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REVIEW ARTICLE

Polyunsaturated fatty acids in marine bacteria – a dogma rewritten

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Prologue

The invention of GC techniques rapidly led to the description of distinct types of fatty acids in plants, animals and micro-organisms and, apart from one group of cyanobacteria, it became accepted wisdom that (unlike plants and animals) bacteria did not contain polyunsaturated fatty acids (PUFAs) as acyl functions of the complex lipids in their membranes (Harwood & Russell, 1984). This fundamental distinction matched perceptions of the evolutionary origins of these groups and was not shaken by the discovery of the *Archaea*, since the hydrophobic moiety of the membrane lipids in that distinctive domain is based not on esterified fatty acids but instead on ether-linked phytanyl (isoprenoid-based) alkyl chains.

Scientific progress is often made through the invention of better analytical techniques, and this fact is exemplified by the use of modern, sensitive techniques for fatty acid analysis, which is illustrated in the following account of the occurrence, biosynthetic mechanism and membrane function of PUFAs in some marine bacteria. We also show how knowledge of their fatty acid compositions has contributed to the rewriting of the phylogeny of marine psychrophilic bacterial genera and discuss their potential biotechnological applications.

To aid readers through the complexities of lipid nomenclature, the structures of the main PUFAs that we shall discuss are shown in Fig. 1, together with their trivial names and the ways of shorthand numbering from either the methyl end (n or ω system) or the carboxyl end (Δ system). Each has its advantages and will be used in this review to illustrate different features of biosynthesis or function. The intricacies of lipid metabolism are generally not described and only sufficient detail is given for the reader to appreciate the particular chemotaxonomic, biosynthetic or biotechnological issue.

Approaching the status quo

Several early studies of bacterial lipids reported the presence of long-chain polyunsaturated fatty acyl components, particularly linoleic acid (18:2Δ9,12 or 18:2n6). However, these reports were either ignored by the mainstream of lipid (bio)chemists, regarded as analytical errors/miscalculations or believed to be due to incorporation from yeast extract or broth-derived animal products taken up from the growth medium (i.e. not the result of *de novo* synthesis). Whilst it is now understood that the majority of bacteria will indeed take up exogenous PUFAs if they are present in the culture medium (e.g. see Watanabe *et al.*, 1994), the beginnings of the exogenous uptake argument were grounded in few facts as limited studies had been conducted to examine the fatty acid composition of common bacteriological media.

By the early 1960s it was known that some bacteria possessed aerobic desaturation mechanisms for unsaturated fatty acid biosynthesis (*vide infra*), but the fact that Scheuerbrandt & Bloch (1962) reported finding no PUFA from a (small) sample of bacterial species, combined with a literature which ' ... does not mention their presence in bacterial sources ... ', led Erwin & Bloch (1964) to extrapolate that '...bacteria lack polyunsaturated fatty acids altogether ... '. However, Fulco (1974) provided ample evidence for aerobic fatty acid desaturation in bacilli and mycobacteria leading to the biosynthesis of such dienoic fatty acids as 16:2Δ5,10. Significantly, these PUFAs do not have the methylene-interrupted double-bond positional pattern that is characteristic of those in eukaryotic micro-organisms, plants and animals. Subsequently, Goldfine (1972) recommended that the doctrine on PUFAs in bacteria should

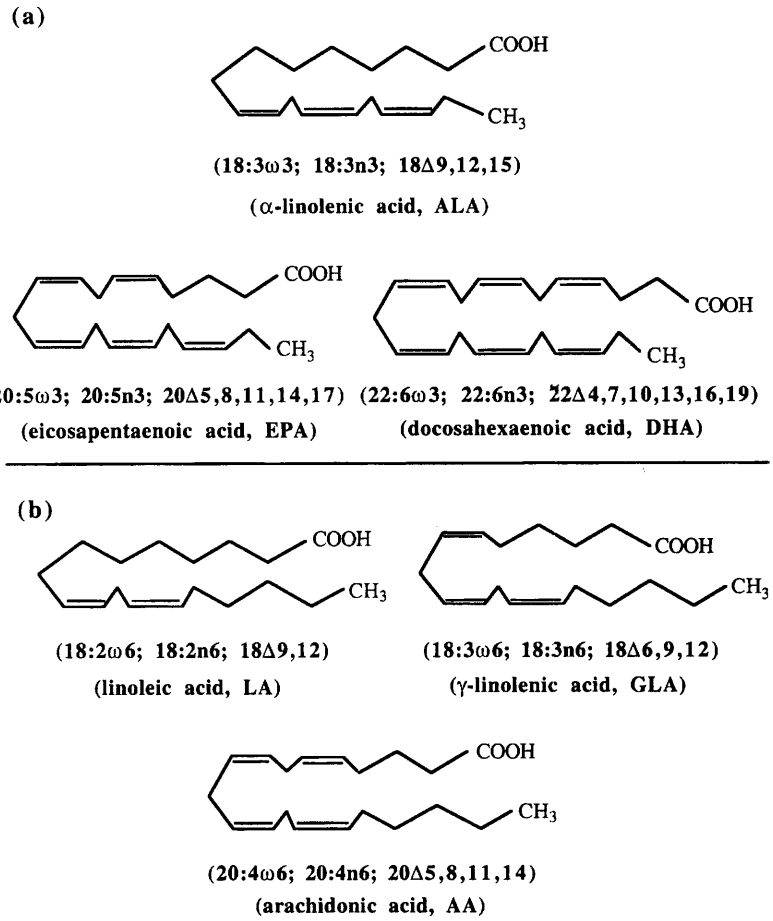

Fig. 1. Chemical structures of the common PUFAs from (a) the n3 family and (b) the n6 family. The conformation of the acyl chain is presented in a stylized format and is not meant to indicate its orientation within a membrane lipid (see text for a discussion of this aspect).

be modified so as to exclude the biosynthesis of methylene-interrupted PUFAs.

Nonetheless, the wrong identification of PUFAs in some bacteria continued to occur and lent weight to the argument of non-PUFA production by prokaryotes. Whilst analytical methods of the time were robust, they were time-consuming and occasionally open to the misidentification of components when unexpected or novel fatty acids were encountered. This was particularly true where identification of fatty acids was based solely on relative retention times and equivalent carbon number calculations determined by GC (e.g. see Drücker & Owen, 1973). An example is that of Huston & Albro (1964), who reported the occurrence of 20:4 from *Micrococcus* (*Sarcina*) *luteus* ATCC 533 based on relative retention time and equivalent carbon number identification. However, Tornabene *et al.* (1967), analysing fatty acids from the same strain using an early model combined GC-MS system, failed to detect 20:4 but did report a small proportion of 18:2 when cultures were grown under similar conditions.

A significant advance was made by Oliver & Colwell (1973), when they reported the existence of PUFAs in marine bacterial isolates; this was followed by the finding of eicosapentaenoic acid (20:5n3; EPA) within the marine bacterium *Flexibacter polymorphus* (Johns & Perry, 1977). The latter study, in particular, changed thinking on the subject because the identity of EPA was confirmed using modern analytical methodology (viz. combined GC-MS), leaving little doubt as to the authenticity of the report. Incidentally, the work of Johns & Perry (1977) also challenged the assumption put forward by Scheuerbrandt & Bloch (1962), and supported by Erwin & Bloch (1964) and Gurr & James (1971), that aerobic and anaerobic pathways of fatty acid biosynthesis (*vide infra*) were mutually exclusive in bacteria. That misconception was finally laid to rest by the extensive studies of Wada *et al.* (1991), who proved conclusively that both pathways could operate within a single species.

That recent work is symptomatic of a surge of interest in bacteria as a source of PUFAs, now that it is accepted that some species do have the metabolic capacity to make EPA, as well as docosahexaenoic acid (DHA; 22:6n3) or arachidonic acid (AA; 20:4n6). Table 1 gives the fatty acid compositions of some marine bacteria having representative patterns of PUFA content, viz. AA (*Shewanella gelidimarina*), high EPA (*Shewanella hanedai*), PUFA-negative (*Shewanella putrefaciens*) and DHA plus EPA (*Colwellia psychrerythraea*). A major reason for interest in these acids stems from their importance in mammalian (human) health (Lees, 1990). For instance, they are the precursors of such potent regulatory molecules as prostaglandins, thromboxanes

Table 1. Fatty acid compositions (wt %) of some representative marine bacteria

Fatty acid	*Shewanella gelidimarina** ACAM 456	*Shewanella hanedai*†	*Shewanella putrefaciens*‡ NCIB 10472	*Colwellia psychrerythraea*§
12:0	0·7	0·2	–	–
i13:0	15·8	6·4	2·1	< 1
13:0	1·3	0·2	2·4	–
i14:0	0·6	0·3	16·2	< 1
14:1n7	0·8	TR	0·7	6·5
14:1n5	0·1	–	–	< 1
14:0	4·4	9·3	1·0	6·4
i15:0	8·3	8·5	9·2	–
15:0	6·7	4·1	3·4	6·7
15:1n8	1·7	0·1	4·7	1·2
15:1n6	1·7	0·3	0·6	0·2
16:0	6·4	13·6	6·1	30·0
16:1n7	27·4	29·0	13·0	33·4
i17:0	0·1	1·4	0·9	< 1
17:0	0·5	0·8	4·0	0·7
17:1n8	4·2	1·0	8·9	0·6
17:1n6	0·8	0·5	2·5	0·4
18:0	0·1	0·3	2·4	1·2
18:1n9	0·3	0·5	2·3	0·6
18:1n7	1·0	3·0	3·6	1·2
18:4n3	0·3	–	–	–
20:4n3	0·3	–	–	–
20:5n3	16·0	20·2	–	0·7
22:6n3	–	–	–	6·8
Other	0·2	0·3	10·2	2·3
Total	100·0	100·0	100·0	–

* Nichols & Russell (1996).

† Mean values from Bowman *et al.* (1997); TR, <0·1%.

‡ Moule & Wilkinson (1987).

§ Mean values from Bowman *et al.* (1998a).

and leukotrienes, and are components of acyl lipids in the membranes of the brain and retina. Humans normally synthesize them from linoleic acid (18:2n6) and α-linolenic acid (18:3n3), essential fatty acids which are derived from plants. Alternatively, DHA and EPA may be provided as lipid components from fish oils. A normal balanced diet should provide the necessary PUFAs but recent medical evidence that the longer-chain acids help to prevent atherosclerosis and heart disease has prompted a large rise in their consumption as dietary supplements. The usual source of acids for these supplements is fish oils but there are problems of flavour taint and worldwide fish stocks are under threat. Therefore, alternative sources are being sought and the ease of culture of bacteria makes them an attractive alternative. However, the levels of PUFAs in bacteria are not as high as in some species of fungi, the main reason being that in fungi the acids are components of stored oils (triacylglycerols) whereas in bacteria they are in membrane lipids. Oleaginous fungi store large amounts of lipid, mainly triacylglycerols, and *Mortierella* spp. are noteworthy for the n3 and n6 PUFA contents of their stored lipid: typically up to 40% of the fungal dry weight may be triacylglycerol in which the acyl chains are up to 15% EPA or 55% AA (Kendrick & Ratledge, 1992; Eroshin *et al.*, 1996; Chen *et al.*, 1997; Singh & Ward, 1997).

Thus bacteria cannot compete with fungi as primary producers of PUFA-rich oils. However, bacteria could be used as feedstock for organisms, such as rotifers, as a way of introducing them into a marine food web for producing PUFA-rich oils by aquaculture (discussed below). Therefore a better understanding of the biosynthetic pathway and its regulation in bacteria would pave the way for increasing levels of PUFAs, and the relative ease of genetic manipulation in bacteria makes them attractive for studying enzyme regulation and also gives the potential for gene transfer into other organisms to enhance or modify their PUFA-producing capacity. This latter approach has already attracted commercial interest (Facciotti *et al.*, 1998).

There are further reasons also for studying bacterial PUFAs. Their occurrence in a selected group of marine psychrophilic, halotolerant bacteria raises questions about their role in membrane structure and stabilization at low temperatures in saline conditions. Their distinctive taxonomic distribution can also be used for classification and identification. Evolutionary relationships may not be confined to bacterial groupings, because the structural similarity of bacterial PUFAs to those that are typical of mammals raises the possibility of lateral gene transfer.

Chemotaxonomy

Early reports (DeLong & Yayanos, 1985, 1986; Wirsen *et al.*, 1987) highlighted the correlation between the distribution of PUFA-producing bacteria and their habitat in deep-sea sediment and water. More recent studies have reinforced the link between fatty acid composition and environmental niche (Hamamoto *et al.*, 1994, 1995). It has been suggested that the combined constraints of constant low temperature and high pressure have acted as the joint selection pressures for the evolution of bacteria with the ability to produce PUFAs (Nichols *et al.*, 1995). Hence, the evidence points to an association of '*Vibrio*'-type isolates from deep-sea sediment that appear to be responsible for the recorded prevalence of both EPA and DHA production by bacterial isolates from this environment. Two recent publications have noted the occurrence of similar PUFA-producing isolates from deep-sea fish and polar invertebrates (Yano *et al.*, 1997; Jøstensen & Landfald, 1997). The fatty acid compositions of isolates in both these studies were characteristic of *Colwellia* and *Shewanella* species. Yazawa *et al.* (1988) also noted a comparatively high association of EPA-producing isolates from the intestines of mackerel species (2·3–2·9% of isolates) in comparison to other temperate fish species studied (0–1·5%). The association of PUFA-producing isolates may not be with any particular marine environment per se, but instead reflect the association of specific bacterial populations (*Colwellia*, *Shewanella* and *Flavobacterium-Cytophaga-Bacteroides* species) with certain environmental niches.

Whilst many reports have gone to great lengths in detailing the production of PUFAs by marine bacteria, little regard appears to have been paid to stringent determinations of their taxonomic identity. Recent advances in this area now allow us to conduct a retrospective appraisal of the taxonomy of PUFA-producing bacteria. Phylogenetic investigations of 5S and 16S rRNA sequences have established a close relationship between the genera of common Gram-negative marine bacteria: *Colwellia*, *Shewanella*, *Alteromonas*, *Pseudoalteromonas* and *Ferrimonas* (DeLong *et al.*, 1993; Gauthier *et al.*, 1995; Rossello-Mora *et al.*, 1995; Bowman *et al.*, 1997, 1998a). Two of these five genera (*Colwellia* and *Shewanella*) are now recognized as central players in the production of PUFAs in the marine environment.

The genus *Colwellia* was created with the reassignment of the phylogenetically distinct taxon '*Vibrio psychroerythrus*' and the description of *Colwellia hadaliensis* (Deming *et al.*, 1988). The chemotaxonomic markers of the genus *Colwellia* became apparent with the report of Bowman *et al.* (1998a) which introduced four new species to the genus (*Colwellia demingiae*, *Colwellia psychrotropica*, *Colwellia rossensis* and *Colwellia hornerae*), all of which were isolated from Antarctic sea ice. Apart from the production of DHA, the *Colwellia* species from this study had membrane lipids which were dominated by fatty acids of the n-even chain-length type. In particular, elevated proportions of 14:0 and 14:1n7*cis* were correlated with DHA production. Careful analysis of this characteristic now distinguishes them from species of the genus *Vibrio* to which many such strains were originally assigned, but where they formed an atypical chemotaxonomic grouping (Wilkinson, 1988).

The majority of strains previously identified as *Vibrio* spp. that contain DHA and possess the *Colwellia* chemotaxonomic profile may be reassigned to the newly described species of *Colwellia*. However, two significant groups are left unaccounted for by this procedure. First, strains that contain the *Colwellia* fatty acid fingerprint but have a much higher proportion of DHA than did recognized species (e.g. see DeLong & Yayanos, 1986), and second, strains that similarly exhibit the *Colwellia* fingerprint but contain EPA instead of DHA (e.g. see Hamamoto *et al.*, 1995). Further, '*Colwellia*' strains that produce EPA do not contain 14:1, unlike other species that do produce DHA (DeLong & Yayanos, 1986; Hamamoto *et al.*, 1994, 1995; Yano *et al.*, 1997). Exceptions to this observation are the isolate Groups IV–VI of Jøstensen & Landfald (1997) and '*Vibrio pelagius*' of Ringø *et al.* (1992). These isolates contain low levels of both 14:1 and EPA. The strains that contain high proportions of DHA and the *Colwellia* markers, together with the similar EPA-producing strains, are probably two undescribed species of *Colwellia*. The full story of the genus *Colwellia*, and its importance to the PUFA story, is therefore yet to be completed.

The genus *Shewanella* was derived from the reclassification of '*Alteromonas putrefaciens*' by MacDonell & Colwell (1985). Previously, Owen *et al.* (1978) had described four distinct genetic groups within this species (Groups I–IV). Early reports of the fatty acid compositions of all *S. putrefaciens* Groups had identified some unusual fatty acyl components. Most notable was the combined presence of n-even-chain, iso-branched-odd-chain and n-odd-chain fatty acids in this bacterium, the major components being 16:1n7*cis*, iso13:0, iso15:0, 17:1n8*cis* and 15:0 (Wilkinson & Caudwell, 1980; Moule & Wilkinson, 1987). This characteristic fatty acid composition differentiated it from other members of the then family *Vibrionaceae* (Wilkinson, 1988) and became a 'fingerprint' for new species of the genus *Shewanella* (Bowman *et al.*, 1997). However, EPA

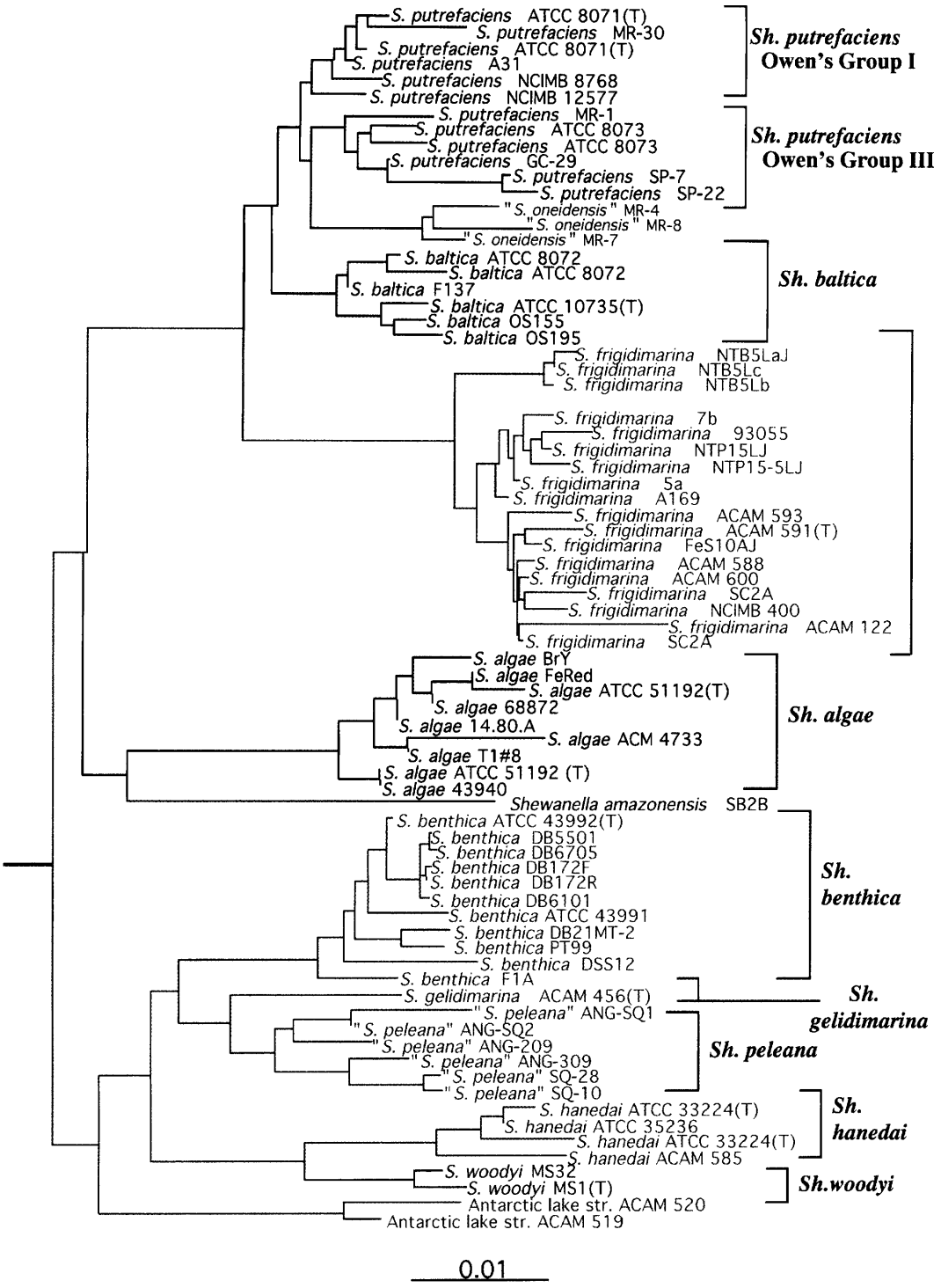

Fig. 2. Schematic representation of the unrooted 16S rRNA phylogenetic tree for the genus *Shewanella*. Species known to produce PUFAs and their lines of descent are coloured green. Species and lines of descent known not to produce PUFAs are coloured black. Species for which no data are available are coloured blue. Adapted from Bowman (1999).

production was not an ability noted in *S. putrefaciens* strains by early investigators of this genus.

The contradictory inference of EPA production appears to have been made first by Wirsen *et al.* (1987), who described an EPA-producing *Alteromonas* isolate, the fatty acid composition of which was characteristic of the '*A. putrefaciens*' fingerprint. A report the following year (Yazawa *et al.*, 1988) described the isolation of an EPA-producing marine bacterium (strain SCRC-2738) which was also considered to be '*A. putrefaciens*'-like (although it was not clear from the data published at

that time). However, by 1996 the reported fatty acid composition of SCRC-2738 had been refined and now resembled that of a *Shewanella* sp. (Yazawa, 1996). Further reports of EPA production attributed to strains of *S. putrefaciens* were also published (e.g. Matsui *et al.*, 1991; Nichols *et al.*, 1994; Jøstensen & Landfald, 1997).

It was obvious that a taxonomic revision of this situation was needed. Early reports indicated that the fatty acid composition of *S. putrefaciens*, whilst being characteristic in a number of aspects, did not possess EPA. However, more recent reports attributed EPA production to *S. putrefaciens* strains (Matsui *et al.*, 1991; Nichols *et al.*, 1994; Jøstensen & Landfald, 1997). During this period *S. putrefaciens* Groups II and IV were renamed as *Shewanella baltica* and *Shewanella alga*, respectively (Ziemke *et al.*, 1998; Simidu *et al.*, 1990; Nozue *et al.*, 1992). A further three new species (*Shewanella benthica*, *S. hanedai* and *Shewanella colwelliana*) had also been described yet their fatty acid compositions had not been determined (MacDonell & Colwell, 1985; Weiner *et al.*, 1988; Coyne *et al.*, 1989). The answers were provided in part by Bowman *et al.* (1997) during their description of subsequent novel taxa isolated from Antarctic sea ice. The newly described species *Shewanella frigidimarina* and *S. gelidimarina*, together with the existing species *S. benthica* and *S. hanedai*, were found to contain varying proportions of EPA. *S. putrefaciens* (Group I) and *S. alga* did not contain EPA (*S. colwelliana* was not examined, as the type strain was not available). Interestingly, the 'fingerprint' of the *Shewanella* genus was maintained in all species, while the relative proportion of EPA appeared to be related to the optimal growth temperature of the species. This allows a presumptive reassessment of many isolates previously described as '*S. putrefaciens*'.

Even more confusion was added by reports from a number of authors who described PUFA-producing isolates as *Vibrio* spp. when their chemotaxonomic profile clearly supported a presumptive identification as *Shewanella* spp. (Ringø *et al.*, 1992; Henderson *et al.*, 1993; Iwanami *et al.*, 1995; Jøstensen & Landfald, 1996). Indeed, a telling comment was made by Jøstensen & Landfald (1997), who stated 'The tentative classification of the present PUFA producers was in keeping with a consistent pattern in preceding literature, where such bacteria, with few exceptions, have been affiliated to the vibrios on one hand....or *S. putrefaciens* on the other.'. Perhaps such misleading statements in the literature can now be put to rest in the light of the newer information given above.

A schematic representation of the current 16S rRNA phylogenetic tree for the genus *Shewanella* is displayed in Fig. 2. Overlaid on this illustration is a summary of known PUFA production by *Shewanella* species. Several points are evident. First, it is implied that the progenitor of the genus possessed the ability to synthesize PUFAs. Second, it appears that this ability has been retained by all the psychrophilic and several of the psychrotolerant species. Third, the ability to synthesize PUFAs, or the expression of PUFA synthesis, has disappeared on two distinct occasions during the divergence of psychrotolerant and/or mesophilic species. This may imply a specific role for PUFAs in cold-temperature adaptation. The conservation of PUFA genes (in this genus at least) may also point to the potential utilization of these 'functional genes' as phylogenetic markers in culture-independent ecological studies.

To date there has been a conspicuous lack in the literature of reports of PUFA-producing isolates from the *Flavobacterium-Cytophaga-Bacteroides* phylum and the genus *Thiothrix*. Isolates from both of these groups were amongst the first reported to produce PUFAs (Johns & Perry, 1977; Jacq *et al.*, 1989). However, further reports of such isolates have not occurred in subsequent studies of marine PUFA-producing bacteria, with one exception. Temara *et al.* (1991) described the isolation of a symbiotic *Thiothrix* sp. from the digestive system of the echinoid *Echinocardium cordatum* which contained an 18:3 fatty acid component. However, longer-chain PUFAs such as that found from the *Thiothrix*-like strain of Jacq *et al.* (1989) were not identified.

Several novel species of *Flavobacterium* and *Flectobacillus* have been isolated from the saline lakes of Antarctica (McGuire *et al.*, 1987; Dobson *et al.*, 1991, 1993) although none were reported to contain PUFAs (Dobson *et al.*, 1993; Nichols *et al.*, 1993). Recent studies of the Antarctic environment have discovered further novel species of this phylum that do produce PUFAs. *Psychroflexus torquis* (Bowman *et al.*, 1998b) is unique amongst all PUFA-producing bacteria yet described, as it produces both EPA and an n6 PUFA component, AA (Nichols *et al.*, 1997a). Such examples indicate that the biosynthetic diversity of bacterial PUFA production has yet to be fully described.

Mechanism of fatty acid biosynthesis in the *Bacteria*

Like all organisms, the *Bacteria* make fatty acids using an organized array of several enzyme activities, known trivially as fatty acid synthase (FAS). In most bacteria the activities are usually arranged as a dissociable multienzyme complex (a Type II FAS; see Harwood & Russell, 1984) around a central acyl carrier protein (ACP) molecule; a similar organization occurs in plant FAS, but not in eukaryotic micro-organisms. The intermediates are covalently bonded to ACP as they undergo successive cycles of condensation, reduction, dehydration and reduction for each 2C-unit added from malonyl-ACP. In contrast, some bacteria have a so-called Type I FAS composed of large multifunctional proteins (i.e. they each have several active sites) to which ACP is covalently bound, as found also in the FAS of animals. The primer molecule for FAS is generally acetyl-ACP, which after seven rounds of 2C-unit addition gives palmitic acid as the straight-chain product (16:0). Many bacteria, particularly Gram-positive gen-

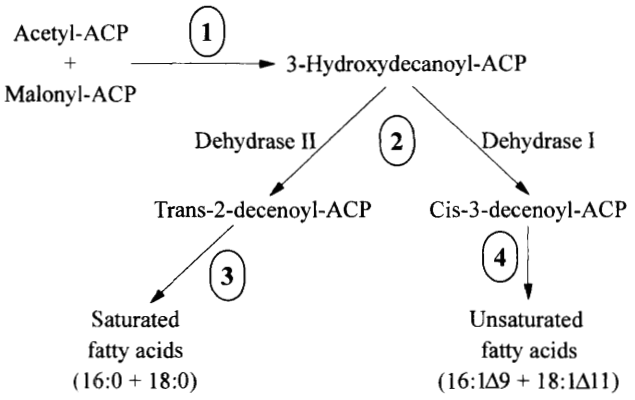

Fig. 3. Pathways of fatty acid biosynthesis in bacteria. The following steps are depicted: 1 → 3, FAS producing only saturated fatty acids; 1 → 4, FAS producing a mixture of saturated and unsaturated fatty acids ('anaerobic pathway'). See text for full explanation. The dehydrase II enzymes in the two FAS are not necessarily the same, but for the sake of simplicity have not been distinguished in the diagram. ACP, acyl carrier protein.

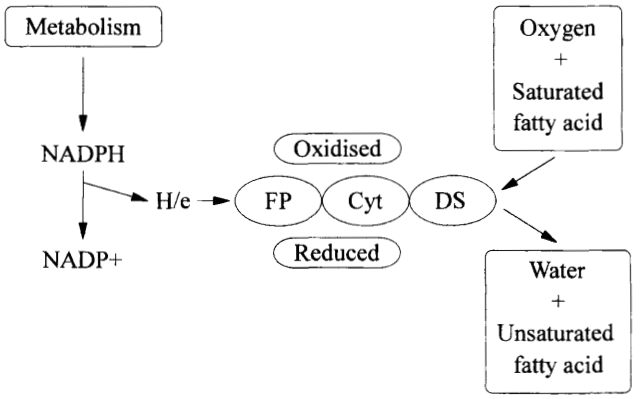

Fig. 4. Generic outline of the mechanism of formation of unsaturated fatty acids by aerobic desaturation. Reducing equivalents from NADPH are passed through a short series of redox proteins ending in the desaturase enzyme, which uses them to reduce molecular oxygen to water. In yeast these proteins are cytochrome b_5 and cytochrome-b_5 reductase; in some bacteria a cytochrome-b_5-like cytochrome is involved, but the identity of the redox proteins in marine Antarctic bacteria is unknown. Note that the cycle may be repeated with monounsaturated fatty acid products being the substrates for further rounds of desaturation with desaturases having different positional specificities to give PUFA products. FP, flavoprotein; Cyt, cytochrome; DS, desaturase enzyme.

era, contain methyl-branched-chain fatty acids. These are also made by the same FAS system except that it utilizes a branched-chain primer molecule, which is derived from amino acids by deamination and activation with CoA. Thus, valine is converted to isobutyryl-CoA, which is the precursor of iso-branched even-carbon-numbered fatty acids; leucine gives iso-branched odd-carbon-numbered products and isoleucine gives anteiso-branched odd-carbon-numbered products (iso-branched fatty acids have the methyl group in the ω-1 position and anteiso-branched acids in the ω-2 position: see Harwood & Russell, 1984, for details). The mechanism of primer selection and the regulation (e.g. by temperature) of FAS which produce branched-chain fatty acids is poorly understood (Kaneda, 1991).

There is a further functional distinction in the bacterial FAS, which may be one of two types, synthesizing either saturated fatty acid products only (like the systems in animals and plants and eukaryotic micro-organisms) or a mixture of saturated and monounsaturated products (summarized in Fig. 3). The latter system is known as the 'anaerobic pathway', but this is less a reflection of the reaction mechanism for fatty acids per se, which does not involve oxygen directly, than of the way in which unsaturated fatty acids are produced. In bacteria which use the anaerobic pathway, the FAS is modified so that a mixture of saturated and monounsaturated products is made. Instead of a simple linear process, there is a branch point at the level of a C10 (or C12) hydroxy intermediate, which is dehydrated stereospecifically to give a *trans*-2,3 or a *cis*-3,4 unsaturated intermediate; the first gives rise to 'normal' saturated products whereas the second gives monounsaturated fatty acids (Fig. 3). The balance of carbon flow down the saturated and unsaturated 'wings' of the split pathway is regulated by temperature because there are distinct condensing enzymes (β-ketoacyl-ACP synthetases) for saturated and unsaturated intermediates and they have different thermal stabilities, giving more unsaturated products at lower temperatures (de Mendoza & Cronan, 1984). None of these reactions require molecular oxygen directly; the oxidative steps are achieved by the transfer of hydrogens to cofactors such as NAD^+, which is reoxidized by other metabolic reactions at the substrate level or by oxidative phosphorylation. In contrast, bacteria which do not have the specific dehydrase for the formation of the *cis*-3-ene intermediate make unsaturated fatty acids by subsequently desaturating the saturated FAS products using separate and mechanistically distinct desaturase enzymes. These are generally located in membranes and utilize as their substrate the acyl chains of intact membrane lipids. Although their reaction mechanism also involves the removal of hydrogens to a cofactor (NAD^+ or $NADP^+$), the reoxidation of the reduced cofactor is achieved by a short electron transfer chain which forms part of the desaturase enzyme complex in the membrane and which uses molecular oxygen as the terminal acceptor (Fig. 4). Therefore, the process of making unsaturated fatty acids involving desaturases is an aerobic one. It follows that anaerobic bacteria must use the anaerobic pathway of fatty acid biosynthesis, but aerobic bacteria may use either route. Until recently, it was believed that the two pathways were mutually exclusive, only one occurring in a particular bacterium. Although that tenet still holds true for most bacterial species, some exceptions have been discovered recently, and the extensive studies of Wada *et al.* (1991) have proved that both biosynthetic pathways could operate simultaneously in the one

bacterium. This work is relevant to PUFA synthesis in bacteria, as discussed below.

A characteristic of the anaerobic pathway is that it is limited to the production of monounsaturated fatty acids only. Similarly, it is generally true that bacteria which use aerobic desaturase only make monounsaturated fatty acids, implying that they have only a single type of desaturase enzyme. Desaturases are specific for the position of the double bond which they 'insert', the position being fixed relative to the carboxyl end of the substrate molecule. So, for example, a Δ9 desaturase will insert a double bond into stearic (18:0) or palmitic (16:0) acids to give oleic (18:1Δ9) or palmitoleic (16:1Δ9) acids. In organisms that make PUFAs, there are distinct desaturases for inserting the different double bonds, which are generally methylene-interrupted – i.e. they are separated by a $-CH_2-$ unit. This gives a distinct pattern to PUFAs, such as in α-linolenic (18:3Δ9,12,15) or arachidonic (20:4Δ5,8,11,14) acids.

In contrast, the anaerobic pathway 'inserts' the double bond as part of the cyclic process of adding two-carbon units and generally produces palmitoleic (16:1Δ9) and *cis*-vaccenic (18:1Δ11) acids. Moreover, because the branch point at which unsaturated and saturated product lines diverge is seldom absolute, it is common to observe minor products alongside the major fatty acid product – e.g. traces of 18:1Δ9 and 18:1Δ13 together with a major proportion of 18:1Δ11. Therefore, the isomeric pattern of unsaturated fatty acids, which can readily be analysed using capillary (but not packed-column) GLC, is diagnostic of whether the anaerobic pathway or desaturase is used to make unsaturated fatty acids in a given bacterium.

Therefore, it was particularly revealing to find an Antarctic marine bacterium such as *S. gelidimarina* in which the monounsaturated fatty acids had a double-bond isomeric pattern that was diagnostic of the anaerobic pathway, whereas the PUFAs were typical of the methylene-interrupted fatty acids found in eukaryotic organisms from yeasts to humans. Clearly, two distinct systems for making fatty acids must be present. Experiments have shown that these systems are regulated by growth substrates or temperature in an independent manner (Nichols & Russell, 1996; Yazawa, 1996; Nichols *et al.*, 1997b).

Mechanism of PUFA biosynthesis in the *Bacteria*

Since the anaerobic pathway of bacterial fatty acid biosynthesis is not capable of introducing more than one double bond per fatty acid product, PUFAs must be synthesized by an aerobic mechanism (but see comment below). It is assumed that a series of Δ5, Δ6 and Δ9 desaturases are involved, since bacterial desaturases (like those from animals and plants) are known to introduce a double bond at a specific position relative to the carboxyl terminus (Quint & Fulco, 1973; Russell, 1978) and bacterial PUFA products also comprise two distinct families (the n3 and the n6 series) that are the same as animal fatty acids. The desaturases would have to act in concert with an elongation system (or a set of specific elongases), unless desaturases with other positional specificities are involved – the latter is a real possibility because 'unusual' positional specificities are known, such as Δ10 in bacilli (Quint & Fulco, 1973). Just such unusual desaturases (Δ15 and Δ17) were proposed by Iwanami *et al.* (1995) in an unidentified '*Vibrio*' species, which was able to convert radiolabelled AA to EPA. A similar Δ17 desaturase had been surmised to occur in the fungus *Saprolegnia parasitica* on the basis of radiolabelling studies (Gellerman & Schlenk, 1979). The enzyme is believed to exist in several fungi, including *Mortierella* spp., which carry out the same conversion (Singh & Ward, 1997). A consequence of Δ17 desaturation of AA (20:4Δ5,8,11,14) to EPA (20:5Δ5,8,11,14,17) is to convert an n6 acid to an n3 acid, whereas the actions of the more common Δ4, Δ5 and Δ6 desaturases on either n3 or n6 families of acids do not.

It should be noted in this context that several interpretations of the published experimental data of Iwanami *et al.* (1995) with *Vibrio* are possible and the existence of the proposed desaturases has not been confirmed in a subsequent publication. These authors proposed a PUFA biosynthetic pathway involving a concerted series of desaturases and elongases on the basis of experiments using radiolabelled oleic acid (18:1n9) and some other polyunsaturated precursors of EPA (Iwanami *et al.*, 1995). However, their data show that the major products from oleic acid contained only two or three double bonds, not five as in EPA, and that cerulenin (an inhibitor of elongation) affected a step in the conversion of triunsaturated to tetraunsaturated fatty acid. Therefore, their proposed pathway to final products containing 20 carbon atoms and up to five double bonds remains at best speculative. They also observed that anaerobiosis had only a marginal effect on desaturation, which is consistent with the data of Nichols *et al.* (1992), who reported the anaerobic synthesis of PUFAs by '*S. putrefaciens*'. More recently, Facciotti *et al.* (1998) have reported that the PUFA-synthesizing systems of several marine bacteria can function under anaerobic conditions. Therefore, the general scheme of (aerobic) desaturation, as illustrated in Fig. 4, may well have to be modified in the light of further investigation to include a terminal acceptor other than oxygen for the reducing equivalents coming from the desaturase enzyme. This would make polyunsaturation in marine bacteria analogous with anaerobic respiration using such acceptors as nitrate or fumarate.

Using *S. gelidimarina* as the test organism, we have been unable to detect the accumulation of polyunsaturated intermediates of the biosynthesis of EPA, even using sensitive radiolabelling techniques (D. S. Nichols & N. J. Russell, unpublished results). Using radiolabelled malonyl-CoA as the precursor, the major radioactive

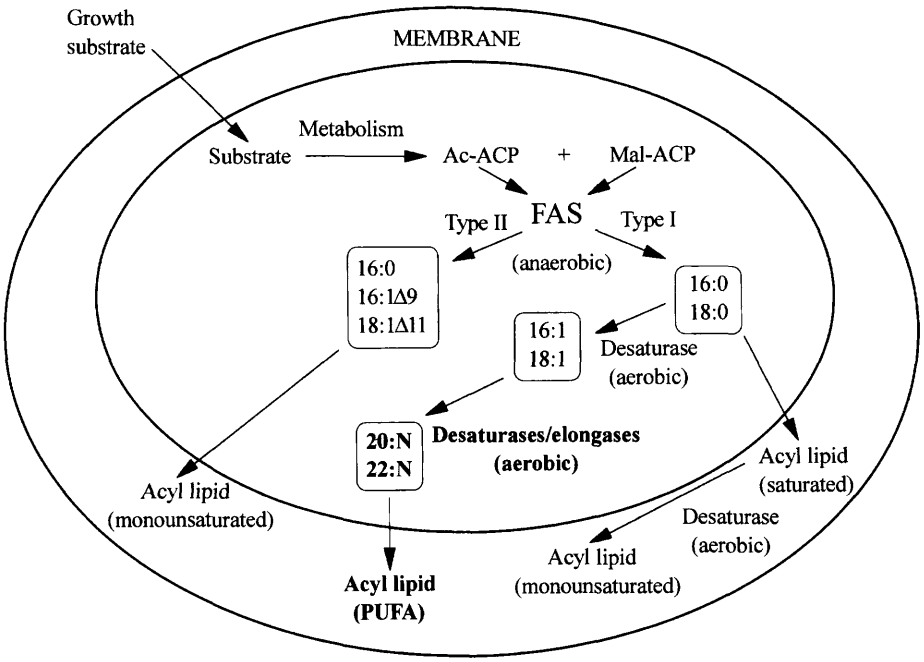

Fig. 5. Summary of possible pathways for the biosynthetic origin of fatty acyl chains in bacterial membrane lipids. The substrate for aerobic desaturation in the cytoplasm could be acyl-CoA or acyl-ACP; the position of the double bond is commonly Δ9. Saturated acyl lipid in the membrane may also be desaturated to give monounsaturated lipid, but neither of the two classes of monounsaturated lipids (i.e. those derived from aerobic or anaerobic desaturation) is capable of direct conversion to PUFAs because of the need for elongation. Nothing is known of the elongation mechanism(s) in marine bacteria, but acyl-CoA or acyl-ACP is probably used (see text for discussion). Ac-ACP, acetyl-acyl carrier protein; Mal-ACP, malonyl-acyl carrier protein; 20:N and 22:N, PUFAs.

product is EPA. Subcellular fractionation failed to separate the desaturation and elongation reactions of the *de novo* fatty acid biosynthetic pathway for EPA from malonyl-CoA. We interpret this finding as evidence that the complete pathway from FAS primer to final PUFA product is organized as an integrated complex. It is likely that, unlike most other systems, the desaturases involved in PUFA biosynthesis utilize acyl-CoA (or acyl-ACP) as their substrate rather than intact acyl lipids in the membranes. Elongases could not use complex lipids as substrate because the two-carbon units must be added to the carboxyl terminus which is blocked in an acyl-lipid and therefore cannot be activated. A model of the potential reaction pathways for the biosynthesis of membrane lipids containing PUFA acyl chains is shown in Fig. 5. These biosynthetic results with *S. gelidimarina* are consistent with fatty acid analyses of EPA-containing bacteria which either lack other PUFAs with two, three or four double bonds or contain only very small (trace) amounts; *Psychroflexus torquis* is an exception as it contains both EPA and AA (Bowman *et al.*, 1998b).

The concept of an integrated complex for PUFA biosynthesis is supported by the elegant experiments of Yazawa and co-workers, who have successfully cloned and expressed a 38 kb genomic DNA fragment from the marine bacterium '*S. putrefaciens*' strain SCRC-2738 in *Escherichia coli* (Yazawa, 1996) and the marine cyanobacterium *Synechococcus* sp. (Takeyama *et al.*, 1997). *E. coli*, of course, does not normally make PUFAs and the cyanobacterium only makes them up to 18:3. In each instance the recombinant organisms produced EPA, albeit in rather small amounts. For example, in *Synechococcus* sp. the amount of EPA made was only $\leqslant 0{\cdot}5\,\%$ of the total fatty acids, but significantly EPA synthesis was up-regulated by a decrease in growth temperature. The 38 kb insert contains eight ORFs, three of which are homologous to FAS enzymes, and the authors speculated that the remaining ORFs may include desaturase genes. It will be interesting to determine whether the FAS component of the gene cluster is a distinct type compared with that which produces saturated and monounsaturated fatty acids.

Role of PUFAs in bacterial membranes

Why should Antarctic marine psychrophilic bacteria have PUFAs as acyl components of their various membrane lipids, when other bacteria equally adapted to grow at low temperatures do not? One answer is that marine psychrophiles, particularly those in sea ice, experience constant low temperature ($-1{\cdot}8$ °C). However, that comment ignores the fact that not all marine psychrophiles contain PUFAs and also fails to explain the functional significance for membrane structure and stability.

That the proportions of PUFAs in membrane lipids change with alterations in growth temperature and may be maximal at the lowest growth temperatures (e.g. see Hamamoto *et al.*, 1994) indicates they are likely to be part of a homeoviscous adaptive response to regulate membrane fluidity. However, it has been demonstrated using model lipid systems that the introduction of more than two double bonds in a membrane lipid has little additional effect on lowering the liquid-crystalline to gel phase transition temperature and even monounsaturated phospholipids of the type found commonly in bacterial membranes remain fluid at temperatures a few degrees below zero (Russell, 1989). In fact the introduction of multiple double bonds restricts acyl-chain rotation and creates a structural element that influences the packing order of the acyl chains, because each double bond 'bends' the acyl chain so that molecules with four or five double bonds form 'hairpins' which reduce the effective acyl chain length (Keough & Kariel, 1987; Keough *et al.*, 1987). The presence of PUFAs may be more relevant to the maintenance of the correct phase of the lipids (i.e. a homeophasic adaptive response), because many common bacterial phospholipids (including unsaturated phosphatidylethanolamine, a major lipid in *Shewanella* and *Colwellia* species) favour the formation of non-bilayer phases (e.g. hexagonal-II phase) which would disrupt membrane packing and permeability with disastrous consequences for cell viability. The multiple double bonds in PUFAs allow sufficient molecular motion and disruption of molecular packing of the acyl chains to lower the transition temperature to the gel state, thereby keeping the membrane fluid, but concomitantly provide a higher degree of packing order (compared to monounsaturated residues) with a shorter acyl chain length which prevents non-bilayer-phase formation and both 'seals' the bilayer and controls passive permeability at low temperatures.

These ideas are consistent with the so-called 'effective chain length' model of Cevec (1991) and the 'dynamic phase behaviour' model of Hazel (1995). Briefly, Cevec (1991) considers that the fluidizing influence of unsaturated fatty acyl chains on the gel to liquid-crystalline phase transition of membrane lipids may be explained by their effective chain length in the bilayer. He states that for monounsaturated acyl chains in which the double bond is closer to either end of the molecule, the shorter of the two chain segments is usually permanently 'fluid'. Hence the fatty acyl chain behaves with an effective chain length of only the longer of the two chain segments. Hazel (1995) considers that the important factor of cell membrane regulation lies not in a single parameter (viz. fluidity) but in the adjustment of membrane lipid composition to balance factors driving the gel to liquid-crystalline transition on the one hand with those favouring the bilayer to non-bilayer phase transition on the other. The latter may be needed for such functions as membrane growth and its partitioning during cell division. Thus considerations of the significance of fatty acyl changes that take into account only fluidity may be misleading.

A recent fast atom bombardment tandem mass spectrometric analysis of EPA distribution in phospholipid classes of *S. gelidimarina* by Nichols *et al.* (1997b) revealed that the PUFA is associated with different fatty acyl chains in the two major phospholipids: in phosphatidylethanolamine (PE) it is paired mainly with branched-chain acids (particularly iso13:0), whereas in phosphatidylglycerol (PG) it is paired mainly with monounsaturated and straight-chain acids, while there is less EPA in PG compared with PE. This distribution appears to be consistent with explanations based upon a homeophasic (rather than a homeoviscous) adaptive response because unsaturated PE tends to form hexagonal phases, whereas PG tends to form lamellar (bilayer) phases. The selective incorporation of PUFAs into PE prevents deleterious formation of hexagonal-II phase domains (which could occur if monounsaturated or branched-chain fatty acyl chains were increased at lower temperatures) whilst continuing to provide sufficient fluidity and order to the membrane lipid phase. However, although all membranes require a balance of bilayer and non-bilayer phase-forming lipids in the membrane, with different lipids serving different functional as well as structural roles (Dowhan, 1997), it is difficult to generalize in relation to the role of PUFAs in the membranes of marine psychrophilic bacteria. For example, Yazawa (1996) found that there was twice as much EPA in PG compared with PE in a strain (SCRC-2738) of '*S. putrefaciens*', although no information was given about how the relative proportions might alter with changes in growth temperature. Biophysical studies of such marine psychrophiles are required in order to determine the phase behaviour of their membrane lipids. These experiments should be backed up by others in which selected PUFA biosynthetic genes are eliminated so as to reveal phenotypic functions. Mutants of *S. putrefaciens* (SCRC-2738) impaired in EPA biosynthesis are unable to grow at 2 °C unless supplemented with EPA or another PUFA, but revertants did not have increased PUFA contents: instead they increased the biosynthesis of 16:1Δ9 and 18:1Δ11 (by the anaerobic pathway), which suggests that PUFAs may not have a unique or vital function in low-temperature adaptation (Facciotti *et al.*, 1998).

Epilogue

The rapid pace of discovery of new marine psychrophiles containing PUFAs has been driven by two main factors: the interest in biodiversity and the search for biotechnological solutions to human concerns about nutrition and health.

The use of fatty acid compositions has become commonplace as a tool in the identification of new organisms and in their taxonomic descriptions. Among the marine bacteria, PUFAs have been particularly significant in redefining old classifications and creating new groupings (e.g. Bowman *et al.*, 1997, 1998a, b). That process will continue but it is the biotechnological production of PUFAs from marine bacteria (and other microorganisms) that will provide a focus for future develop-

ments in the elucidation of the biosynthetic pathway and the characterization of the genes, including their transfer into industrially relevant producer organisms, probably both microbial and plant. It is perhaps fitting that bacterial genes, which possibly were acquired through natural horizontal transfer from higher organisms, should artificially be transferred back in the creation of recombinant organisms for the beneficial biotechnological production of useful compounds.

PUFA supplements from fish oils are widely used personally on a daily basis, generally taken in the form of capsules. The oils are also added to infant milk formula, and other uses are emerging, including additions to chocolate and bread, sometimes with exaggerated claims for their effect on human mental ability ('Einstein milk'!). No doubt other nutriceutical (Linko & Hayakawa, 1996) and pharmaceutical uses will be developed, and, as fish stocks continue to dwindle worldwide, there will be ever more pressure to seek alternative, microbiological solutions to the supply of PUFAs.

The potential role of marine bacteria as a significant *de novo* source of PUFAs in marine food webs (Yazawa *et al.*, 1992) mirrored the concept of using such organisms as an alternative primary PUFA-rich feedstock in the artificial food chains used in the aquaculture industry. By varying the primary bacterial feedstock, specific PUFA enrichment can be achieved, and several studies have now demonstrated the trophic transfer of both EPA and DHA from bacteria to the rotifer *Brachionus plicatilis*, which is used in the cultivation of small-mouthed larval fish (Watanabe *et al.*, 1992; Nichols *et al.*, 1996; Lewis *et al.*, 1998). With further development, this concept may represent the most 'natural' biotechnological application of PUFA-producing marine bacteria.

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