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## **A vertebrate fatty acyl desaturase with $\Delta 4$ activity**

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## **Abstract**

Biosynthesis of the highly biologically active long-chain polyunsaturated fatty acids, arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, in vertebrates requires the introduction of up to three double bonds catalyzed by fatty acyl desaturases (Fad). Synthesis of ARA is achieved by  $\Delta 6$  desaturation of 18:2n-6 to produce 18:3n-6 that is elongated to 20:3n-6 followed by  $\Delta 5$  desaturation. Synthesis of EPA from 18:3n-3 requires the same enzymes and pathway as for ARA, but DHA synthesis reportedly requires two further elongations, a second  $\Delta 6$  desaturation and a peroxisomal chain shortening step. This paper describes cDNAs, *fad1* and *fad2*, isolated from the herbivorous, marine teleost fish (*Siganus canaliculatus*) with high similarity to mammalian Fad proteins. Functional characterization of the cDNAs by heterologous expression in the yeast *Saccharomyces cerevisiae* showed that Fad1 was a bifunctional  $\Delta 6/\Delta 5$  Fad. Previously, functional dual specificity in vertebrates had been demonstrated for a zebrafish *Danio rerio* Fad and baboon Fad so the present report suggests bifunctionality may be more widespread in vertebrates. However, Fad2 conferred on the yeast the ability to convert 22:5n-3 to DHA indicating that this *S. canaliculatus* gene encoded an enzyme having  $\Delta 4$  Fad activity. This is the first report of a Fad with  $\Delta 4$  activity in any vertebrate species and indicates that there are two possible mechanisms for DHA biosynthesis, a direct route involving elongation of EPA to 22:5n-3 followed by  $\Delta 4$  desaturation, as well as the more complicated pathway as described above.

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## **Introduction**

Polyunsaturated fatty acids (PUFA) cannot be synthesized de novo by vertebrates and so must be obtained in the diet. However, the most biologically active essential fatty acids (EFA) including arachidonic (ARA; 20:4n-6), eicosapentaenoic (EPA; 20:5n-3)

and docosahexaenoic (DHA; 22:6n-3) acids are long-chain PUFA (LC-PUFA) that can be synthesized in vertebrates through sequential desaturation and elongation of C<sub>18</sub> PUFA, 18:2n-6 and 18:3n-3. Synthesis of ARA is achieved by  $\Delta$ 6 desaturation of 18:2n-6 to produce 18:3n-6 that is elongated to 20:3n-6 followed by  $\Delta$ 5 desaturation (1). Synthesis of EPA from 18:3n-3 requires the same enzymes and pathway as for ARA, but DHA synthesis reportedly requires two further elongation steps, a second  $\Delta$ 6 desaturation and a peroxisomal chain shortening step (2). The extent to which any species can convert C<sub>18</sub> PUFA to LC-PUFA varies, associated with their complement of fatty acyl desaturase (Fad) and elongase (Elovl) enzymes. Some animals, notably extreme carnivores, have very limited ability to synthesize LC-PUFA, and consequently have a dietary requirement for pre-formed C<sub>20</sub> and C<sub>22</sub> PUFA (3-6).

As with all vertebrates, PUFA are essential nutrients in fish, but requirements vary with C<sub>18</sub> PUFA being the EFA for freshwater and diadromous species, whereas marine fish have a dietary requirement for LC-PUFA (7). The molecular basis of LC-PUFA synthesis in fish is understood as well as any vertebrate, driven by the crucial role fish play as the primary source of n-3 LC-PUFA in the human diet (8,9). Evidence suggests that the dependence of marine fish on dietary LC-PUFA is due to deficiency in one or more enzymes required for their biosynthesis (7-10). Like mammals (11), Atlantic salmon possesses separate genes for  $\Delta$ 5 and  $\Delta$ 6 Fads (12-14). Distinct  $\Delta$ 6 Fad cDNAs have been isolated from all fish species studied to date including freshwater and marine species (15-18). Other than salmon, the only other fish Fad with  $\Delta$ 5 activity is the bifunctional  $\Delta$ 6/ $\Delta$ 5 Fad previously isolated from zebrafish (19). Thus the inability of some species to produce DHA might be explained by the lack of a gene encoding  $\Delta$ 5 activity, a deficiency that has no significant consequence in the DHA-rich marine ecosystem. In contrast, the bifunctional zebrafish gene and multiple subfunctionalized salmon genes have enabled these species that spend all, or a significant part, of their lifecycles in relatively nutrient-poor freshwater environments, to produce essential LC-PUFA (10). However, the influence of trophic level on LC-PUFA biosynthesis capability has never been fully explored. Thus, all the marine species studied to date consume mainly animals, often

other fish (carnivores/piscivores) with trophic levels greater than 2.8 (20). In contrast, the freshwater species so far examined consume either mainly plant/detritus (herbivores) or plants/detritus plus animals (omnivores) with trophic levels between 2.0 and 2.79 (20).

The present study was initiated to study a marine species, *Siganus canaliculatus* (Park, 1797) (white-spotted spinefoot or rabbitfish), which is truly herbivorous consuming algae and seagrasses with a trophic level below 2.8 (21). Previously, the cDNA for a Fad had been cloned (*fad1*) but not functionally characterized (22). Here we report the cloning of the cDNA for a further *S. canaliculatus* gene (*fad2*) and the results of heterologous expression of both *fad* cDNAs in the yeast *Saccharomyces cerevisiae*. The results show the two *S. canaliculatus* Fads display bifunctional  $\Delta 6/\Delta 5$  and  $\Delta 4/\Delta 5$  activities, respectively. This is the first report of a vertebrate Fad with  $\Delta 4$  activity and suggests that there is potentially more than one pathway for the synthesis of DHA in vertebrates.

## Results

**Sequence and phylogenetic analysis of Fad2.** The *fad2* cDNA was 1831 bp in length (excluding polyA tail) and contains a 1338 bp open-reading frame (ORF) (GenBank accession number GU594278). The deduced protein has 445 amino acids and is 82.7 % identical to the previously isolated *S. canaliculatus* Fad1 (EF424276), and 67.8 %, 57.8 % and 63.6 % identical to *Danio rerio*  $\Delta 6/\Delta 5$  (AF309556), *Homo sapiens*  $\Delta 5$  (AF199596) and  $\Delta 6$  (AF126799), respectively. The deduced Fad2 polypeptide sequence has a number of characteristic features of microsomal Fad proteins, including three histidine boxes, an N-terminal cytochrome *b5* domain containing the heme-binding motif, and two transmembrane regions. Phylogenetic analysis of Fad1 and Fad2 with a variety of Fads of other species shows that *S. canaliculatus* desaturases are most closely to marine teleost  $\Delta 6$  Fads, and more distantly from lower eukaryotes  $\Delta 4$  and  $\Delta 5$  Fads (Fig. 1).

**Functional characterization.** The *S. canaliculatus* Fad proteins were functionally characterized by determining the fatty acid (FA) profiles of yeast transformed with either the pYES2 vector alone or the vector with the putative *fad* cDNA inserts (pYES2fad1 and pYES2fad2), and grown in the presence of potential FA substrates including those for  $\Delta 6$  (18:3n-3 and 18:2n-6),  $\Delta 5$  (20:4n-3 and 20:3n-6) and  $\Delta 4$  (22:5n-3 and 22:4n-6). Yeast transformed with pYES2 vector alone showed the main FA normally found in *S. cerevisiae*, namely 16:0, 16:1 isomers, 18:0 and 18:1n-9, together with the exogenously added FA. This is consistent with *S. cerevisiae* not possessing  $\Delta 4$ ,  $\Delta 5$  or  $\Delta 6$  desaturase activities (19). Additional peaks were observed in the FA profiles of pYES2fad1 grown in the presence of  $\Delta 6$ , 18:3n-3 and 18:2n-6, and  $\Delta 5$ , 20:4n-3 and 20:3n-6, substrates. Similarly, the FA profile of yeast transformed with pYES2fad2 also showed additional peaks when grown in presence of  $\Delta 4$  substrates, 22:5n-3 and 22:4n-6, and  $\Delta 5$  substrates. The GC traces obtained with n-3 FAs are shown in Fig. 2A-I. Based on GC retention times, the additional peaks observed with the presence of the *S. canaliculatus* cDNAs were identified as 18:4n-3 (Fig. 2B), 20:5n-3 (Fig. 2E-F) and 22:6n-3 (Fig. 2I).

FAME from transgenic yeast incubated with PUFA were derivatized to picolinyl esters and subjected to electron ionization (EI) GC-MS to confirm the structures of PUFA produced in the presence of *fad* constructs (Fig. 3). The samples all showed prominent ions at  $m/z = 92, 108, 151$  and  $164$ , which are characteristic of picolinyl derivatives, representing pyridine ring fragments (23). The EI spectra of the additional peak in pYES2fad1-transformed yeast incubated with 18:3n-3 showed a fragmentation pattern with a mass ion of  $367 m/z$  and prominent peaks at  $352, 338, 312, 298, 272, 258, 232, 218$  and  $192$  (Fig. 3A). The initial interval of 15 represented the terminal methyl and was followed by an interval of 14, indicating one methylene group. The subsequent intervals of 26 denoted the positions of four double bonds indicating that this FA is  $\Delta^{15,12,9,6}18:4 = 18:4n-3$  (Fig. 3A). The EI spectra of the additional FA from both pYES2fad1- and pYES2fad2-transformed yeast incubated with 20:4n-3 showed mass ions of  $393 m/z$  with prominent ions at intervals of 26 ( $364-338, 324-298, 284-258, 244-218, \text{ and } 204-178 m/z$ ), confirming that the product

FAs are  $\Delta^{17,14,11,8,5}20:5 = 20:5n-3$  (Fig. 3B & C). The spectra of the additional peak observed in yeast transformed with pYES2fad2 and incubated with 22:5n-3 showed a mass ion of 419  $m/z$ , with prominent ions at intervals of 26 (390-364, 350-324, 310-284, 270-244, 230-204, and 190-164  $m/z$ ), confirming that this FA is  $\Delta^{19,16,13,10,7,4}22:6 = 22:6n-3$  (Fig. 3D). The GC-MS data confirmed that the *S. canaliculatus fad1* cDNA is a Fad that introduces double bonds into 18:3n-3 at the  $\Delta 6$  position and also into 20:4n-3 at the  $\Delta 5$  position. Additionally, *fad2* cDNA encodes a Fad that introduces double bonds at the  $\Delta 4$  position of 22:5n-3 and, to a lesser extent, the  $\Delta 5$  position of 20:4n-3.

The analyses indicated that the yeast cells transformed with pYES2fad1 acquired functional  $\Delta 6$  and  $\Delta 5$  desaturation activity, whereas cells transformed with pYES2fad2 had functional  $\Delta 4$  desaturation and  $\Delta 5$  desaturation capability. On the basis of the percentages of substrate FA converted to product, the *S. canaliculatus fad1* cDNA is more active on  $\Delta 6$  than on  $\Delta 5$  substrates, with *fad2* showing higher specificity on  $\Delta 4$  than  $\Delta 5$  substrates (Table 1). Both Fads preferentially converted n-3 rather than n-6 FAs.

## Discussion

This paper is the first report of  $\Delta 4$  Fad activity in a vertebrate. Historically, production of DHA from EPA was thought to occur via an elongation followed by  $\Delta 4$  desaturation (see 1,2). However, in the late 1990's, Sprecher and co-workers indicated an alternative pathway in rats with sequential elongations of EPA or ARA to  $C_{24}$  substrates followed by desaturation at the  $\Delta 6$  position (2). Biochemical studies indicated that this pathway also likely operated in rainbow trout (24,25), and that mammalian  $\Delta 6$  Fads were capable of desaturating both  $C_{18}$  and  $C_{24}$  substrates (26,27). Molecular studies showed that the zebrafish  $\Delta 6/\Delta 5$  and salmon  $\Delta 6$  Fad cDNAs could utilize  $C_{24}$  FA in addition to  $C_{18}$  FA when expressed in *S. cerevisiae* (28). Furthermore, no Fad with  $\Delta 4$  activity had been isolated from a vertebrate species and, thus, it became the paradigm that vertebrates in general produced DHA from EPA via the alternative "Sprecher" pathway and did not possess a  $\Delta 4$  Fad (1,2). The present

study has clearly demonstrated a teleost fish capable of  $\Delta 4$  desaturation and that two alternative pathways for the production of DHA from EPA are thus possible in at least some vertebrates (Fig. 4).

The rate of DHA synthesis could be faster in the more direct  $\Delta 4$  pathway that only requires endoplasmic reticulum, whereas the “Sprecher” pathway also involves peroxisomes, translocation of PUFA intermediates and limited fatty acid oxidation, a catabolic step. Previously,  $\Delta 4$  Fads have been demonstrated in other organisms including protozoan trypanosomes (29) and marine microalgae *Pavlova lutheria* and *Thraustochytrids* (30, 31), the latter of which is particularly interesting as it also has two alternative pathways for DHA synthesis and contains a polyketide (PKS) pathway not requiring aerobic desaturases (32,33). However, as expected, the phylogenetic analysis showed that the *S. canaliculatus*  $\Delta 4$  Fad was more closely related to other vertebrate  $\Delta 6$  and  $\Delta 5$  Fads than the algal and protozoan  $\Delta 4$  desaturases (34).

The first bifunctional desaturase isolated from a vertebrate was the zebrafish Fad with  $\Delta 6/\Delta 5$  activity (19), and subsequently desaturases with dual function have also been described in moths ( $\Delta 11$  and  $\Delta 10,12$ ) (35), and fungus ( $\Delta 12$  and  $\Delta 15$ ) (36). Recently, a baboon Fad was reported to possess both  $\Delta 6$  and  $\Delta 8$  activity (37). Phylogenetic analysis showed that the *S. canaliculatus* Fads clustered with Fads from taxonomically similar species rather than with the zebrafish bifunctional desaturase a pattern that has been observed previously (17,18). The multiple desaturase encoding genes in Atlantic salmon shared more than 90% identity to each other but were strictly mono-functional, with three  $\Delta 6$  Fads and a  $\Delta 5$  Fad (12-14). Gene duplication and environmental pressure may underpin the diversity of Fad isoforms and functions. Recently, molecular evolution and functional diversification of Fads after recurrent gene duplication was shown in *Drosophila* (38). Fish possess varied and ‘plastic’ genomes as a result of frequent genomic changes including polyploidy, gene and chromosomal duplications, and gain of introns, making them interesting models for environmental genomics (39). Indeed, the evolution of stearoyl-CoA desaturases in teleost fishes has been described in relation to ancient and modern duplication events

(40). Thus it is likely that environmental pressure and genome duplication has led to molecular evolution and functional diversification of other Fads in teleosts.

This is also the first report of a marine fish expressing  $\Delta 5$  desaturase activity as previously no  $\Delta 5$  activity or gene had been reported in any marine fish. Indeed lack of  $\Delta 5$  activity (8,10) formed the biochemical and molecular basis for the difference in EFA requirements between freshwater/diadromous species (e.g. zebrafish, carp, trout and salmon) whose EFA requirements can be satisfied by 18:3n-3 and 18:2n-6, and marine species (e.g. cod, turbot, sea bream and sea bass) whose EFA requirements are not satisfied by C<sub>18</sub> PUFA and instead require preformed LC-PUFA (7). The demonstration of genes encoding enzymes with  $\Delta 6/\Delta 5$  and  $\Delta 4$  Fad activities in the marine species *S. canaliculatus* is thus interesting in relation to the determinants of LC-PUFA biosynthesis in fish and vertebrates in general. Previously, it was unclear whether the above distinction between marine and freshwater species was related simply to environment. The underpinning hypothesis was actually based on nutrient supply in the two environments, i.e higher levels of EPA and, especially, DHA in the marine environment (8), and so could also be related to feeding habits and trophic level (41). The trophic level of the marine fish studied were generally > 3.0 (carnivores) compared to the lower trophic level (<2.8) of the freshwater species investigated (20). *S. canaliculatus* consumes exclusively benthic algae and seagrasses and thus is a rare example of an exclusively herbivorous species inhabiting the marine environment (21,22). This study therefore is the first evidence that trophic level can prevail over environment and that the above distinction between freshwater/marine species is too simplistic (10). The presence of  $\Delta 6/\Delta 5$  Fad indicates that *S. canaliculatus* has the desaturation activities necessary for the production of EPA and ARA from 18:3n-3 and 18:2n-6, respectively, and furthermore the presence of  $\Delta 4$  Fad enables the subsequent endogenous production of DHA from EPA.

The above discussion is of far more than simple scientific interest as it is also highly relevant to aquaculture and the provision of n-3 LC-PUFA to the burgeoning human population. Fish are the major dietary source of n-3 LC-PUFA (20) and, with declining fisheries worldwide (42), farmed fish constitute an ever-increasing

proportion of the fish in the human food basket amounting to one half in 2009 (43). Until now, high n-3 LC-PUFA levels in flesh of farmed fish have been obtained by the use in the feeds of fish oils, paradoxically themselves derived from marine fisheries, but this is not sustainable and will constrain continuing growth of aquaculture activities (44). Alternatives to fish oil are urgently required but the prime candidates, vegetable oils, are rich in C<sub>18</sub> PUFA but devoid of the n-3 LC-PUFA abundant in fish oil (9). Feeding fish on vegetable oil can thus have important consequences for the human consumer as it lowers the n-3 LC-PUFA content of the flesh compromising nutritional value (45). The problem is particularly acute in fish species that do not have the capability of endogenous production of LC-PUFA and this has included all marine species investigated to date (7-10). *S. canaliculatus*, the white-spotted spinefoot or rabbitfish, is a reef-associated, perciform, oceanodromous species that inhabits tropical marine or brackish water, and is a common food fish in its region and a prime candidate for aquaculture (22). As such it is the first example of a marine species that would likely thrive on feeds formulated with C<sub>18</sub>-rich vegetable oils and endogenously produce the healthful LC-PUFA, ARA, EPA and DHA.

In conclusion, this work has demonstrated for the first time the presence of  $\Delta 4$  Fad activity in a vertebrate species indicating an alternative, simpler pathway for the production of DHA from EPA. Furthermore the demonstration of further examples of bifunctional Fads suggests these may be more common among vertebrate species. The isolation and characterization of these Fad activities in an herbivorous, marine teleost demonstrates that trophic level has likely taken precedent over environment in the evolution of LC-PUFA biosynthesis pathways in fish.

## **Materials and Methods**

**Molecular cloning of *S. canaliculatus*  $\Delta 4$  *fad* and sequence analysis.** As with the previously cloned *S. canaliculatus* desaturase *fad1* cDNA (gb|EF424276|) (22), the highly conserved sequences of vertebrate  $\Delta 6$  or  $\Delta 5$  Fads were used to design primers for the amplification of *fad2*. A fragment was obtained by polymerase chain reaction

(PCR) and further extended by 5' and 3' rapid amplification of cDNA ends (RACE) (GeneRacer™ Kit, Invitrogen) to produce full-length *fad2* cDNA (gb| GU594278|).

The amino acid (AA) sequence deduced from the *S. canaliculatus fad2* cDNA was compared with mammalian and teleost Fad proteins using EMBOSS Pairwise Alignment Algorithms tool (<http://www.ebi.ac.uk/Tools/emboss/align/>). A phylogenetic tree was constructed on the basis of the AA sequence alignments between the *S. canaliculatus* Fads and those from other organisms, using the neighbor joining method (46).

**Heterologous expression of *fad* ORFs.** PCR fragments corresponding to the ORFs of *S. canaliculatus fad1* (gb|EF424276|) and *fad2* (gb|GU594278|) cDNAs were amplified from brain cDNA (Pfu Turbo polymerase, Stratagene). Briefly, gene-specific primers 5'-GGAGGATGGGGATGTGAGTA-3' (forward) and 5'-ATAAAACCATGTGGGCAGGT-3' (reverse) for *fad1*, and 5'-GAAGACGGAGGATGAGGATG-3' (forward) and 5'-TGCTCAGCACAGGATTGAGT-3' (reverse) designed on the untranslated regions (UTRs) were used in first-round PCR. The isolation of the *fad1* and *fad2* ORFs was achieved in second-round (nested) PCR using first round PCR products primed with 5'-CCCAAGCTTAGGATGGGAGGTGGAGGTC-3' and 5'-CCGTCTAGATCATTATGGAGATATGC-3' containing restriction sites (underlined) for *Hind*III (forward) and *Xba*I (reverse). The amplified DNA fragments were digested with the corresponding restriction endonucleases (New England BioLabs) and ligated into pYES2 vector (Invitrogen). The resulting plasmid constructs, pYES2fad1 and pYES2fad2, were transformed into *S. cerevisiae* (strain INVSc1) using the S.c. EasyComp Transformation kit (Invitrogen). Yeast transformed with pYES2 (control), or the constructs pYES2fad1 or pYES2fad2, were grown in *S. cerevisiae* minimal medium<sup>-uracil</sup> using galactose induction of gene expression as described previously (19). Recombinant yeast cultures were supplemented with one of the following FA substrates:  $\alpha$ -linolenic (18:3n-3), linoleic (18:2n-6), eicosatetraenoic (20:4n-3), dihomo- $\gamma$ -linolenic (20:3n-6), docosapentaenoic (22:5n-3) and

docosatetraenoic (22:4n-6) acids. FA substrates were added to the yeast cultures at final concentrations of 0.5 (C<sub>18</sub>), 0.75 (C<sub>20</sub>) and 1.0 (C<sub>22</sub>) mM as uptake efficiency decreases with increasing chain length. After 2-days, approximately equal amounts of yeast cells were transferred to glass test tubes, and collected by centrifugation (500 x g for 2 min), washed twice with 5 ml Hanks' balanced salt solution (Invitrogen), and lipid extracted by homogenization in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, as described previously (19).

**GC-MS analysis.** FA methyl esters (FAME) were prepared, extracted, and purified by thin-layer chromatography (19), and picolinyl esters were prepared from FAME (47). Briefly, FAME samples dissolved in 1 ml dry dichloromethane were added to a mixture of 0.1 ml potassium *tert*-butoxide in tetrahydrofuran (1.0 M) and 0.2 ml 3-pyridylcarbinol. After incubation at 40°C for 30 min, the picolinyl esters were extracted by adding 2 ml water and 4 ml iso-hexane, and the organic phase collected and evaporated under dry nitrogen. Picolinyl derivatives were subjected to electron ionization (EI) GC-MS using a Fisons GC8000 gas chromatograph coupled to an MD800 mass spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK). The gas chromatograph was equipped with a fused silica capillary column (30 m x 0.32 mm i.d.) coated with Zebron ZB-Wax (Phenomenex, Macclesfield, UK) and used helium as carrier gas. Samples were applied using on-column injection with the oven temperature programmed to rise from 80 to 250°C at 40°C/min. Proportions of substrate FA converted to desaturated FA product were calculated as [product area/(product area +substrate area)] x 100 as described previously (19).

**Materials.** Eicosatetraenoic, docosapentaenoic and docosatetraenoic acids (>98-99% pure) were from Cayman Chemical Co. (Ann Arbor, USA) and the remaining FA substrates (>99% pure) were from Sigma-Aldrich Co. (Dorset, UK). Chemicals used to prepare the *S. cerevisiae* minimal medium<sup>-uracil</sup>, BHT, potassium *tert*-butoxide in tetrahydrofuran and 3-pyridylcarbinol were from Sigma-Aldrich Co. TLC (20 x 20 cm x 0.25 mm) plates precoated with silica gel 60 (without fluorescent indicator) were

from Merck (Darmstadt, Germany). All solvents were HPLC grade and obtained from Fisher Scientific (Loughborough, UK).

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### **References**

1. Cook HW, McMaster RCR (2004) in *Biochemistry of Lipids, Lipoproteins and Membranes*, eds Vance DE, Vance JE (Elsevier, Amsterdam). pp 181-204.
2. Sprecher H (2000) *Biochim Biophys Acta* 1486:219-231.
3. Rivers JPW, Sinclair AJ, Crawford MA (1975) *Nature (London)* 258:171-173.
4. Rivers, JPW, Hassam AG, Crawford MA, Brambell MR (1976) *FEBS Lett* 67:269-270.
5. Hassam AG, Rivers JPW, Crawford MA (1977) *Nutr Metab* 21:321-328.
6. Sinclair AJ, McLean JG, Monger EA (1979) *Lipids* 14:932-36.
7. Tocher DR (2010) Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquaculture Res* 41:717-732.
8. Bell MV, Tocher DR (2009) in *Lipids in Aquatic Ecosystems*, eds Arts MT, Brett M, Kainz M (Springer-Verlag, New York). pp 211-236.
9. Tocher DR (2009) Issues surrounding fish as a source of  $\omega$ 3 long-chain polyunsaturated fatty acids. *Lipid Technol* 21:13-16.
10. Leaver MJ, et al. (2008) Towards fish lipid nutrigenomics: current state and prospects for fin-fish aquaculture. *Rev Fisheries Sci* 16(S1):71-92.
11. Marquardt A, Stohr H, White K, Weber BHF (2000) cDNA cloning, genomic structure, and chromosomal localization of three members of the human fatty acid desaturase family. *Genomics* 66:175-183.

12. Hastings N, et al. (2005) Molecular cloning and functional characterization of fatty acyl desaturase and elongase cDNAs involved in the production of eicosapentaenoic and docosahexaenoic acids from  $\alpha$ -linolenic acid in Atlantic salmon (*Salmo salar*). *Mar Biotechnol* 6:463-474
13. Zheng X, et al. (2005) Highly unsaturated fatty acid synthesis in vertebrates: new insights with the cloning and characterization of a  $\Delta 6$  desaturase of Atlantic salmon. *Lipids* 40:13-24.
14. Monroig O, et al. (2010) Multiple fatty acyl desaturase (FAD) genes in Atlantic salmon: cloning and functional expression of cDNAs confirm presence of three  $\Delta 6$  FADs. *Biochim Biophys Acta* In press.
15. Seiliez I, Panserat S, Kaushik S, Bergot P (2001) Cloning, tissue distribution and nutritional regulation of a delta 6-desaturase-like enzyme in rainbow trout. *Comp Biochem Physiol* 130B:83-93.
16. Seiliez I, Panserat S, Corraze G, Kaushik S, Bergot P (2003) Cloning and nutritional regulation of a delta 6-desaturase-like enzyme in the marine teleost gilthead seabream (*Sparus aurata*). *Comp Biochem Physiol* 135B:449-460.
17. Zheng X, et al. (2004) Characterization and comparison of fatty acyl  $\Delta 6$  desaturase cDNAs from freshwater and marine teleost fish species. *Comp Biochem Physiol* 139B:269-279
18. Tocher DR, et al. (2006) Highly unsaturated fatty acid synthesis in marine fish: cloning, functional characterization, and nutritional regulation of fatty acyl  $\Delta 6$  desaturase of Atlantic cod (*Gadus morhua* L.). *Lipids* 41:1003-1016.
19. Hastings N, et al. (2001) A vertebrate fatty acid desaturase with  $\Delta 5$  and  $\Delta 6$  activities. *Proc Natl Acad Sci USA* 98:14304-14309.
20. Tacon AGJ, Metian M, Turchini GM, DeSilva SS (2010) Responsible aquaculture and trophic level implications to global fish supply. *Rev Fisher Sci* 18:94-105.
21. Woodland DJ (1990) Revision of the fish family Siganidae with descriptions of two new species and comments on distribution and biology. *Indo-Pacific Fishes* 19:136 p.

22. Li Y, et al. (2008) The effects of dietary fatty acids on liver fatty acid composition and delta 6-desaturase expression differ with ambient salinities in *Siganus canaliculatus*. *Comp Biochem Physiol* 151B:183-190.
23. Christie WW (2003) *Lipid Analysis* (Oily Press, Bridgwater). p 289.
24. Buzzi M, Henderson RJ, Sargent JR (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.
25. Buzzi M, Henderson RJ, Sargent JR (1997) Biosynthesis of docosahexaenoic acid in trout hepatocytes proceeds via 24-carbon intermediates. *Comp Biochem Physiol* 116B:263–267.
26. D'Andrea S, et al. (2002) The same rat  $\Delta$ 6-desaturase not only acts on 18- but also on 24-carbon fatty acids in very-long-chain polyunsaturated fatty acid biosynthesis. *Biochem J* 364:49–55.
27. De Antueno RJ, et al. (2001) Activity of human  $\Delta$ 5 and  $\Delta$ 6 desaturases on multiple n-3 and n-6 polyunsaturated fatty acids. *FEBS Lett* 509:77–80.
28. Tocher DR, Agaba M, Hastings N, Teale AJ (2003) In *The Big Fish Bang – Proceedings of the 26th Annual Larval Fish Conference*, eds Browman HI, Skiftesvik AB (Institute of Marine Nutrition, Bergen). pp 211-227.
29. Tripodi KEJ, Buttigliero LV, Altabe SG, Uttaro AD (2006) Functional characterization of front-end desaturases from trypanosomatids depicts the first polyunsaturated fatty acid biosynthetic pathway from a parasitic protozoan. *FEBS J* 273:271–280.
30. Tonon T, Harvey D, Larson TR, Graham IA (2003) Identification of a very long chain polyunsaturated fatty acid  $\Delta$ 4-desaturase from the microalga *Pavlova lutheri*. *FEBS Lett* 553:440-444.
31. Qiu X, Hong H, MacKenzie SL (2001) Identification of a  $\Delta$ 4 fatty acid desaturase from *Thraustochytrium* sp. involved in the biosynthesis of docosahexanoic acid by heterologous expression in *Saccharomyces cerevisiae* and *Brassica juncea*. *J Biol Chem* 276:31561-31566.

32. Metz JG, et al. (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* 293:290–293.
33. Lippmeier JC, et al. (2009) Characterization of both polyunsaturated fatty acid biosynthetic pathways in *Schizochytrium* sp. *Lipids* 44:621-630.
34. Sperling P, Ternes P, Zank TK, Heinz E (2003) The evolution of desaturases. *Prostaglandins Leukot. Essent. Fatty Acids* 68:73–95.
35. Serra M, Gauthier LT, Fabrias G, Buist PH (2006)  $\Delta 11$  desaturases of *Trichoplusia ni* and *Spodoptera littoralis* exhibit dual catalytic behaviour. *Insect Biochem Mol Biol* 36:822–825.
36. Zhang S, Sakuradani E, Ito K, Shimizu S (2007) Identification of a novel bifunctional  $\Delta 12/\Delta 15$  fatty acid desaturase from a basidiomycete, *Coprinus cinereus* TD#822-2. *FEBS Lett* 581:315-319.
37. Park WJ, Kothapalli KSD, Lawrence P, Tyburczy C, Brenna JT (2009) An alternate pathway to long-chain polyunsaturates: the FADS2 gene product  $\Delta 8$ -desaturates 20:2n-6 and 20:3n-3. *J Lipid Res* 50:1195–1202.
38. Fang S, et al. (2009) Molecular evolution and functional diversification of fatty acid desaturases after recurrent gene duplication in *Drosophila*. *Mol Biol Evol* 26:1447–1456.
39. Cossins AR, Crawford DL (2005) Fish as models for environmental genomics. *Nat Rev Genet* 6:324–333.
40. Evans H, et al. (2008) Ancient and modern duplication events and the evolution of stearoyl-CoA desaturases in teleost fishes. *Physiol Genomics* 35:18–29.
41. Sargent JR, Tocher DR, Bell JG (2002) in *Fish Nutrition*, eds Halver JE, Hardy RW (Academic Press, San Diego). pp 181-257.
42. Worms B, et al. (2006) Impacts of biodiversity loss on ocean ecosystem services. *Science* 314:787-790.
43. Food and Agricultural Organization of the United Nations (FAO) (2009) *The State of World Fisheries and Aquaculture 2008 (SOFIA)* (FAO Fisheries and Aquaculture Department, Rome). 176 pp.

44. Naylor RL, et al. (2009) Feeding aquaculture in an era of finite resources. *Proc Natl Acad Sci USA* 106:15103-15110.
45. Bell JG, Tocher DR (2009) in *Oils and Fats Handbook Volume 4; Fish Oils*, ed Rossell B (Leatherhead Food International, Leatherhead). pp 171-184.
46. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425.
47. Destailats F, Angers P (2002) One-step methodology for the synthesis of FA picolinyl esters from intact lipids. *J Am Oil Chem Soc* 79:253-256

### Legends to Figures

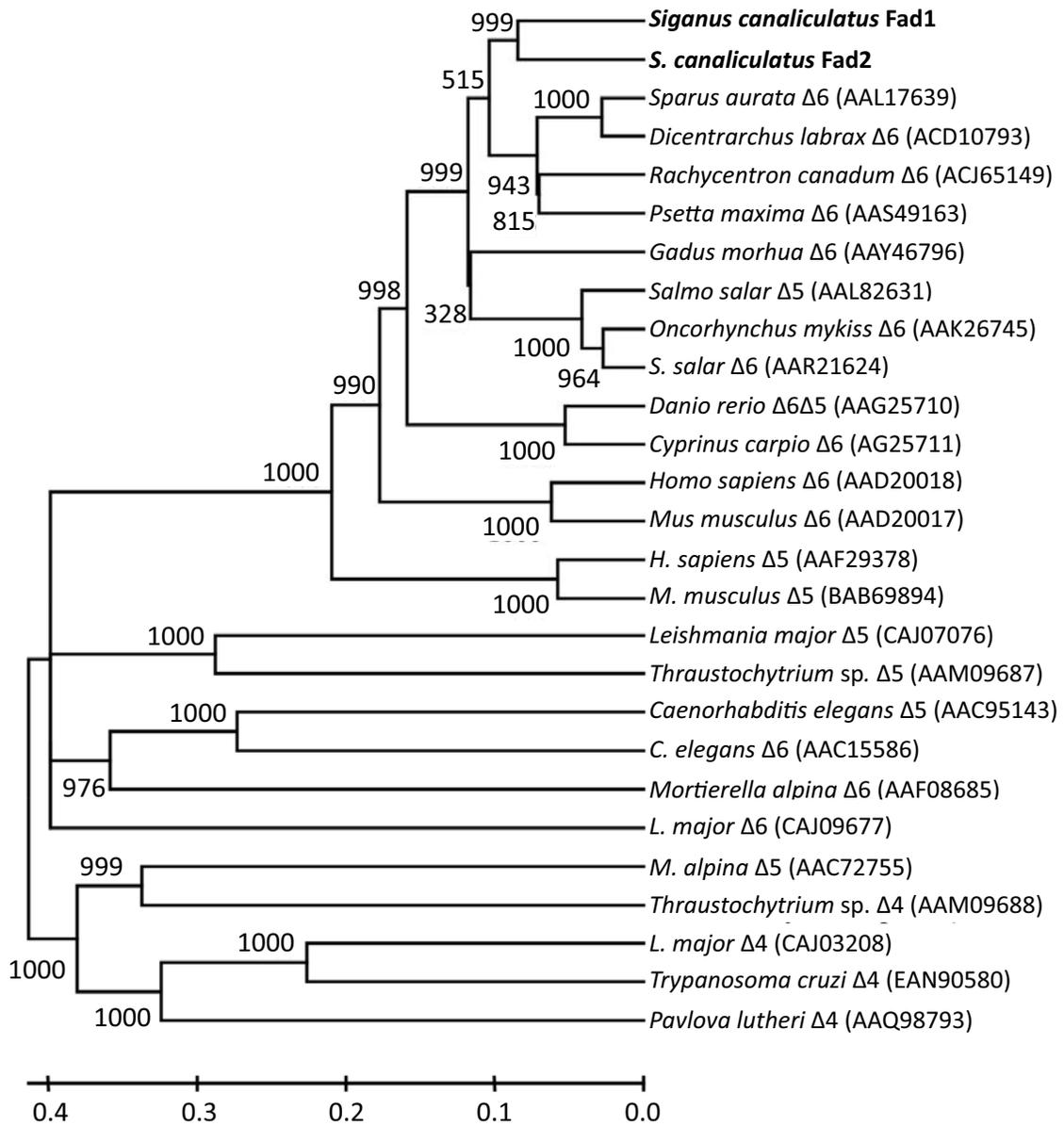
Fig. 1. Phylogenetic tree comparing the deduced AA sequences of *Siganus canaliculatus* Fad1 and Fad2 with desaturase proteins from fish and other organisms. The tree was constructed using the neighbor joining method (46) with MEGA4. The horizontal branch length is proportional to AA substitution rate per site. The numbers represent the frequencies with which the tree topology presented was replicated after 1000 iterations.

Fig. 2. Functional characterization of the *Siganus canaliculatus* putative fatty acyl desaturases in transgenic yeast (*Saccharomyces cerevisiae*). FAME were extracted from yeast transformed with pYES2 vector alone (A, D, G) or the constructs pYES2fad1 (B, E, H) and pYES2fad2 (C, F, I), and grown in the presence of FA substrates (\*) 18:3n-3 (A-C), 20:4n-3 (D-F), and 22:5n-3 (G-I). Peaks 1-4 represent the main endogenous FAs of *S. cerevisiae*, namely 16:0, 16:1 isomers, 18:0 and 18:1n-9, respectively. Based in retention times, additional peaks (arrowed) were identified as 18:4n-3 (B), 20:5n-3 (E and F) and 22:6n-3 (I). Vertical axis, FID response; horizontal axis, retention time.

Fig. 3. Mass spectra of the arrowed peaks in Fig. 2. Picolinyl esters were prepared from FAME extracted from yeast transformed with pYES2fad1 and grown in the presence of 18:3n-3 (A) and 20:4n-3 (B), or pYES2fad2 and grown in the presence of 20:4n-3 (C) and 22:5n-3 (D). FAME were analyzed by GC-MS as described in Materials and Methods. The identities of the peaks were confirmed as 18:4n-3 (A),

20:5n-3 (B and C) and 22:6n-3 (D). Vertical axis, % abundance; horizontal axis, mass-to-charge ( $m/z$ ) ratio.

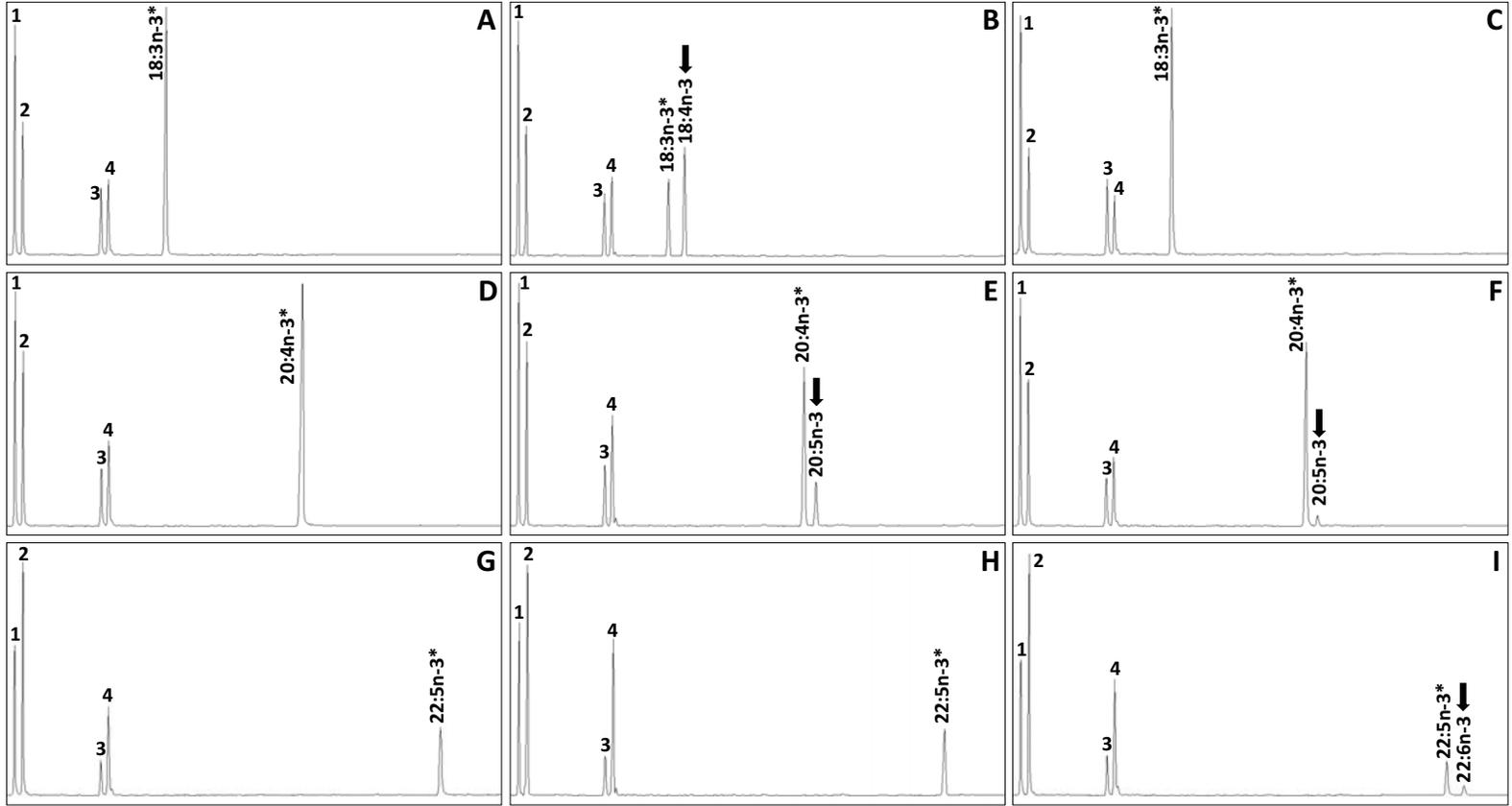
Fig. 4. PUFA biosynthesis pathways. Solid lines indicate pathways confirmed in teleosts, while broken lines indicate pathways shown in other organisms but unconfirmed in teleosts.

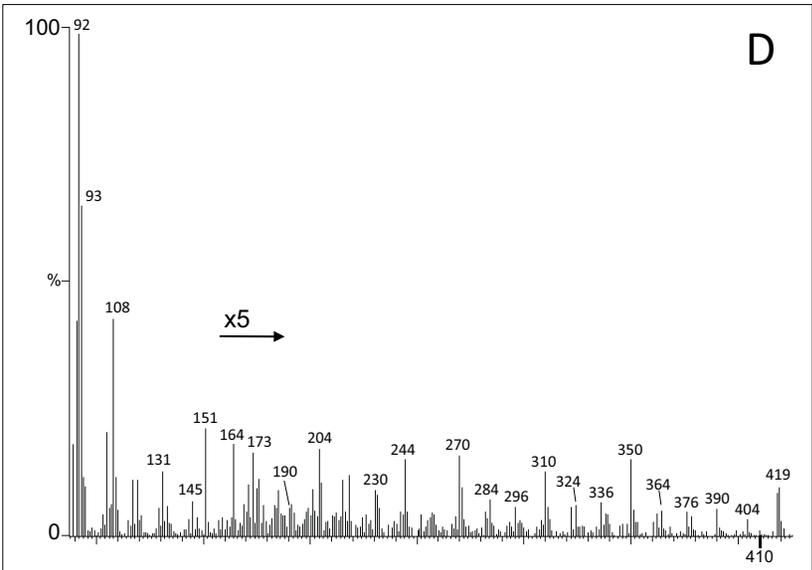
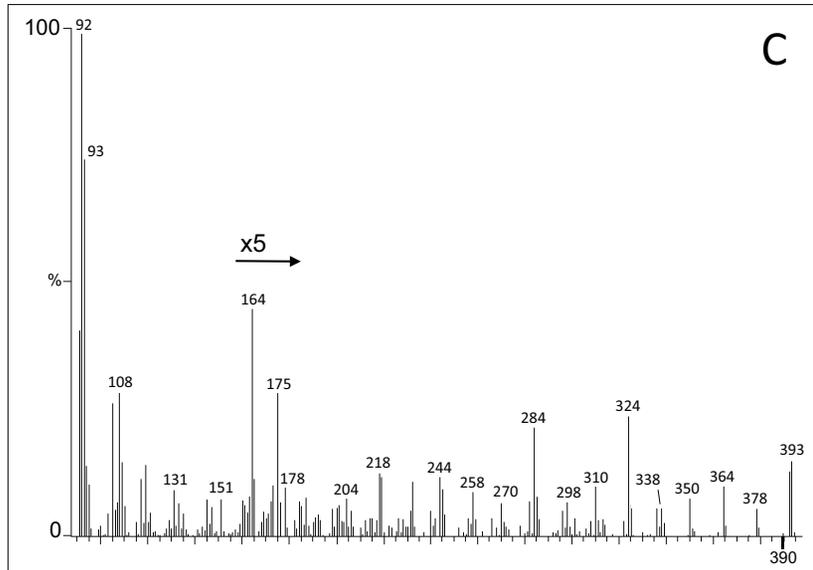
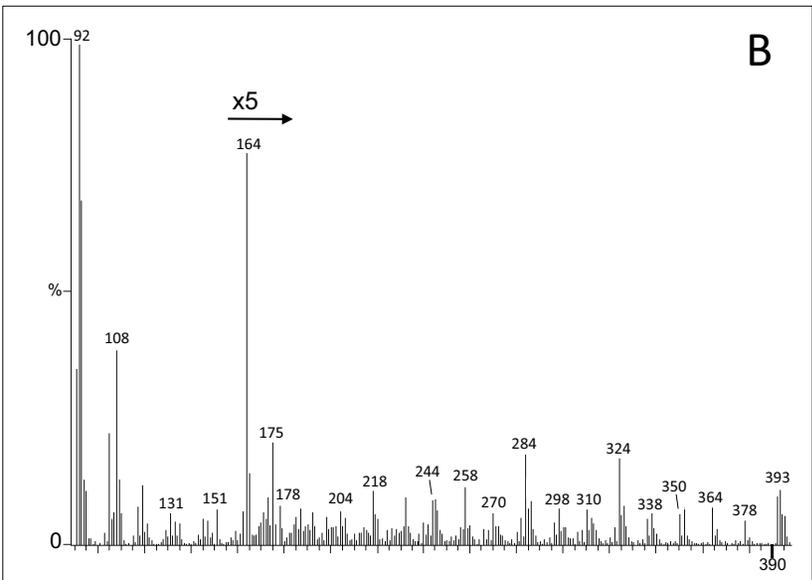
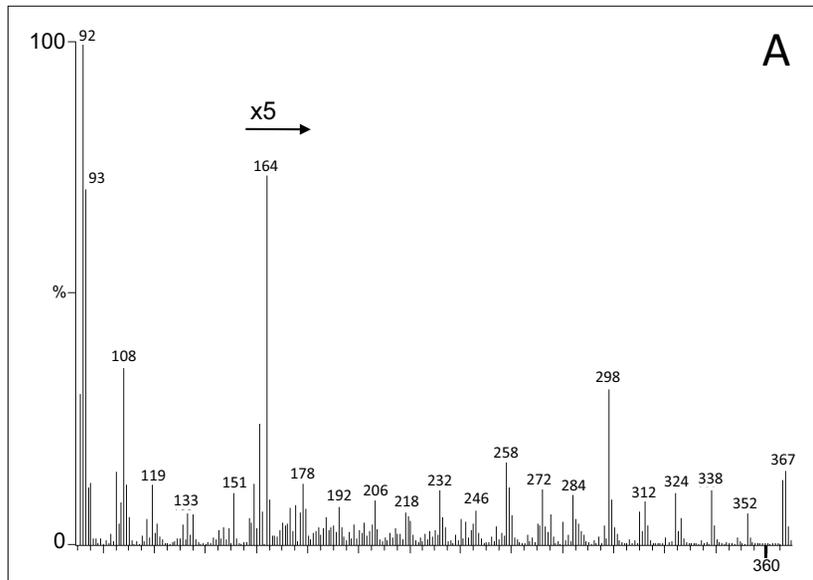


pYES2

pYES2fad1

pYES2fad2





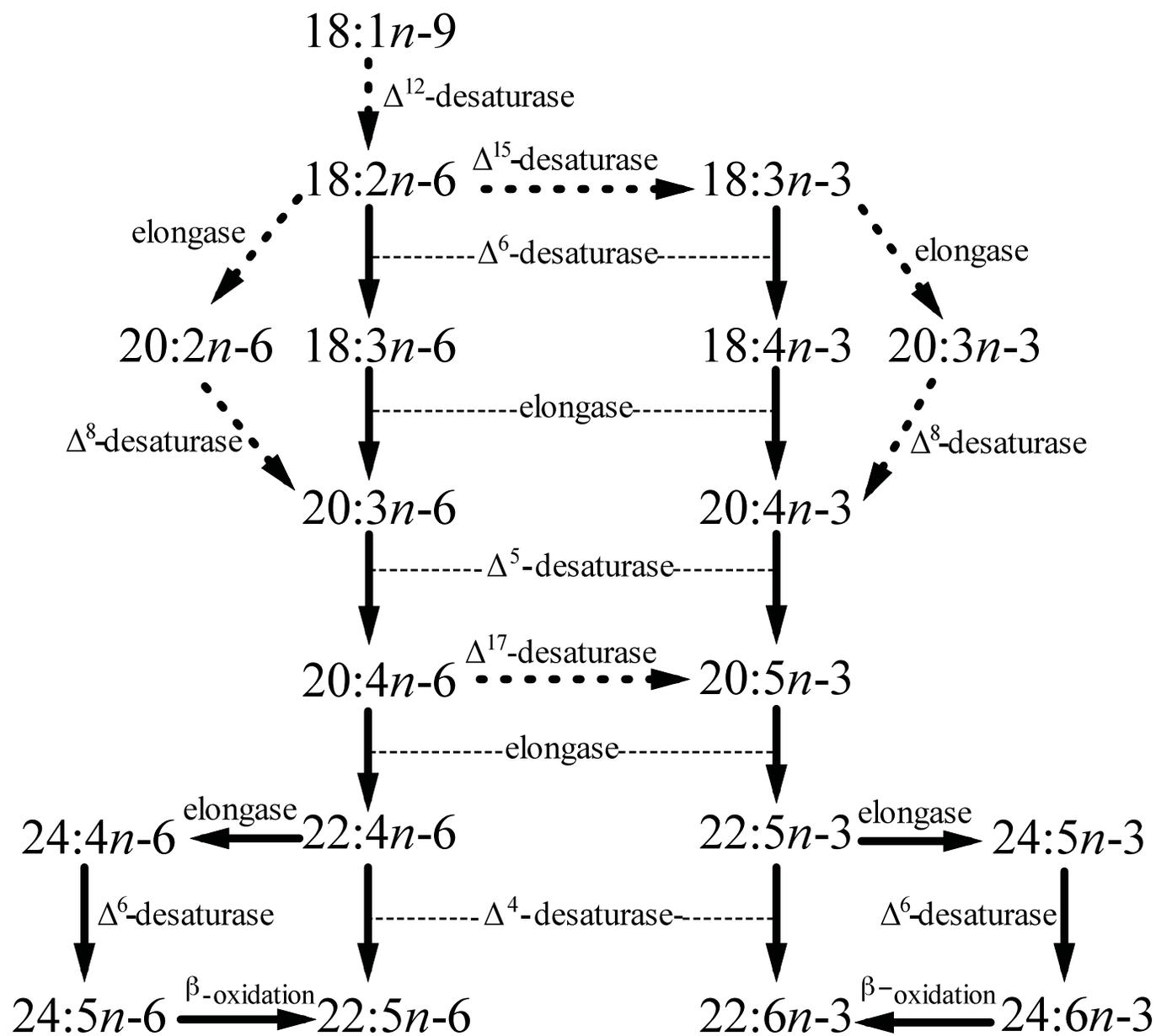


Table 1. Substrate conversions of pYES2fad1 and pYES2fad2-transformed yeast grown in presence of  $\Delta 6$ ,  $\Delta 5$  and  $\Delta 4$  fatty acid (FA) substrates.

FA substrate	Product	Conversion (%)		Activity
		pYES2fad1	pYES2fad2	
18:3n-3	18:4n-3	59	0	$\Delta 6$
18:2n-6	18:3n-6	35	0	$\Delta 6$
20:4n-3	20:5n-3	22	6	$\Delta 5$
20:3n-6	20:4n-6	12	2	$\Delta 5$
22:5n-3	22:6n-3	1	23	$\Delta 4$
22:4n-6	22:5n-6	0	14	$\Delta 4$