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MARINE BIOTECHNOLOGY

Molecular Cloning and Functional Characterization of Fatty Acyl Desaturase and Elongase cDNAs Involved in the Production of Eicosapentaenoic and Docosahexaenoic Acids from α-Linolenic Acid in Atlantic Salmon (*Salmo salar*)

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Abstract: Fish are the only major dietary source for humans of α-3 highly unsaturated fatty acids (HUFAs) and with declining fisheries farmed fish such as Atlantic salmon (*Salmo salar*) constitute an increasing proportion of the fish in the human diet. However, the current high use of fish oils, derived from wild capture marine fisheries, in aquaculture feeds is not sustainable in the longer term and will constrain continuing growth of aquaculture activities. Greater understanding of how fish metabolize and biosynthesize HUFA may lead to more sustainable aquaculture diets. The study described here contributes to an effort to determine the molecular genetics of the HUFA biosynthetic pathway in salmon, with the overall aim being to determine mechanisms for optimizing the use of vegetable oils in Atlantic salmon culture. In this paper we describe the cloning and functional characterization of 2 genes from salmon involved in the biosynthesis of HUFA. A salmon desaturase complementary DNA, SalDes, was isolated that include an open reading frame of 1362 bp specifying a protein of 454 amino acids. The protein sequence includes all the characteristics of microsomal fatty acid desaturases, including 3 histidine boxes, 2 transmembrane regions, and an N-terminal cytochrome b_5 domain containing a heme-binding motif similar to that of other fatty acid desaturases. Functional expression in the yeast *Saccharomyces cerevisiae* showed SalDes is predominantly an α-3 Δ5 desaturase, a key enzyme in the synthesis of eicosapentaenoic acid (20:5n-3) from α-linolenic acid (18:3n-3). The desaturase showed only low levels of Δ6 activity toward C_{18} polyunsaturated fatty acids. In addition, a fatty acid elongase cDNA, SalElo, was isolated that included an open reading frame of 888 bp, specifying a protein of 295 amino acids. The protein sequence of SalElo included characteristics of microsomal fatty acid elongases, including a histidine box and a transmembrane region. Upon expression in yeast SalElo showed broad substrate specificity for polyunsaturated fatty acids with a range of chain lengths, with the rank order being $C_{18} > C_{20} > C_{22}$. Thus this one polypeptide product displays all fatty acid elongase activities required for the biosynthesis of docosahexaenoic acid (22:6n-3) from 18:3n-3.

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INTRODUCTION

Generally declining catches from wild fisheries have resulted in an increasing proportion of fish for human consumption being provided by aquaculture, which is expanding at over 10% per annum (Tidwell and Allan, 2002). However, much aquaculture, including salmonid (salmon and trout) culture, is itself dependent upon wild capture fisheries for the provision of fish meals and oils that have traditionally been the predominant protein and lipid sources (Sargent and Tacon, 1999). Both are readily accepted and digested by fish and, until now, were readily available and inexpensive (Wilson, 1989; Sargent et al., 1989; Barlow, 2000). However, stagnation in industrial fisheries, along with the increased demand for fish oils, has dictated that alternatives to fish oil must be found if aquaculture is to continue to expand and supply more of the global demand for fish (Barlow, 2000).

The only sustainable alternative to fish oils are plant (vegetable) oils, some of which are rich in C_{18} polyunsaturated fatty acids (PUFAs) such as 18:2n-6 and 18:3n-3. However, vegetable oils are devoid of the n-3 highly unsaturated fatty acids (HUFAs), eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3) which are abundant in fish oils (Sargent et al., 2002).The extent to which animals, including fish, can convert C_{18} PUFA to $C_{20/22}$ HUFA varies with species and is associated with their capacity for microsomal fatty acyl desaturation and elongation. Salmonid fish, including Atlantic salmon (*Salmo Salar*) are capable of producing DHA from 18:3n-3 (Buzzi et al., 1996; Tocher et al., 1997; Bell et al., 2001a) and so express all the desaturase and elongase activities necessary for this biosynthetic pathway (Sargent et al., 2002). Salmon fed diets containing vegetable oils show significantly increased activity of the fatty acyl desaturation/elongation pathway compared with fish fed standard diets containing fish oil (Bell et al. 1997, 2001b, 2002; Tocher et al., 1997, 2000, 2001). Nonetheless, tissue fatty acid compositions in salmon fed vegetable oils are characterized by increased levels of C_{18} PUFA and decreased levels of $C_{20/22}$ HUFA, compromising their nutritional value to the human consumer (Bell et al., 2001b, 2002). Even in salmon fed linseed oil containing high levels of 18:3n-3, the increased activity of the desaturation/elongation pathway cannot convert 18:3n-3 to EPA and DHA efficiently enough to prevent the accumulation of 18:3n-3 and depletion of EPA and DHA in the tissues (Bell et al., 1997; Tocher et al., 2000, 2001).

An understanding of the molecular basis of HUFA biosynthesis would underpin efforts to address this problem. However, until recently, although the biochemical pathways involved in PUFA synthesis were described, little was known of the enzymes involved and of the factors affecting their functions. Significant progress has now been made in characterizing the desaturases and elongases involved in HUFA synthesis (Tocher et al., 1998). Full-length complementary DNAs for Δ6 desaturases have been isolated from the filamentous fungi *Morterella alpina* (Huang et al., 1999), the nematode *Caenorhabditis elegans* (Napier et al., 1998), rat (Aki et al., 1999), and mouse and human (Cho et al., 1999a). Fatty acid Δ5 desaturase genes have been isolated from *M. alpina* (Michaelson et al., l998a), *C. elegans* (Michaelson et al., 1998b; Watts and Browse, 1999), and human (Cho et al., 1999b; Leonard et al., 2000a). The genes encoding putative PUFA elongase enzymes have been cloned from *M. alpina* (Parker-Barnes et al., 2000), *C. elegans* (Beaudion et al., 2000), and human (Leonard et al., 2000b). When expressed in *S. cerevisiae* all of these elongase genes were shown to encode enzymes that elongated C_{18} PUFA to C_{20}, and the human elongase also elongated C_{20} PUFA to C_{22}.

The availability of sequence data has made it possible to study the genes of the HUFA biosynthesis pathway in fish, and recently we isolated 2 cDNAs from zebrafish (*Danio rerio*), one with high similarity to mammalian Δ6 desaturase genes (Hastings et al., 2001) and one with high similarity to the human elongase (Agaba et al., 2003). Heterologous expression in *S. cerevisae* indicated that the zebrafish genes were unique in that the desaturase cDNA encoded an enzyme having both Δ6 and Δ5 desaturase activities and the elongase cDNA encoded an enzyme with activity towards C_{18}, C_{20} and C_{22} PUFA. Our objective is to determine the molecular genetics of the HUFA biosynthetic pathway in salmon, with the overall aim being to determine mechanisms for optimizing the use of vegetable oils in Atlantic salmon culture. As a first step, this paper describes the cloning and functional characterization of 2 genes of Atlantic salmon involved in the biosynthesis of EPA and DHA.

Materials and Methods

Cloning of a Salmon PUFA Desaturase cDNA and Sequence Analysis

Desaturase sequences of fish, including the zebrafish Δ6/Δ5 desaturase (GenBank accession number AF309556) and the putative Δ6 desaturases from rainbow trout (AF301910) and carp (AF309557), were aligned to enable the design of the degenerate primer, Fish 6for (5′-CCCAAGCTTGAGGATGGGAGTGG-3′). This was used in conjunction, with T7PolyT (5′-TACGACTCACTATAGGGCGTGCAGTTTTTTTTTTT-3′) for the polymerase chain reaction (PCR) isolation of the salmon desaturase using cDNA produced from total RNA isolated from liver tissue of Atlantic salmon fed a standard pellet diet based on fish meal and oil. Briefly, cDNA was synthesized from salmon liver total RNA using Moloney murine leukemia virus reverse transcriptase (Promega,) primed with T7PolyT. A portion of this cDNA was then subjected to PCR amplification (Ready-to-Go PCR beads; Amersham Biosciences UK Ltd.) with the T7PolyT primer and the degenerate Fish 6for primer, which contains the predicted initiation codon. Amplification involved an initial denaturation step at 95°C for 60 seconds, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 2 minutes. The products were cloned into the Bluescript KSII$^+$ vector (Stratagene) using standard methods, and nucleotide sequences were determined by standard dye terminator chemistry using a PerkinElmer ABI-377 DNA sequencer following the manufacturers protocols. Deduced amino acid sequences were aligned using CLUSTAL X, and sequence phylogenies were predicted using the neighbor joining method of Saitou and Nei (1987). Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations.

Cloning of Salmon PUFA Elongase cDNA

The PUFA elongase sequence (AF206662) of *M. alpina* was used to query the GenBank EST database (at NCBI) for homologues using the tblastn program. Several vertebrate ESTs, including one from zebrafish (GeneBank accession number BF157708), were identified that had high similarity to the query sequence. Degenerate oligonucleotides were designed from the conserved motifs (GPRD, LWWYYF, EFMDSFF) identified by aligning the deduced amino acid sequences of PUFA elongases from human (ELOVL5, AF231981), *Xenopus laevis* (BG813561), and zebrafish (BF157708, AF532782). The primer sequences were UniElo1A (CCTGTGGTGGTAYTAYTT) and UniElo1AA (GRTTY ATGGACACNTTCTTCTT), and they targeted the LWWYYF and EFMDSFFF motifs, respectively. These primers were used in 3′ rapid amplification of CDNA ends (RACE) PCR to clone the partial transcript from Atlantic salmon liver cDNA using the SMART RACE system (Clonetech Laboratories UK Ltd.). PCR fragments were cloned into Bluescript KSII$^+$ vector, and the sequences were determined and analyzed as described above. The sequence of the partial cDNA was then used to design primers for 5′ RACE PCR and so clone the 5′end of the transcript.

Heterologous Expression of Desaturase and Elongase ORFs in Saccharomyces cerevisae

Functional characterization of the genes was by expression of the open reading frames (ORFs) of the salmon putative fatty acid desaturase (SalDes) and elongase (SalElo) sequences in *Saccharomyces cerevisiae*. Expression primers were designed for amplification of the ORFs from salmon liver cDNA. These primers also carried restriction sites to enable cloning into the appropriate expression vectors. For SalDes, the sequence of the sense primer SalpYESFor (CCC AAGCTT ACTATGGGGGGCGGAGGCG) contained an *Hin*dIII site (underlined) and the antisense primer SalPYESRev (CCG CTCGAG TCATTTATGGAGATATGCAT) contained an *Xho*I site (underlined). For SalElo, the sequence of the sense primer SalEloXA (AA GAATTC AAGCTTCTAGGGTCAG AAATGGAG) contained on *Eco*RI site (underlined), and the antisense primer SalEloX1B (AA CTCGAG ACA GTATTCAAGCTTCAGTCCC) contained an *Xho*I site (underlined). PCR was performed using high-fidelity DNA polymerase (Roche Diagnostics Ltd.) following the manufacturer's instruction. After PCR, the DNA fragments were restricted with the appropriate enzymes, *Hin*dIII and *Xho*I for SalDes, and *Eco*RI and *Xho*I for SalElo, and ligated into similarly digested yeast expression vectors pYES2 (Invitrogen Ltd.) and pYX222 (R & D Systems Europe Ltd.,), respectively. These were then used to transform Top10F' Escherichia coli competent cells (Invitrogen Ltd.), which were screened for the presence of recombinant plasmids. Transformation of the yeast. *S. cerevisiae* (strain InvSc1) with the recombinant plasmids was carried out using the S.c.EasyComp Transformation Kit (Invitrogen Ltd.). Selection of

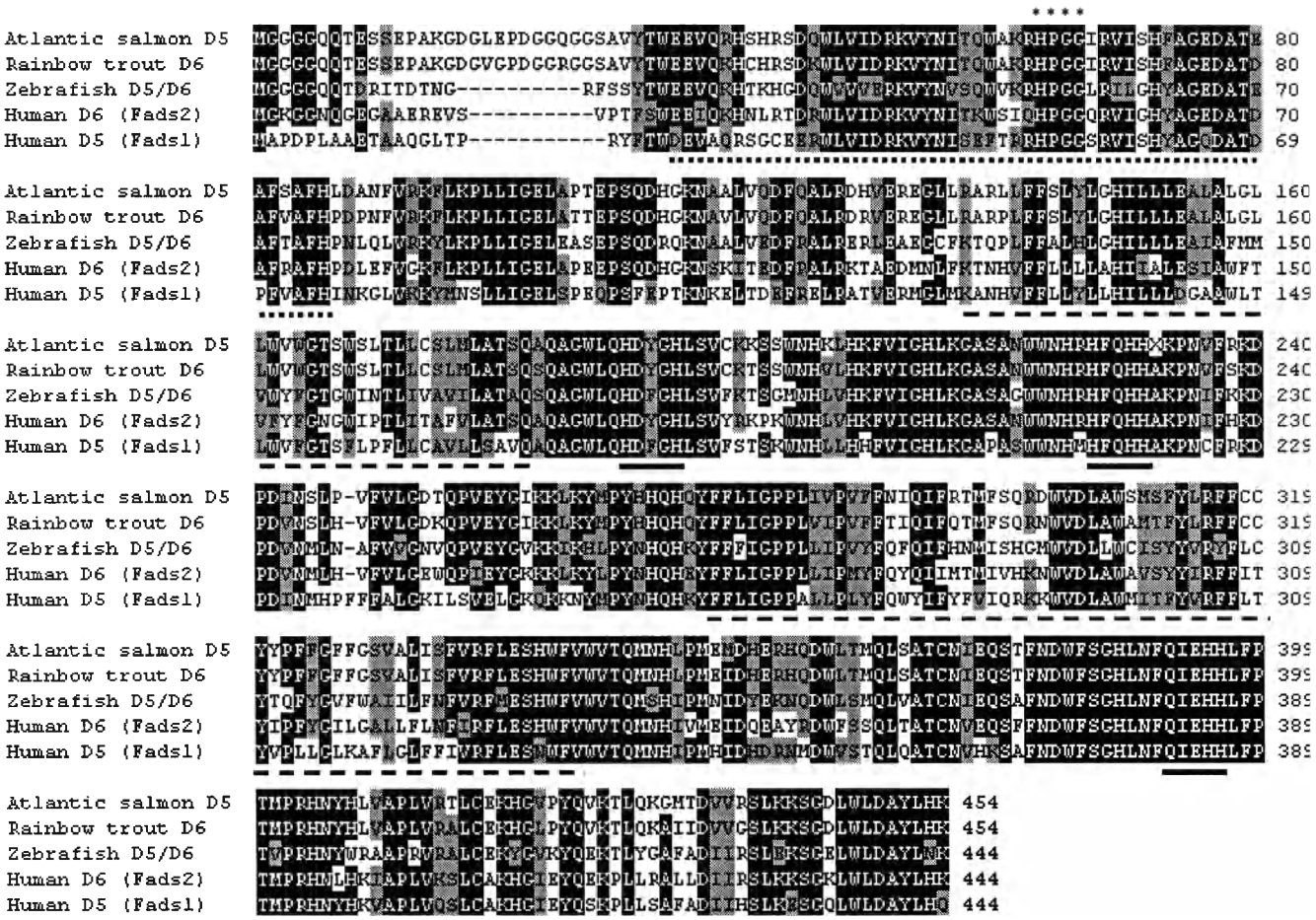

Figure 1. Comparison of the deduced amino acid sequence of a polyunsaturated fatty acyl desaturase from Atlantic salmon with that of desaturases from trout (*O. mykiss*), zebrafish (*Danio rerio*), and human. Identical residues are shaded black and similar residues are shaded grey. Identity and similarity shading was based on the BLOSUM62 matrix, and the cutoff for shading was 75%. Characteristic features are denoted thus: histidine boxes, underlined: cytochromde b_5 domain dotted underline with asterisks denoting the heme-binding motifs and transmembrane regions, dashed underline. Transmembrane regions were predicted from a hydropathy plot taking peaks with scores above 1.6 using a scan window size of 18 (Kyte and Doolittle, 1982).

yeast transformants containing the SalDes/pYES2 and SalElo/pYX222 constructs was by growth on uracil and histidine dropout media, respectively.

Culture of the recombinant yeast SalDes/pYES2 was carried out in SCMM$^{-uracil}$ broth as described previously (Hastings et al., 2001), using galactose induction of gene expression. Expression of SalElo/pYX222, which was under control of a constitutive promoter, was grown on SCMM$^{-histidine}$ broth containing 2% glucose. Each culture was supplemented with one of the following PUFA substrates: for the SalDes cDNA, α-linolenic acid (I8:3n-3), linoleic acid (18:2n-6), eicosatetraenoic acid (20:4n-3), di-homo-λ-linoleic acid (20:3n-6), docosapentaenoic acid (22:5n-3), docosatetraenoic acid (22:4n-6), tetracosapentaenoic acid (24:5n-3), and tetracosatetraenoic acid (24:4n-6); and for the SalElo cDNA, stearidonic acid (18:4n-3), λ-linolenic acid (18:3n-6), eicosapentaenoic acid (20:5n-3), arachidonic acid (20:4n-6), docosapentaenoic acid (22:5n-3), and docosatetraenoic acid (22:4n-6). Yeast cells were harvested, washed, and dried, and fatty acid methyl esters (FAMES) prepared by incubating the cells with 1 ml of methylation reagent (10% v/v, 5% v/v 2,2-dimethylpropane) and 85% v/v dry methanol) at 85°C for 1 hour. The FAMEs were extracted, purified by thin-layer chromatography (TLC), and analyzed by gas chromatography (GC), all as described previously (Hastings et al., 2001). The proportion of substrate fatty acid converted to the longer chain fatty acid product was calculated from the gas chromatograms as 100· [Product Area/(Product Area + Substrate Area)]. Unequivocal confirmation of fatty acid

Table 1. Pairwise Comparison Between Identities and Similarities of Amino Acid Sequences of Fish and Human fatty Acid Desaturases[a]

	Human Δ5	Salmon SalDes	Trout Des	Zebrafish Δ6/Δ5
Human Δ6	62 (76)	60 (75)	61 (76)	58 (75)
Human Δ5		63 (78)	65 (79)	64 (78)
Salmon SalDes			92 (95)	64 (80)
Trout Des				66 (81)

[a]Data are percentages of amino acid residues that are identical or similar (in parentheses). Similar amino acid residues are defined as having the same physico-chemical characteristics (e.g., basic, acidic, hydrophobic, hydrophilic). Trout Des is putative Δ6 desaturase from rainbow trout.

products was performed by GC–mass spectrometry (MS) of the picolinyl derivatives as described in detail previously (Hastings et al., 2001).

Materials

Eicosatetraenoic (20:4n-3), docosapentaenoic (22:5n-3), and docosatetraenoic (22:4n-6) acids (all >98%–99% pure) were purchased from Cayman Chemical Company. Tetracosapentaenoic (24:5n-3) and tetracosatetraenoic (24:4n-6) acids (both >98% pure) were provided by Dr. A.E.A. Porter, Department of Biological Sciences, University of Stirling, and were chemically synthesized from 22:5n-3 and 22:4n-6, respectively, by successive C-l additions, linoleic (18:2n-6), α-linolenic (18:3n-3), λ-linolenic (18:3n-6), stearidonic (18:4n-3), eicosatrienoic (20:3n-6), arachidonic (20:4n-6), and eicosapentaenoic (20:5n-3) acids (all >99% pure), butylated hydroxytoluene (BHT), 2,2-dimethoxypropane, galactose, nitrogen base, raffinose, tergitol NP-40, and uracil dropout medium were obtained from Sigma Chemical Co. Ltd. TLC (20 × 20 cm × 0.25 mm) plates precoated with silica gel 60 (without fluorescent indicator) were purchased from Merck. All solvents were HPLC grade and were from Rathburn Chemicals.

Results

Salmon Desaturase

Sequencing revealed that the salmon desaturase cDNA, SalDes (GenBank accession number AF478472), included an ORF of 1362 bp specifying a protein of 454 amino acids. The protein sequence possessed all the characteristics, of microsomal fatty acid desaturases, including 3 histidine boxes and 2 transmembrane regions (Figure 1). The protein sequence also contained an N-terminal cytochrome b_5 domain containing the heme binding motif, H-P-G-G (alignment positions 63–66), similar to that of other fatty acid desaturases including the zebrafish desaturase. However, the salmon, as with the trout sequence, has an insertion of 10 amino acid residues at the N-terminal end. A pairwise comparison was made between the identities and similarities of the fish and the human amino acid sequences (Table 1). The amino acid sequence predicted by the salmon ORF indicated that the desaturase candidate possessed 92% identity and 95% similarity to the amino acid sequence predicted by the trout Des cDNA (GenBank accession number, AF301910) and 64% identity and 80% similarity to the zebrafish Δ6/Δ5 desaturase (AF309556). Comparisons between the salmon cDNA and the human Δ6 (GenBank accession number AF199596) and Δ5 (AF126799) cDNAs, gave 60% and 63% identities with 75% and 78% similarities, respectively. Phylogenetic analysis comparing a variety of Δ5 and Δ6 desaturases clustered SalDes closely with other, as yet uncharacterized, salmonid genes (rainbow trout and cherry salmon) (Figure 2). SalDes clustered slightly farther from the zebrafish desaturase, which is known to possess both Δ6 and Δ5 activity. All the fish desaturase genes clustered together and closer to the mammalian (mouse and human) Δ6 desaturases than to the Δ5 desaturases (Figure 2).

The salmon desaturase cDNA was functionally characterized by determining the fatty acid profiles of transformed *S. cerevisiae* containing either the p YES vector alone or the vector with the SalDes insert (SalDes/pYES), grown in the presence of a variety of possible fatty acid substrates including 18:2n-6, 18:3n-3, 20:3n-6, 20:4n-3, 22:5n-3, 22:4n-6, 24:5n-3, and 24:4n-6. The fatty acid composition of the yeast transformed with the vector alone

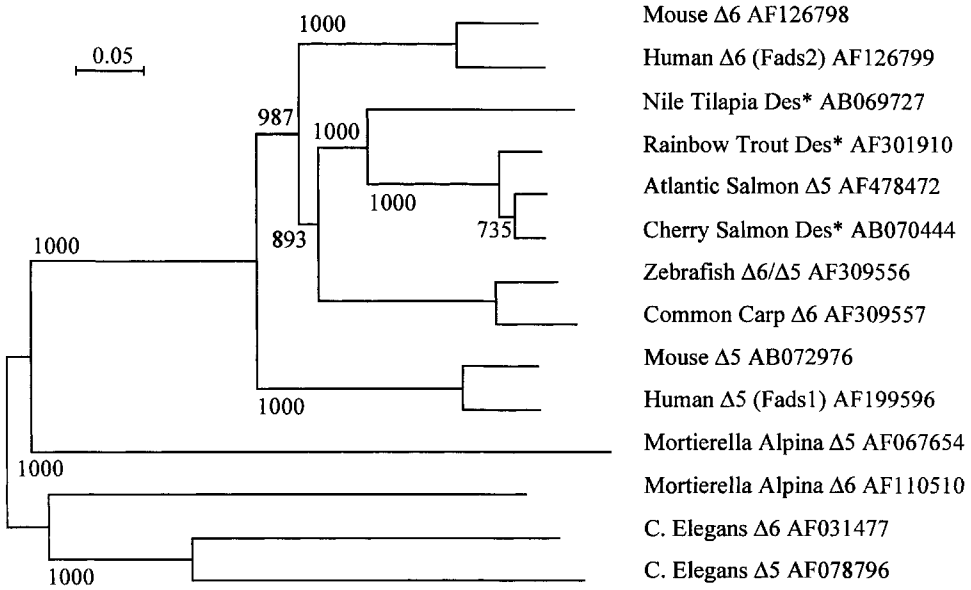

Figure 2. Phylogenetic tree of desaturases from salmon, other fish species (zebrafish, trout, carp, cherry salmon, and tilapia), mammals (mouse and human), yeast (*Mortierella alpina*) and nematode (*Caenorhabditis elegans*). The tree was constructed using the neighbor-joining method with CLUSTAL X NJPLOT, The horizontal branch length is proportional to the amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations. An asterisk denotes a desaturase sequence that has not yet been functionally characterized.

showed the 4 main fatty acids normally found in *S. cerevisiae*—namely, 16:0, 16:1n-7, 18:0, and 18:1n-9—together with the exogenously derived substrate fatty acids, consistent with *S. cerevisiae* not possessing Δ5 or Δ6 fatty acid desaturase activities. Of all the fatty acid substrates tested, the most prominent additional peak was observed in the profile of SalDes/pYES-transformed yeast grown in the presence of the Δ5 desaturase substrate, 20:4n-3 (Figure 3, B). Based on GC retention time, the additional peak associated with the presence of the salmon cDNA was identified as 20:5n-3. Approximately 10% of 20:4n-3 was converted to 20:5n-3 in yeast transformed with the salmon desaturase (Table 2). Small amounts (<1%) of 18:3n-3 (Figure 3, A), 18:2n-6, and 20:3n-6 (Figure 3, C) were also desaturated by the salmon clone. The identities of all product fatty acids were confirmed by GC-MS of the picolinyl derivatives. No desaturated products of C_{22} fatty acid substrates were detected, indicating there was no Δ4 desaturase activity (Table 2). Similarly, no traces of 24:6n-3 and 24:5n-6 were detected by GC-MS in yeast transformed with the salmon desaturase and grown in the presence of 24:5n-3 and 25:4n-6, respectively, indicating no Δ6 activity toward C_{24} substrates (Δ6*). These data indicate that the salmon enzyme is primarily an n-3 Δ5 desaturase with a low level of Δ6 and n-6 Δ5 activity.

Salmon Elongase

Sequencing revealed that the salmon fatty acid elongase cDNA, SalElo (GenBank accession number AY 170327), included an ORF of 888 bp specifying a protein of 295 amino acids. The protein sequence included characteristics of microsomal fatty acid elongases, including a histidine box, endoplasmic reticulum (ER) retention signal, and 2 transmembrane regions (Figure 4). Comparison of the deduced amino acid sequence of the salmon elongase with that of elongases from zebrafish (ZfElo), mouse (Elov12), and human (ELOVL2 and ELOVL5) showed that SalElo shared 75% 55% 53% and 71% identity with ZfElo, Elovl2, ELOVL2, and ELOVL5, respectively. Phylogenetic analyses of the salmon, zebrafish, human, and mouse elongases showed divergence from a common ancestor (Figure. 5). The fish elongases and human ELOV5 have diverged less from a common ancestor than have the 2 mammalian elongases ELOV2 and Elov2.

The salmon elongase cDNA was functionally characterized by determining the fatty acid profiles of transformed *S. cerevisiae* containing either empty vector alone or the vector with the salmon cDNA insert, grown in the presence of a variety of possible fatty acid substrates including 18:3n-6, 18:4n-3, 20:4n-6, 20:5n-3, 22:4n-6, and

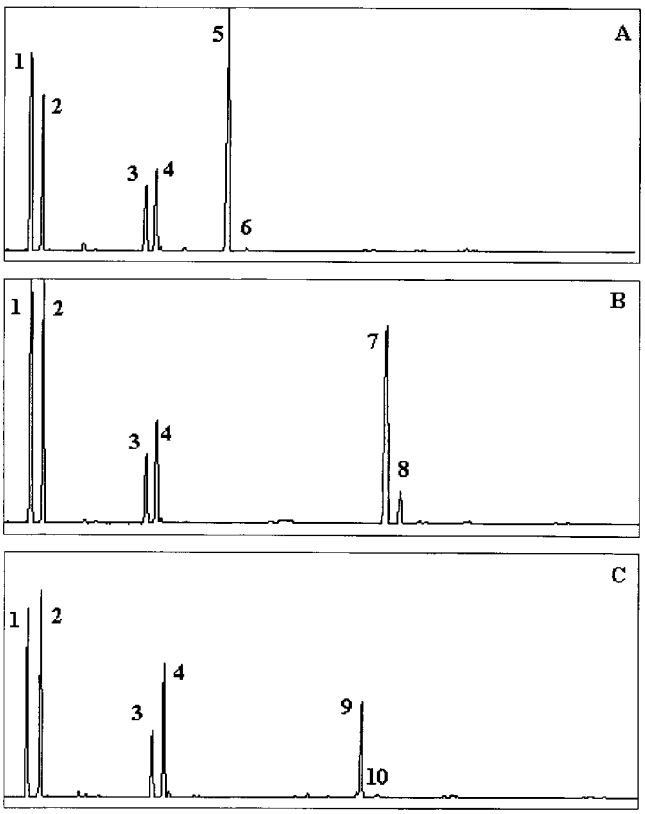

Figure 3. Identification of fatty acid desaturation products in transgenic yeast (*Saccharomyces cerevisiae*). Fatty acids were extracted from yeast transformed with SalDes/pYES grown in the presence of 18:3n-3 (**A**), 20:4n-3 (**B**), or 20:3n-6 (**C**). The first 4 peaks in each panel are the main endogenous fatty acids of *S. cerevisiae*, namely, 16:0(1), 16:1n-7(2), 18:0(3), and 18:1n-9(4). The additional peaks in each panel are the exogenously added substrate fatty acids and the resultant desaturated products, namely, 18:3n-3 (5), 18:4n-3 (6), 20:4n-3 (7), 20:5n-3 (8), 20:3n-6 (9) and 20:4n-6 (10).

22:5n-3. The results showed that the salmon elongase had high C_{18-20} elongase activity, converting 71% of 18:4n-3 to 20:4n-3 (Figure 6, A) and 42% of 18:3n-6 to 20:3n-6. The salmon clone also elongated 39% of 20:5n-3 (Figure 6, B) and 23% of 20:4n-6, thus demonstrating significant C_{20-22} activity. The salmon clone was also able to elongate C_{22} substrates such as 22:5n-3 and 22:4n-6, although at a much lower level than with C_{18} and C_{20} fatty acids (Figure 6, C). These data indicate that the salmon elongase is a PUFA elongase active on a range of fatty acids with activity decreasing with chain length and with a preference for n-3 fatty acid substrates (Table 3). However, the salmon elongase also elongated monounsaturated fatty acids as evidenced by the presence of 18:1n-7, the elongation product of 16:1n-7, and small amounts of 20:1n-9 and 20:1n-7, the elongated products of the respective C_{18} monoenes (Figure 6).

Table 2. Functional Characterization of Atlantic Salmon Desaturase (SalDes) in *Saccharomyces cerevisiae*[a]

Fatty acid substrate	Product	Conversion (%)	Activity
18:3n-3	18:4n-3	0.6	Δ6
18:2n-6	18:3n-6	0.4	Δ6
20:4n-3	20:5n-3	10.2	Δ5
20:3n-6	20:4n-6	0.9	Δ5
22:5n-3	22:6n-3	ND	Δ4
22:4n-6	22:5n-6	ND	Δ4
24:5n-3	24:6n-3	ND	Δ6*
24:4n-6	24:5n-6	ND	Δ6*

[a]Results are expressed as a percentage of substrate fatty acid converted to desaturated product. Δ6* indicates Δ6 desaturase activity towards C_{24} fatty acid substrates; ND, not detected.

Discussion

The extent to which animals, including fish, can convert the plant-derived C_{18} PUFAs, 18:3n-3 and 18:2n-6, to long-chain $C_{20/22}$ HUFAs-varies with species and correlates with their complement of the microsomal fatty acyl desaturase and elongase enzymes. EPA is synthesized from 18:3n-3 by desaturation at the Δ6 position, followed by a 2-carbon elongation, which is in turn followed by a further desaturation at the Δ5 position (Cook, 1996). Synthesis of DHA from EPA has been suggested to proceed via a C_{24}intermediate, requiring 2 successive elongations to 22:5n-3 and then 24:5n-3, which is then desaturated at the Δ6 position (Sprecher et al., 1995). Here we report the sequences and functions of 2 cDNAs from Atlantic salmon that are involved in this fatty acid desaturation/elongation pathway. The salmon elongase cDNA (SalElo) encodes a protein that is very similar to mammalian elongases and has all the main structural characteristics possessed by elongases from other systems, notably the predicted transmembrane domains, the so-called histidine box (HXXHH), and the canonical C-terminal ER retention signal. Similarly, the protein sequence of the salmon desaturase (SalDes) has all the characteristics of microsomal fatty acid desaturases including 3 histidine boxes, a transmembrane region, and the N-terminal cytochrome b_5 domain containing the heme-binding motif.

Figure 4. Comparison of the deduced amino acid sequence of a polyunsaturated fatty acyl elongase from Atlantic salmon elongase (SalElo) with that of elongases from zebrafish (ZfElo), mouse (Elovl2), and human (ELOVL2 and ELOVL5). Identical residues are shaded black, and similar residues are shaded gray. Identity and similarity shading was based on the BLOSUM62 matrix, and the cutoff for shading was 75%. Characteristic features are denoted thus: histidine boxes, underlined; ER retention signal, asterisks; and transmembrane regions, dashed underline. Transmembrane regions were predicted from a hydropathy plot taking peaks with scores above 1.6 using a scan window size of 18 (Kyte and Doolittle, 1982).

The salmon desaturase cloning was based on similarity to Δ6 desaturases, and indeed it is slightly more similar at the protein sequence level to the human Δ6 desaturase than to human Δ5 desaturase. However, functional characterization showed that the only significant activity that the desaturase had was toward 20:4n-3, producing 20:5n-3, and that it is, therefore, primarily a fatty acid Δ5 desaturase. A small amount of Δ6 desaturase activity was also detected, but this is probably physiologically insignificant. The only other functionally characterized fish desaturase to date is that from zebrafish, which was shown to be bifunctional, having both Δ6 and Δ5 activities, with Δ6 > Δ5 (Hastings et al., 2001). The zebrafish gene was the first, and only, fatty acid desaturase reported in any species with both Δ5 and Δ6 activity, and it was speculated that it may represent a component of a prototypic vertebrate PUFA biosynthesis pathway. The results presented here on salmon, a teleost fish more evolutionarily primative than zebrafish, showing that fish can also have monofunctional desaturases indicates that the evolution of the HUFA biosynthetic pathway is possibly more complex. However, much more information will be required to elucidate this question, and further desaturase cDNAs need to be isolated from fish species. It is probable that further desaturases remain to be isolated from Atlantic salmon. Indeed it is highly unlikely that the desaturase characterized in the present study, despite

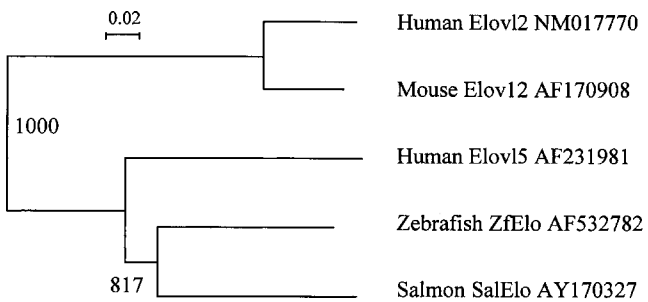

Figure 5. Phylogenetic tree of enlongases from salmon (SalElo), zebrafish (ZfElo), human (ELOVL2, ELOVL2) and mouse (Elovl2).The tree was constructed using the neighbor-joining method with CLUSTALX and NJPLOT. The horizontal branch length is proportional to the amino acid substitution rate per site. The numbers represent the percentage frequencies with which the tree topology presented here was replicated after 10,000 bootstrap iterations.

showing low levels of Δ6 desaturase activity, is the only PUFA desaturase in salmon. In all our previous biochemical studies investigating fatty acid desaturation in salmon cells, Δ6 activity was far greater than Δ5 activity both in freshly isolated primary hepatocytes (Bell et al., 2001b, 2002; Tocher et al., 1997, 2000, 2001) and in established cell cultures (Tocher and Sargent, 1990). Therefore Atlantic salmon should possess an enzyme with high Δ6 desaturase activity, whether it is monofunctional or bifunctional like the pre-

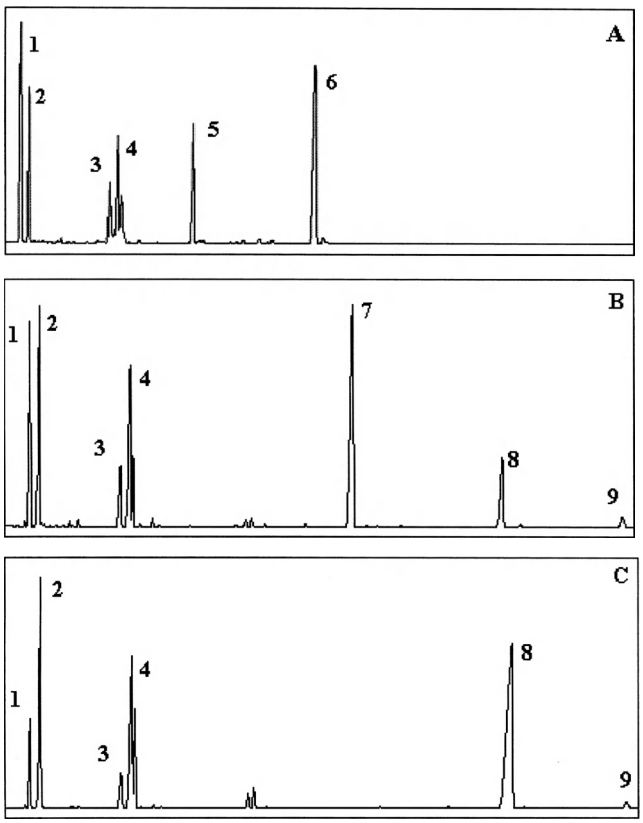

Figure 6. Identification of fatty acid elongation products in transgenic yeast (*Saccharomyces cerevisiae*). Fatty acids were extracted from yeast transformed with SalElo/pYX222 grown in the presence of l8:4n-3 (A) 20:5n-3 (B) or 22:5n-3 (C). The first 4 peaks in each panel are the main endogenous fatty acids of *S. cerevisiae*, namely, 16:0 (1), 16:1n-7 (2), 18:0 (3), and 18:1n-9 (4). The additional peaks in each panel are the exogenously added substrate fatty acids and the resultant elongated products, namely, l8:4n-3 (5), 20:4n-3 (6), 20:5n-3 (7), 22:5n-3 (8), and 24:5n-3 (9).

viously characterized zebrafish gene. It was also acknowledged previously that other, monofunctional desaturases could be present in zebrafish (Hastings et al., 2001).

That salmon possess a Δ5 desaturase is consistent with the fact that salmonids, including the anadromous Atlantic salmon that lives for much of its life in the marine environment, have all the enzymic activities required for the production of DHA. True marine fish are unable to produce DHA at a physiologically significant rate, and this has been attributed to the lack of Δ5 desaturase activity in some species, such as sea bream (Tocher and Ghioni, 1999). In other marine species such as turbot, a deficiency in C_{18-20} elongation has been suggested (Ghioni et al., 1999), and clearly salmon also possess very active C_{18-20} fatty acid elongating activity. Indeed, this study has shown

Table 3. Functional Characterization of Atlantic Salmon Elongase (SalElo) in *Saccharomyces cerevisiae*[a]

Fatty acid substrate	Product	Conversion (%)	Activity
18:4n-3	20:4n-3	71.8	C_{18-20}
18:3n-6	20:3n-6	42.1	C_{18-20}
20:5n-3	22:5n-3	38.9	C_{20-22}
20:4n-6	22:4n-6	22.6	C_{20-22}
22:5n-3	24:6n-3	0.7 (2.9)	C_{22-24}
22:4n-6	24:5n-6	0.3 (2.0)	C_{22-24}

[a]Results are expressed as a percentage of substrate fatty acid converted to elongated product. Two values are shown for conversion of the C_{22} fatty acid substrates, with the first values being the percentage conversion when the C_{22} substrates themselves were used, whereas the values in parentheses represent the percentage conversion of C_{22} produced when the yeast were grown in C_{20} substrates.

that all the fatty acid elongating activities, C_{18-20}, C_{20-22} and C_{22-24}, required for the production of DHA are contained in one polypeptide in Atlantic salmon. In contrast to the desaturase activities, the fatty acid specificity of the salmon elongase is similar to that of the recently characterized zebrafish elongase (Agaba et al., 2004). Broad substrate specificity is a feature of vertebrate fatty acid elongases characterized to date. In contrast to the C_{18-20} elongases of *M. alpina* and *C. elegans*, which show virtually no activity toward C_{20} PUFA (Beaudoin et al., 2000; Parker-Barnes et al., 2000), the human elongase (ELOVL5) and rat elongase (rELO1) have high activity on 20:5n-3 and 20:4n-6 in addition to C_{18-20} elongase activity (Leonard et al., 2000b; Inagaki et al., 2002). However, the ELOVL5 and rELO1 elongases do not have the capacity to elongate C_{22} PUFA. More recently, 2 further mammalian genes have been cloned and characterized, a second human elongase (ELOVL2) and a mouse elongase (elovl2), both of which are able to elongate 22:5n-3 and 22:4n-6 to 24:5n-3 and 24:4n-6, respectively (Leonard et al., 2002). Like the salmon enzyme, the mouse elovl2 product is also able to elongate $C_{18}$and C_{20} PUFA in addition to C_{22} PUFA, whereas the human ELOVL2 clone is only active towardC_{20} and C_{22} PUFA (Leonard et al., 2002). Thus the salmon, zebrafish, and mouse elovl2 elongases have similar PUFA specificities, broader than the specifitities of the human and rat elongases cloned so far. However, the presence of more than one PUFA elongase in human suggests that other elongases may yet be isolated from rat and mouse. Similarly, although the salmon elongase has a broader

substrate specificity compared with either of the human genes that have overlapping substrate specificities, we cannot exclude the possibility that other fatty acid elongases will be isolated from salmon.

It is perhaps noteworthy that the salmon elongase could convert 22:5n-3 to 24:5n-3, as 24:5n-3 is an important intermediate in the biosynthesis of DHA in rats (Sprecher et al., 1995; Sprecher and Chen, 1999). Previously biochemical studies had suggested that DHA synthesis in rainbow trout also proceeds via C_{24} intermediates (Buzzi et al., 1996, 1997). The present study has revealed that a closely related salmonid, Atlantic salmon, possess is a gene whose product is capable of producing C_{24} from C_{22} PUFA. Furthermore, direct synthesis of DHA from 22:5n-3 would require desaturation at the Δ4 position, and thus a Δ4 desaturase, as described in the marine microheterotroph, *Thraustochytrium* sp. (Qui et al., 2001). However, desaturation of PUFA at the Δ4 position has not been demonstrated in any vertebrate, and the desaturase gene isolated in the present study showed no Δ4 desaturating activity.

Together with other published data, the results presented here prompt a question; Do the differences between species in primary structures of desaturases and elongases contribute directly to the variation observed in the ability of the different species to biosynthesize HUFA? The protein sequences of the salmon Δ5 desaturase, zebrafish bifunctional Δ6/Δ5 desaturase, and human Δ6 and Δ5 desaturases are all similar, with large areas of conserved sequence. The catalytic activities are, of course, identical, but the substrate specificities are quite different, indicating that the differences in sequence have a significant impact. Similarly, the sequences of mammalian and fish elongases are similar, but those differences that occur clearly affect the substrate specificities of the enzymes. The accumulating sequence data present an opportunity to study the structural features that have shaped PUFA desaturase and elongase substrate specificities during the evolution of vertebrates, and the possibility to artificially select for desaturases and elongases with superior specification. This may be supported by evidence from biochemical studies, which indicated that the inability to synthesize EPA and DHA in sea bream cells was due to very low Δ5 desaturase activity (Tocher and Ghioni, 1999), whereas in turbot cells it was related to a deficiency in C_{18-20} elongase (Ghioni et al., 1999), and that biosynthesis of arachidonic acid from 18:2n-6 in *M. alpina* may be rate-limited by the activity of the elongase (Wynne and Ratledge, 2000).

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References

Agaba, M., Tocher, D.R., Dickson, C., Dick, J.R., and Teale, A.J. (2004). A zebrafish cDNA encoding a multifunctional enzyme involved in the elongation of polyunsaturated, monounsaturated and saturated fatty acids. *Mar Biotechnol* 6:251–261.

Aki, T., Shimada, Y., Inagaki, K., Higashimoto, H., Kawamoto, S., Shiget, S., Ono, K., and Suzuki, O. (1999). Molecular cloning and functional characterisation of rat Δ6 fatty acid desaturase. *Biochem Biopyhs Res Commun* 255:575–579.

Barlow, S. (2000). Fishmeal and fish oil: sustainable feed ingredients for aquafeeds. *Global Aquacult Advocate* 4:85–88.

Beaudoin, F., Michaelson, L.V., Hey, S.J., Lewis, M.L., Shewry, P.R., Sayanova, O., and Napier, J.A. (2000). Heterologous expression in yeast of the polyunsaturated fatty acid biosynthetic pathway. *Proc Natl Acad Sci USA* 97:6421–6426.

Bell, J.G., Tocher, D.R., Farndale, B.M., Cox, D.I., McKinney, R.W., and Sargent, J.R. (1997). The effect of dietary lipid on polyunsaturated fatty acid metabolism in Atlantic salmon (*Salmo salar*) undergoing parr-smolt transformation. *Lipids* 32:515–525.

Bell, J.G., McEvoy, J, Tocher, D.R., McGhee, F., Campbell, P.J., and Sargent, J.R. (2001). Replacement of fish oil with rape seed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid compositions and hepatocyte fatty acid metabolism. *J Nutr* 131:1535–1543.

Bell, J.G., Henderson, R.J., Tocher, D.R., McGhee, F., Dick, J.R., Porter, A., Smullen, R., and Sargent, J.R. (2002). Substituting fish oil with crude palm oil in the diet of Atlantic salmon (*Salmo salar*) affects tissue fatty acid compositions and hepatic fatty acid metabolism. *J Nutr* 132:222–230.

Bell, M.V., Dick, J.R., and Porter, A.E.A. (2001a). Biosynthesis and tissue deposition of docosahexaenoic acid (22:6n-3) in rainbow trout (*Oncorhynchus mykiss*). *Lipids* 36:1153–1159.

Buzzi, M., Henderson, R.J., and Sargent, J.R. (1996). The desaturation and elongation of linolenic acid and eicosapentaenoic acid

by hepatocytes and liver microsomes from rainbow trout (*Oncorhynchus mykiss*) fed diets containing fish oil or olive oil. *Biochim Biophys Acta* 1299:235–244.

Buzzi, M., Henderson, R.J., and Sargent, J.R. (1997). Biosynthesis of docosahexaenoic acid in trout hepatocytes proceeds via 24-carbon intermediates. *Comp Biochem Physiol* 116B:263–267.

Cho, H.P., Nakamura, M.T., and Clarke, S.D. (1999a). Cloning expression and nutritional regulation of the human Δ6 desaturase. *J Biol Chem* 274:471–477.

Cho, H.P., Nakamura, M.T., and Clarke, S.D. (1999b). Cloning expression and nutritional regulation of the human Δ5 desaturase. *J Biol Chem* 274:37335–37339.

Cook, H.W. (1996). Fatty acid desaturation and chain elongation in eukaryote. In: *Biochemistry of Lipids, Lipoproteins and Membranes*, Vance, D.E., and Vance, J.E. (eds.). Amsterdam, Netherlands: Elsevier, pp 129–152.

Ghioni, C., Tocher, D.R., Bell, M.V., Dick, J.R., and Sargent, J.R. (1999). Low C_{18} to C_{20} fatty acid elongase activity and limited conversion of stearidonic acid, 18:4n-3, eicosapentaenoic acid, 20:5n-3, in a cell line from the rurbot, *Scophthalmus maximus*. *Biochim Biophys Acta* 1437:170–181.

Hastings, N., Agaba, M., Tocher, D.R., Leaver, M.J., Dick, J.R., Sargent, J.R., and Teale, A.J. (2001). A vertebrate fatty acid desaturase with Δ5 and Δ6 activities. *Proc Natl Acad Sci USA* 98:14304–14309.

Huang, Y.-S., Chaudhary, S., Thurmond, J., Bobik, E.G., Yuan, L., Chan, G.E., Kirchner, S.J., Mukerji, P., and Knutson, D.S. (1999). Cloning of Δ12- and Δ5-desaturases from *Mortierella alpina* and recombinant production of λ-linolenic acid in *Saccharomyces cerevisiae*. *Lipids* 34:649–659.

Inagaki, K., Aki, T., Fukuda, Y., Kawamoto, S., Shigeta, S., Ono, K., and Suzuki, O. (2002). Identification and expression of a rat fatty acid elongase involved the biosynthesis of C18 fatty acids. *Biosci Biotechnol Biochem* 66:613–621.

Kyte, J., and Doolittle, R. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157:105–132.

Leonard, A.E., Kelder, B., Bobik, E.G., Kroeger, P.E., Chuang, L.-T., Thurmond, J.M., Parker-Barnes, J.M., Kopchick, J.J., Huang, Y.-S., and Murkerji, P. (2000a). cDNA cloning and characterisation of human Δ5 desaturase involved in the synthesis of arachidonic acid. *Biochem J* 347:719–724.

Leonard, A.E., Bobik, E.G., Dorado, J., Kroeger, P.E., Chuang, L.-T., Thurmond, J.M., Parker-Bames, J.M., Das, T., Huang, Y.-S. Murkerji, and Murkerji, P. (2000b). Cloning of a human cDNA encoding a novel enzyme involved in the elongation of long chain polyunsaturated fatty acids. *Biochem J* 350:765–770.

Leonard, A.E., Kelder, B., Bobik, E.G., Chuang, L.-T., Lewis, C.J., Kopchick, J.J., Murkerji, P., and Huang, Y.-S. (2002). Identification and expression of mammalian long-chain PUFA elongation enzymes. *Lipids* 37:733–740.

Michaelson, L.V., Lazarus, C.M., Griffiths, G., Napier, J.A., and Stobart, A.K. (1998a). Isolation of a Δ5 fatty acid desaturase gene from *Mortierrela alpina*. *J Biol Chem* 273:19055–19059.

Michaelson, L.V., Napier, J.A., Lewis, M., Griffiths, G., Lazarus, C.M., and Stobart, A.K. (1998b). Functional identification of a fatty acid Δ^5 desaturase gene from *Caenorhabditis elegans*. *FEBS Lett* 439:215–218.

Napier, J.A., Hey, S.J., Lacey, D.J., and Shewry, P.R. (1998). Identification of a *Caenorhabditis elegans* Δ6 fatty acid desaturase by heterologous expression in *Saccharomyces cereviciae*. *Biochem J* 330:611–614.

Parker-Barnes, J.M., Das, T., Bobik, E., Leonard, A.E., Thurmond, J.M., Chuang, L., Huang, Y.S., and Mukerji, P. (2000). Identification and characterization of an enzyme involved in the elongation of n-6 and n-3 polyunsaturated fatty acid. *Proc Natl Acad Sci USA* 97:8284–8289.

Qui, X., Hong, H, and MacKenzie, S.L. (2001). Identification of a Δ4 fatty acid desaturase from *Thraustochytrium* sp. involved in the synthesis of docosahexaenoic acid by heterologous expression in *Saccharomyces cerevisiae* and *Brassica juncea*. *J BiolChem* 276:31561–31566.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425.

Sargent, J.R., and Tacon, A. (1999). Development of farmed fish: a nutritionally necessary alternative to meat. *Proc Nutr Soc* 58:377–383.

Sargent, J.R., Henderson, R.J., and Tocher, D.R. (1989). The lipids. In: *Fish Nutrition*, 2nd ed. Halver, J.E. (ed.). San Diego, Calif: Academic Press, pp 153–218.

Sargent, J.R., Tocher, D.R., and Bell, J.G. (2002). The lipids. In: *Fish Nutrition*, 3rd ed. Halver, J. E., and Hardy, R.W. (eds.). San Diego; Calif: Acadeinic Press, pp 181–257.

Sprecher, H., and Chen, Q. (1999). Polyunsaturated fatty acid biosynthesis: a microsomal-peroxisomal process. *Prostagland Leukotr Essent Fatty Acids* 60:317–321.

Sprecher, H., Luthria, D.L., Mohammed, B.S., and Baykousheva, S.P. (1995). Re-evaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *J Lipid Res* 36:2471–2477.

Tidwell, J.H., and Allan, G.L. (2002). Fish as food: aquaculture's contribution. *World Aquaculture* 33:44–48.

Tocher, D.R., and Ghioni, C. (1999). Fatty acid metabolism in marine fish: low activity of Δ5 desaturation in gilthead sea bream (*Sparus aurata*) cells. *Lipids* 34:433–440.

Tocher, D.R., and Sargent, J.R. (1990). Incorporation into phospholipid classes and metabolism via desaturation and elongation of various ^{14}C-labelled (n-3) and (n-6) polyunsaturated fatty acids in trout astrocytes in primary culture. *J Neurochem* 54:2118–2124.

Tocher, D.R., Bell, J.G., Dick, J.R., and Sargent, J.R. (1997). Fatty acyl desaturation in isolated hepatocytes from Atlantic salmon (*Salmo salar*): stimulation by dietary borage oil containing λ-linolenic acid. *Lipids* 32:1237–1247.

Tocher, D.R., Bell, J.G., Henderson, R.J., McGhee, F., Mitchell, D., and Morris, P.C. (2000). The effect of dietary linseed and rapeseed oils on polyunsaturated fatty acid metabolism in Atlantic salmon (*Salmo salar*) undergoing parr-smolt transformation. *Fish Physiol Biochem* 23:59–73.

Tocher, D.R., Bell, J.G., MacGlaughlin, P., McGhee, F., and Dick, J.R. (2001). Hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition of liver in salmonids: effects of dietary vegetable oil. *Comp Biochem Physiol* 130:257–270.

Tocher, D.R., Leaver, M.J., and Hodgson, P.A. (1998). Recent advances in the biochemistry and molecular biology of fatty acyl desaturases. *Prog Lipid Res* 37:73–117.

Watts, J.L., and Browse, J. (1999). Isolation and characterisation of a Δ5 fatty acid desaturase from *Caenorhabditis elegans*. *Arch Biochem Biophys* 362:175–182.

Wilson, R.P. (1989). Amino acids and proteins. In: *Fish Nutrition*, 2nd ed. Halver, J.E. (ed.). San Diego, Calif: Academic Press.

Wynn, J.P., and Ratledge, C. (2000). Evidence that the rate limiting step for the biosynthesis of arachidonic acid in *Mortierella alpina* is at the level of the 18:3 to 20:3 elongase. *Microbiology* 146:2325–2331.