	-	-	-	-	-	4.5
16:1ω7c	25.7	11.4	-	-	2.5	16.7
16:1ω7t	TR	1.8	-	-	-	0.1
16:1ω5c	-	-	-	-	1.8	TR
17:1ω8c	0.2	0.5	TR	0.7	0.9	0.6
17:1ω6c	0.2	TR	TR	-	0.1	-
18:1ω9c	0.6	6.6	0.7	-	3.8	2.5
18:1ω7c	0.1	1.5	-	-	TR	1.0
Σ monounsat.:	27.9	21.8	0.8	1.6	11.9	25.3
18:2ω6	0.7	3.4	-	-	2.5	TR
18:3ω3	TR	TR	-	-	0.1	TR
18:4ω3	-	-	-	-	TR	-
Σ PUFA:	0.7	3.4	-	-	2.6	TR
Total:	100.0	100.0	100.0	100.0	100.0	100.0

* Double bond position not determined TR = < 0.05%

Thus, *F. gondwanense*, which has a very similar fatty acid composition to that of strains JOO1 and JOO2 (Fig. 3), is distinctive from other members of the genus *Flavobacterium* (Skerratt *et al.* 1991). However, two significant differences were apparent between the fatty acid compositions of *F. gondwanense* and strains JOO1 and JOO2. Firstly, the minor branched C_{14}

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Table IX. Percentage fatty acid composition of Antarctic strains possessing a high abundance of saturated fatty acids

	Strains									
	298	AOY	JCO	PBY1	302	PBY2	113	167	JCW	BAB
Fatty acid										
12:0	-	6.3	-	-	-	-	-	0.9	-	-
13:0	-	-	3.6	-	-	-	-	-	-	-
14:0	2.9	13.1	0.6	2.8	3.5	3.1	3.5	6.4	3.1	7.0
15:0	0.5	1.2	42.8	0.8	0.3	1.4	5.4	7.5	1.0	3.6
16:0	37.9	28.3	0.8	36.1	33.9	45.9	26.5	39.4	22.4	22.4
17:0	0.4	0.5	0.8	1.0	0.6	1.4	0.7	1.3	0.7	1.1
18:0	29.2	16.1	27.2	23.8	39.2	36.0	16.7	15.7	15.8	6.8
Σ saturates:	70.9	65.6	75.7	64.5	77.6	87.8	52.8	71.2	43.1	40.9
i13:0	-	-	-	0.4	-	-	-	-	-	-
i14:0	-	-	TR	-	-	-	4.6	0.3	-	0.1
i15:0	TR	4.5	TR	0.1	0.3	TR	3.5	12.5	0.3	0.7
a15:0	TR	2.3	TR	TR	0.2	TR	14.7	12.8	0.5	-
i15:1*	-	2.0	-	-	-	-	-	-	-	-
i16:0	-	-	-	-	-	-	23.9	1.2	0.3	-
i17:0	-	-	TR	TR	0.0	-	-	0.2	0.1	0.2
i17:1*	-	-	-	-	-	-	-	-	TR	0.5
Σ branched:	TR	8.7	TR	0.6	0.5	TR	46.8	27.1	1.3	1.5
14:1ω7c	-	-	-	-	-	-	-	-	TR	0.8
14:1ω5c	-	-	-	-	TR	TR	-	-	TR	-
15:1ω8c	-	-	-	-	-	-	-	-	-	1.5
16:1ω9c	1.0	-	0.3	-	-	0.3	-	-	-	-
16:1ω7c	3.6	9.2	0.8	4.4	1.5	0.3	-	-	15.7	13.7
16:1ω5c	1.5	-	-	-	-	-	-	-	-	-
17:1ω8c	TR	0.1	0.2	0.4	TR	0.6	TR	1.3	0.4	2.5
17:1ω6c	TR	TR	-	TR	-	-	-	0.4	-	-
18:1ω9c	17.7	10.4	15.9	19.6	18.3	7.4	-	-	5.8	15.6
18:1ω7c	0.8	1.2	0.3	3.6	0.3	0.2	0.4	-	31.3	19.6
18:1ω7t	-	-	-	-	TR	-	-	-	-	0.5
Σ monounsat.:	24.6	20.9	17.5	27.9	20.0	8.8	0.4	1.8	53.3	54.1
18:2ω6	4.4	4.6	6.7	7.0	1.9	3.4	-	-	2.3	2.6
18:3ω3	-	0.2	-	-	TR	-	-	-	TR	0.2
18:4ω3	-	-	-	-	-	-	-	-	-	0.6
20:5ω3	-	-	-	-	-	-	-	-	-	-
Sum PUFA:	4.4	4.8	6.7	7.0	1.9	3.4	-	-	2.3	3.4
Total:	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

* Double bond position not determined TR = < 0.05%

monounsaturated component from *F. gondwanense* was identified as i14:1ω8c by Skerratt *et al.* (1991), whereas strains JOO1 and JOO2 contained the positional isomer i14:1ω9c. Secondly, the present strains produced a series of three C_{16}–C_{18} straight-chain monounsaturated fatty acids; 16:1ω7c, 17:1ω8c and 18:1ω9c as well as 18:2ω6 (Table V). These components were not reported from *F. gondwanense* (Skerratt *et al.* 1991), but have been identified as fatty acid components from a range of other flavobacteria (Dees *et al.* 1985, 1986). This evidence suggests strains JOO1 and JOO2 represent new strains or species closely related to *F. gondwanense*.

Strain 171 has been characterized as the new species *Flectobacillus glomeratus* (McGuire *et al.* 1987) and this study reports the first fatty acid composition of this species (Table V). As for most species within the *Flavobacterium–Cytophaga* group, the fatty acid composition of *Flectobacillus glomeratus* is dominated by i15:0. However, significant levels of i15:1ω10c are also present, enabling *Flectobacillus glomeratus* to be

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distinguished from other members of this group. Such monounsaturated branched C_{15} components have only been identified from the Antarctic flavobacteria *F. salegens* , *F. gondwanense* (Skerratt *et al.* 1991) and now *Flectobacillus glomeratus* within the *Flavobacterium–Cytophaga* group. This may suggest that the biosynthesis of these unsaturated branched fatty acids is a direct response to the lower temperature Antarctic environment.

16s RNA sequencing has shown a close relationship between *Flectobacillus glomeratus* and the Antarctic flavobacteria *F. salegens* and *F. gondwanense* discussed above (S.J. Dobson, personal communication 1991). However, while *Flectobacillus glomeratus* was characterized by i15:0 as the major fatty acid, a15:0 predominated in *F. gondwanense*. This distribution was also reflected in the levels of branched C_{15} monounsaturates. The synthesis of branched-chain fatty acids relies upon the substitution of specific primer molecules which replace acetyl-CoA as the chain initiator for fatty acid synthesis. The majority of these branched-chain primers are derived from the amino acids leucine, isoleucine and valine yielding the three series of branched-chain fatty acids *iso*-$C_{15/17}$, *anteiso*-$C_{15/17}$ and *iso*-$C_{14/16}$ respectively. The relative ratios of branched-chain fatty acids produced are dependent on the substrate specificity exhibited by the acetyl-CoA:ACP transacylase involved (Kaneda 1991 & references therein). The predominance of *iso*-C_{15} fatty acids and the near absence of *anteiso*-C_{15} components suggests that *Flectobacillus glomeratus* may preferentially utilize leucine rather than isoleucine as a branched-chain fatty acid precursor. This may reflect a high substrate specificity for leucine exhibited by the acyl-CoA:ACP transacylase. Significant levels of i16:1ω6c were also present, however, indicating a possible substrate preference for this enzyme of:

leucine>>valine>>isoleucine.

Within *F. gondwanense* it would appear that this pattern of substrate preference may be reversed, such that:

isoleucine>>valine>>leucine.

Flectobacillus glomeratus also exhibited a greater relative level and diversity of monounsaturated components, with 15:1ω6c the major unsaturated fatty acid present. Levels of hydroxy fatty acids, however, were much lower than for *F. gondwanense*. Thus, the fatty acid composition of *Flectobacillus glomeratus* generally reflects that of other species within the *Flavobacterium– Cytophaga* group, but may be chemotaxonomically differentiated by the production of high levels of the branched C_{15} monounsaturated i15:1ω10c. The similar fatty acid composition of *Flectobacillus glomeratus* to the remainder of the *Flavobacterium–Cytophaga* group also supports the extremely characteristic nature of that reported for *F. gondwanense* (Skerratt *et al.* 1991) (Fig. 3).

Conclusions

It is evident that, while the strains analysed do not contain the high levels of PUFA found in some algae and fish oils, it is possible that manipulation of culture conditions will increase these levels. Similarly, modern technologies are available to enrich PUFA from oils (Ackman *et al.* 1988). These facts, together with the previously discussed advantages of bacterial PUFA production, and the possibility of isolating further higher producing strains, indicate Antarctic bacteria are worthy of further research in this area. The results of this study also illustrate that while many microbial systematic studies are now placing greater emphasis on modern molecular biology techniques, fatty acid composition also provides important chemotaxonomic information for the identification of closely related organisms.

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