3 CLONING AND CHARACTERISATION OF A *FATTY ACYL Δ6DESATURASE* AND A *FATTY ACYL ELONGASE* cDNA FROM SOUTHERN BLUEFIN TUNA

3.1 Introduction

3.1.1 Long chain polyunsaturated fatty acid (LCPUFA) synthesis enzymes

3.1.1.1 The desaturase enzymes

Desaturase enzymes modify C-H bonds at saturated or monounsaturated carbons in all animals (Sperling *et al.* 2003). Higher animals are able to insert double bonds at the Δ4, Δ5, Δ6 and Δ9 positions, while insects and plants can also insert double bonds at the Δ12 and Δ15 positions (Marquardt *et al.* 2000). Animal desaturases are extra-plastidial, membrane-bound enzymes, usually located in the endoplasmic reticulum (ER) (Sperling and Heinz 2001; Whelan and Rust 2006). An N-terminal membrane-bound cytochrome b_5 domain receives electrons from NADH or NADPH (Sperling and Heinz 2001). The di-iron complex in the centre of the cytochrome b_5 domain is kept in place by histidine rich regions containing histidine, glutamine, glutamic acid and aspartic acid (Sperling and Heinz 2001). The active sites of desaturases may interact with substrates at the membrane surface due to their di-iron centre being surrounded by residues of intermediate polarity/hydrophobicity (Sperling and Heinz 2001). Desaturases are predicted to contain multiple transmembrane spanning domains (Knutzon *et al.* 1998). The histidine-rich regions I (HX_3X) and II (HX_2HH) are located between the two transmembrane domains and region III (HH) can be found at the C-terminus of the protein. In mammals the most C-terminal histidine box has been found to vary from the consensus HXXHH, with the first histidine residue being replaced with a glutamine residue, QXXHH (Sperling *et al.* 2003; Aki *et al.* 1999; Knutzon *et al.* 1998; Napier *et al.* 1998). The human *Δ5desaturase* and *Δ6desaturase* genes consist of 12 coding exons (Zheng *et al.*

2009a). In contrast, the fish *Δ6desaturase* genes consist of 13 exons, with the first exon being non-coding, followed by 12 coding exons (Zheng *et al.* 2009a).

Δ6desaturase genes have been cloned and/or functionally characterised from the fungus *Mortierella alpina* (Huang *et al.* 1999), the nematode *Caenorhabditis elegans* (Napier *et al.* 1998), rat (Aki *et al.* 1999), mouse, human (Cho *et al.* 1999a) and fish species, including Atlantic salmon (*Salmo salar*) (Hastings *et al.* 2005; Zheng *et al.* 2005), zebrafish (*Danio rerio*) (Hastings *et al.* 2001), carp (*Cyrinus carpio*), turbot (*Psetta maxima*) (Zheng *et al.* 2004), rainbow trout (*Oncorhynchus mykiss*) (Seiliez *et al.* 2001; Zheng *et al.* 2004), gilthead sea bream (*Sparus aurata*) (Seiliez *et al.* 2003; Zheng *et al.* 2004), white-spotted spinefoot (*Siganus canaliculatusi)* (Li *et al.* 2008), cobia (*Rachycentron canadum*) (Zheng *et al.* 2009), Atlantic cod (*Gadus morhua*) (Tocher *et al.* 2006) and barramundi (*Lates calcarifer*) (Mohd-Yusof *et al.* 2010). The zebrafish desaturase is the only characterized enzyme with both Δ5desaturase and Δ6desaturase activities (Hastings *et al.* 2001). The other desaturases have predominately Δ6desaturase activity. The only characterised fish desaturase with predominately Δ5desaturase activity is from Atlantic salmon (Hastings *et al.* 2005).

Expression of *Δ5desaturase* and *Δ6desaturase* in mammals is high in the liver, brain and heart (Cho *et al.* 1999; Cho *et al.* 1999a). Expression of *Δ5desaturase* and *Δ6desaturase* in fish tissues varies with species. The Atlantic salmon *Δ5desaturase* and *Δ6desaturase* and the zebrafish *Δ5/Δ6desaturase* are most highly expressed in the intestine, liver and brain (Zheng *et al.* 2005; Monroig *et al.* 2009; Morais *et al.* 2009). European sea bass have the highest *Δ6desaturase* expression in the heart, brain and ovary (González-Rovira *et al.* 2009), while cobia have the highest *Δ6desaturase* expression in the brain, liver and heart (Zheng *et al.* 2009).

3.1.1.2 The elongase enzymes

Elongase enzymes use the substrates malonyl-CoA and fatty acyl-CoA for two carbon additions to the chain length of fatty acids (Wang *et al.* 2005). In the mouse, rat and human genomes there are six elongases identified (Elovl1-Elovl6) which are categorized by substrate specificity (Wang *et al.* 2005; Jakobsson *et al.* 2006). Elovl1

and Elovl6 elongate saturated and monounsaturated fatty acids (Wang *et al.* 2005). Elovl2 elongates C_{20-22} PUFA, Elovl3 and Elovl4 elongate fatty acids with $\leq C_{26}$, and Elovl5 elongates C_{16-20} PUFA (Leonard *et al.* 2004; Wang *et al.* 2005). Elongation occurs in the ER (Wang *et al.* 2005). The characteristic structural features include the conserved motifs KxxExxDT (Box 1), QxxFLHxYHH containing the single histidine box redox centre motif HxxHH (Box 2), NxxxHxxMYxYY (Box 3) and TxxQxxQ (Box 4) (Agaba *et al.* 2005; Jakobsson *et al.* 2006). The histidine residues in the histidine box may act as iron-chelating ligands to form Fe-O-Fe clusters where the oxygen can then be used for electron transfer (Inagaki *et al.* 2002). Elongases contain multiple transmembrane regions and lysine or arginine residues close to the C terminus which may function as an ER retention signal (Agaba *et al.* 2005; Jakobsson *et al.* 2006).

Fatty acyl elongase genes have been cloned and/or functionally characterised from the fungus *M. alpina* (Parker-Barnes *et al.* 2000), the nematode *C. elegans* (Beaudion *et al.* 2000), rat (Inagaki *et al.* 2002), mouse (Leonard *et al.* 2002), human (Leonard *et al.* 2000) and fish species, including Nile tilapia (*Oreochromis nilotica*), North African catfish (*Clarius gariepinus*), Atlantic cod (*Gadus morhua*), gilthead sea bream (*Sparus aurata*), turbot (*Psetta maxima*) (Agaba *et al.* 2005), zebrafish (*Danio rerio*) (Agaba *et al.* 2004), Atlantic salmon (*Salmo salar*) (Hastings *et al.* 2005; Morais *et al.* 2009), rainbow trout (*Oncorhynchus mykiss*) (Meyer *et al.* 2004), cherry salmon (*Onchorhynchus masou*) (Alimuddin *et al.* 2008), cobia (*Rachycentron canadum*) (Zheng *et al.* 2009) and barramundi (*Lates calcarifer*) (Mohd-Yusof *et al.* 2010). Until recently, all of the characterised fish fatty acyl elongases were considered to be Elovl5-like, as they prefer $C_{18/20}$ PUFA substrates. However, Morais *et al.* (2009) and Monroig *et al.* (2009) have recently characterised Elovl2 elongases in Atlantic salmon and zebrafish which prefer $C_{20/22}$ PUFA substrates.

Expression of the elongase genes can be found in a variety of fish tissues, with highest expression not always being in the same tissues as the desaturases. The expression of the *Elovl2* and *Elovl5* from Atlantic salmon and zebrafish was highest in the same tissues as the respective *Δ5desaturase*, *Δ6desaturase* or *Δ5/Δ6desaturase* (Zheng *et al.* 2005; Monroig *et al.* 2009; Morais *et al.* 2009). Two Elovl5 genes have

been characterised from Atlantic salmon, which are referred to as *Elovl5α* and *Elovl5b* (Morais *et al.* 2009). The Elovl5α and Elovl5b have similar activity but Elovl5α prefers n-3 PUFA substrates (Morais *et al.* 2009). The Atlantic salmon *Elovl5α* was more highly expressed in a range of tissues than *Elovl5b* and *Elovl2* (Morais *et al.* 2009). However, the Atlantic cod *Elovl5* was highly expressed in the brain and gill, with the lowest expression from the nine tissues studied in the liver (Tocher *et al.* 2006).

3.1.2 The ability of fish species to use the LCPUFA synthesis pathway

The freshwater food chain consists of C_{18} and C_{20} fatty acids found in microalgae, plants and insects (Sargent *et al.* 2002). As a generalization, freshwater fish are considered herbivorous or omnivorous. Freshwater fish in a natural habitat have a fillet n-3:n-6 ratio of 1-4:1 (de Souza *et al.* 2007). Freshwater fish are considered to have the ability to convert C_{18} PUFA to C_{20} and C_{22} LCPUFA using the LCPUFA synthesis pathway (Sargent *et al.* 2002). The marine food chain consists of C_{20} and C_{22} LCPUFA found in smaller fish, ultimately derived from phytoplankton and zooplankton at the base of the food chain (Sargent *et al.* 2002; Tocher 2003). Furthermore, many marine fish are considered carnivorous. Marine fish have a much higher n-3:n-6 ratio than freshwater fish (de Souza *et al.* 2007). As an evolutionary consequence of consuming an n-3 LCPUFA rich carnivorous diet, marine fish seem to have lost the ability to convert the limited dietary ALA to EPA and DHA (Sargent *et al.* 2002). This is probably due to one or more of the enzymes in the LCPUFA synthesis pathway not being well expressed, rather than being absent all together (Sargent *et al.* 2002). Similarly, top terrestrial predators such as the domestic cat and lion show limited Δ6desaturase activity, as an evolutionary response to their natural diet (Rivers *et al.* 1975; Rivers *et al.* 1976). Fish oil is essential in the diets of marine fish to satisfy the requirement for EPA and DHA (Sargent *et al.* 2002). The impact of replacing fish oils in marine fish aquaculture feeds with vegetable oils is currently being investigated with many species, in many countries. However, the impact of changing the dietary fatty acids is dependent on the functionality of the enzymes in the LCPUFA synthesis pathway, as well as on the PUFA composition of the diet.

The capacity of the LCPUFA synthesis enzymes varies from one marine fish species to the next (Agaba *et al.* 2005; Zheng *et al.* 2004; Zheng *et al.* 2009). The marine fish species of particular interest to us is SBT. SBT consume carnivorous diets due to their position at the top of the food chain. Their unique ability to selectively accumulate and retain a large quantity of DHA originating from their diet positions them in a different ecological niche to the fish species used in previous characterisation studies of fatty acyl desaturases and elongases (Saito *et al.* 1997; Mourente and Tocher 2003). Therefore, to elucidate the enzymatic regulation in the LCPUFA synthesis pathway this study aimed first of all to characterise the SBT fatty acyl desaturase and elongase genes.

3.2 Methods

3.2.1 Materials

All reagents used were obtained from Invitrogen™ Australia Pty. Ltd. (Mount Waverley, Vic, Aust.) unless otherwise stated. The PUFA supplemented to the yeast were obtained from Nu-Chek Prep, Inc. (MN, USA) or Cayman Chemical Company (Sapphire Bioscience, Australia). The pYES2 and pYES2/CT vectors were a kind gift from Prof Michael James and Dr Rebecca Cook-Johnson (Rheumatology Unit, Royal Adelaide Hospital, SA, Australia).

3.2.2 Bioinformatics and sequence analysis

All nucleotide and amino acid sequences were obtained from the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) database GenBank, unless otherwise stated. The Bioinformatics analysis tool BioManager version 2.0 provided by the Australian National Genomic Information Service (ANGIS, www.angis.org.au) was used to perform all sequence and bioinformatics analysis. Multiple sequence alignments were carried out using ClustalX 2.0 (www.clustal.org) and visualization based on colour shading was done with GeneDoc software (www.nrbsc.org/gfx/genedoc/).

Maximum parsimony (MP) analysis was conducted with phylogenetic analysis using parsimony (*and other methods) (PAUP*) version 4.0b 10 (Swofford 2003). MP analyses were performed with heuristic searches using 100 random sequence additions and tree bisection reconnection (TBR) branch swapping. Node support was assessed using 1000 bootstrap replicates. The choice of out-group was limited by the availability of fatty acyl elongase and desaturase sequences relating to the basal animals in terms of evolutionary progression of animal phylogeny in the GenBank database. Therefore, *M. alpina* and *C. elegans* were chosen to represent basal phyla.

3.2.3 Isolation of total RNA from SBT tissue

Mortars, pestles, spatulas and tweezers were soaked in 0.1% (v/v) diethyl pyrocarbonate (DEPC) (Sigma-Aldrich®, Australia) for 24 h to inactivate RNases. The DEPC solution was decanted and equipment was autoclaved at 121°C for 20 min. Autoclaving inactivated the DEPC by causing hydrolysis of diethyl pyrocarbonate, releasing carbon dioxide and ethanol. Plastic consumables, like pipette tips, were subjected to two autoclave cycles at 121°C for 20 min to inactivate RNases.

SBT liver, kidney, gonad and spleen tissue was obtained from a commercial farm located near Port Lincoln in SA on 19/04/07. The tissue was stored in RNA*later*® (Ambion, TX, USA) at -80°C until the RNA isolation was performed. One hundred mg of tissue was excised and excess RNA*later*® (Ambion) was blotted from the sample. The tissue was immersed in liquid nitrogen in a DEPC-treated mortar and pestle and ground to a fine powder. Total RNA was extracted from the ground tissue using the RiboPure™ Kit (Ambion), according to the manufacturer's protocol.

3.2.4 DNase treatment of total RNA

Total RNA was treated with RQ1 RNase-Free DNase (Promega, WI, USA) to degrade double-stranded and single-stranded DNA. Each total RNA extract was treated with 5 µl of RQ1 RNase-Free DNase and 20 µl of 10X reaction buffer H. The DNase treatment was allowed to proceed at 37°C for 30 min.

3.2.5 Clean up of DNase treated RNA

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich®) was added to the DNase-treated RNA and mixed. The sample was centrifuged at 17,000 *g* for 5 min. The top layer was transferred to a clean 1.5 mL microcentrifuge tube. One-tenth volumes of 3 M sodium acetate (Sigma-Aldrich®) and 2 volumes of 99% (v/v) molecular grade ethanol (Sigma-Aldrich®) were added and mixed by inversion. The sample was centrifuged at 8,000 *g* for 15 min. The supernatant was

then removed and the pellet resuspended in one volume of 70% (v/v) ethanol. The sample was centrifuged at 8,000 *g* for 15 min. This process of resuspending the pellet in 70% (v/v) ethanol and centrifuging was repeated. The supernatant was removed and the pellet was allowed to dry. The pellet was resuspended in 100 µl of nuclease-free H_2O. Samples were then run on a 1% (w/v) agarose gel to confirm the presence and integrity of the RNA. The RNA was aliquoted into small volumes to avoid repeated freeze-thawing and stored at -80°C.

3.2.6 Quantification of RNA/DNA

A Thermo Scientific NanoDrop^{TM} 1000 Spectrophotometer was used to assess RNA/DNA integrity and quality. Two µl of RNA/DNA was used to measure OD_{260} and OD_{280}. The concentration of RNA/DNA was determined using the formula below:

Concentration of RNA/DNA (ng μl^{-1}) = $OD_{260/280}$ * extinction coefficient (ng μl^{-1})

Extinction coefficient for nucleic acids (ng μl^{-1}):
Double-stranded DNA = 50
Single-stranded DNA = 33
RNA = 40

An $OD_{260}/_{280}$ ratio of 1.8 – 2.1 was desirable to ensure good RNA purity.

3.2.7 Primers

All primers were supplied by GeneWorks Pty. Ltd. (Thebarton, SA, Aust.). The primers were checked for dimer formation, hairpins, GC content and melting temperature using NetPrimer (www.premierbiosoft.com/netprimer) and the Sigma-Genosys DNA calculator (www.sigma-genosys.com/calc/DNACalc.asp). All primer sequences used for *Δ6desaturase* or *Elovl5* amplification can be seen in Table 3.1 and Table 3.2. The bases in italics represent the restriction enzyme sites and the bases which are underlined represent the start or stop codons. The bases which flank

the restriction enzyme sites ensure the restriction enzyme cuts the PCR product efficiently.

3.2.8 Polymerase chain reaction (PCR)

A Perkin Elmer GeneAmp® PCR System 2400 thermo cycler (Applied BioSystems, USA) was used for all PCR amplifications. PCR was performed in a volume of either 25 or 50 µl, in 0.2 mL thin wall clear domed cap tubes (Axygen® Scientific, Inc., CA, USA). PCR products were visualised via gel electrophoresis.

3.2.8.1 Clean up of PCR products

PCR products were excised from the agarose gel and gel purified using the Wizard® SV Gel and PCR Clean-Up System (Promega), according to the manufacturer's centrifugation protocol.

3.2.8.2 PCR screening transformed colonies

Half a colony was picked from the plate with a toothpick and swirled in a PCR tube containing 50 µl of sterile H_2O. The other half of the colony was used to inoculate 5 mL of LB medium (Appendix B.1) for growth overnight at 37°C and subsequent plasmid purification. The tube for PCR screening was vortex mixed to evenly suspend the bacteria. The cells were lysed at 99°C for 5 min. The cell debris was pelleted by centrifuging at 12,000 *g* for 1 min. The supernatant was used as template for PCR screening.

3.2.9 Quantitative real time polymerase chain reaction (qRT-PCR) using SBT tissues

Total RNA was isolated from SBT liver, kidney, gonad and spleen tissue as described in section 3.2.3. Total RNA (2 µg) was reverse transcribed into cDNA using SuperScript™ III Reverse Transcriptase according to the manufacturer's protocol, with the addition of 40 U RNaseOUT Ribonuclease Inhibitor. To identify

the exon-exon junctions in the *Elovl5* and *Δ6desaturase* sequences they were annotated against the genomic sequence of the pufferfish *Takigugu rubripes* and zebrafish, respectively, from the ENSEMBL database (www.ensembl.org). The primers were designed based on the full length sequences at intron-spanning regions to avoid amplification of genomic DNA. Target sequences, approximately 100 to 200 bp, were amplified using the following primers. *Elovl5* was amplified using the *Elovl5* F - 5'-CCACGCTAGCATGCTGAATA-3' and the *Elovl5* R - 5'-ACATCACGACATGGACGAAG-3' primers, while *Δ6desaturase* was amplified using the *Δ6des* F - 5'-TGGGCGGTGATTCTGTTTAA-3' and the *Δ6des* R - 5'-GGTGCTCGATCTGGAAGTTGAG-3' primers. Plasmid standards containing *Δ6desaturase* or *Elovl5* cDNAs were used at different concentrations (10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 copies) for absolute quantification. qRT-PCR was performed on a Rotor Gene RG-3000 (Corbett Research, Cambridge, UK) machine using Platinum® SYBR® Green qPCR SuperMix-UDG and 7.5 pmol of each primer in a 25 µl final volume. Each amplification was carried out in duplicate with a systematic negative control (no template control; containing no cDNA) and a no reverse transcriptase control (RNA samples were not reverse transcribed to cDNA). The PCR cycling used was a hold at 50°C for 2 min followed by 95°C for 2 min; 40 cycles of 95°C for 20 s, 58°C for 20 s, 72°C for 20 s; 1 cycle of 72°C for 20 s; melt curve from 72°C to 99°C. Threshold fluorescence was set using the automatic settings of the Rotor-Gene 6 program. Agarose gel electrophoresis of the PCR products and melt curve analysis was used to determine that a single specific product, generally devoid of primer dimers, was obtained. The *Δ6desaturase* and *Elovl5* amplicons were verified by sequencing.

3.2.9.1 Calculation of gene transcript levels

The absolute copy number of the target gene in each sample was calculated from the standard curve generated during each run.

3.2.10 Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described by Sambrook *et al.* (2001). Analytical grade agarose (Promega) was used to prepare 1% (w/v) gels in 1X TAE

buffer (Appendix B.4). The agarose was dissolved in a microwave. After allowing time for the liquefied agarose to cool, 0.6 µl of 10 mg mL^{-1} ethidium bromide per 10 mL of TAE was added. The agarose was poured into the gel tray and allowed to set. The gel systems used included the Mini-Sub Cell GT and Wide Mini-Sub Cell GT (BioRad Laboratories, Inc., CA, USA). The gel was submerged with 1X TAE buffer and the samples were loaded after mixing with 6X loading dye (Promega) in a 6:1 ratio. The gel was run at 100 V for approximately 30-40 min. The gel was visualised under ultra violet light on a BioRad Transilluminator and imaged using a BioRad DigiDoc®, powered by a Canon® digital camera and the Canon® Utilities Zoom Browser Ex Version 5.5 Software.

3.2.11 Nucleic acid sequencing

Sequencing was performed by the Australian Genome Research Facility (AGRF) (Brisbane, Australia) using Big Dye Terminator sequencing technology and capillary separation on an AB 3730*xl* 96-capillary sequencer. Purified DNA samples for sequencing were supplied according to the requirements of AGRF. Double stranded plasmid DNA was supplied at 400-1000 ng and the quantity of a PCR product was supplied according to its length, together with 6.4 pmol of the appropriate primer. The samples were mailed overnight at room temperature.

The results of the sequencing were analysed using Sequencher 4.1.4 software (Gene Codes, MI, USA).

3.2.12 Ligation of a PCR product into a plasmid

PCR products were ligated into the pGEM-T® Easy Vector (Promega), pYES2 or pYES2/CT vectors using 3 Weiss units of T4 DNA Ligase (Promega). The ligations were incubated overnight at 4°C.

3.2.13 Transformation of *E. coli* DH5α cells

Subcloning Efficiency™ DH5α™ Competent Cells were heat-shock transformed with purified plasmid or linear DNA, according to the manufacturer's protocol. Cells were spread plated on LB media plates (Appendix B.1) with antibiotic selection if appropriate. The plates were allowed to dry and then incubated overnight at 37°C. Following the overnight incubation the plates were stored at 4°C until required.

3.2.14 Glycerol stocks of bacterial cells

Glycerol stocks were prepared from overnight cultures of *E. coli* DH5α. Stocks were stored in 1.5 mL tubes and consisted of 800 µl of overnight culture and 200 µl of autoclaved 80% (v/v) glycerol. The stocks were stored at -80°C. For revival of bacterial cells, the glycerol stocks were scraped and streak plated onto a LB medium plate.

3.2.15 Plasmid DNA purification

Plasmid DNA was purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega), according to the manufacturer's centrifugation protocol.

3.2.16 Cloning of an internal fragment of the sbt*Elovl5* and sbt*Δ6des* cDNA

Within this section, the cloning of an internal fragment of the sbt*Elovl5* cDNA was performed by Valene H.L. See.

An internal fragment of the sbt*Elovl5* and sbt*Δ6des* cDNA was cloned using reverse transcriptase-polymerase chain reaction (RT-PCR). First strand cDNA was synthesised using 0.3 µg of total RNA with 4 U Omniscript™ RT (Qiagen, Australia), according to the manufacturer's protocol. The cDNA was stored at -20°C for up to 3 months.

PCR primers to amplify an internal fragment of the sbt*Elovl5* cDNA were designed based on an alignment of Atlantic cod (AY660881), Atlantic salmon (AY170327), cherry salmon (DQ067616), rainbow trout (AY605100), turbot (AF465520) and zebrafish (AF532782) fatty acyl elongase sequences. The PCR amplification of the sbt*Elovl5* cDNA used primer set A (Table 3.1). PCR primers to amplify two internal overlapping fragments of the sbt*Δ6des* cDNA were designed based on an alignment of Atlantic cod (DQ054840), Atlantic salmon (NM_001123575), cherry salmon (AB074149), rainbow trout (NM_001124287), turbot (AY546094), Gilthead sea bream (AY055749), Nile tilapia (AB069727), zebrafish (AF309556) and carp (AF309557) *Δ6desaturase* sequences. The PCR amplification of the sbt*Δ6des* cDNA was performed using primer sets A and B (Table 3.2).

The amplification of the sbt*Elovl5* cDNA was performed with Platinum® *Taq* DNA Polymerase High Fidelity and an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 68°C for 1 min, followed by a final extension at 72°C for 5 min. The amplification of the sbt*Δ6des* cDNA was performed with Bioneer *Pfu* DNA polymerase (Pacific Laboratory Products Pty. Ltd., Australia) and an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C (primer set A) or 54°C (primer set B) for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. Primer sets A and B were designed with an overlapping region of ≈120 bp.

The gel purified PCR products (section 3.2.8.1) were cloned into the pGEM®-T Easy Vector (Promega) (section 3.2.12). Positive colonies were selected (section 3.2.8.2), plasmid purified (section 3.2.15) and sequenced (section 3.2.11).

3.2.17 Cloning of the 3' and 5' ends of the sbt*Elovl5* cDNA

The cloning of the 3' and 5' ends of the sbt*Elovl5* cDNA was performed by Valene H.L. See. The 3' and 5' ends of the sbt*Elovl5* cDNA were cloned using 3' and 5' rapid amplification of cDNA ends (RACE)-PCR with the SMART™ RACE cDNA Amplification Kit and the Advantage® 2 PCR Kit (Clontech, CA, USA). The primers for RACE-PCR (Table 3.1, primer set B) were designed based on the known

sequence of the internal fragment of the sbt*Elovl5* cDNA. RACE-PCR cycling conditions were 5 cycles of 94°C for 30 s and 72°C for 2 min, followed by 5 cycles of 94°C for 30 s, 70°C for 30 s and 72°C for 2 min, followed by 30 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 2 min.

The gel purified RACE-PCR products (section 3.2.8.1) were cloned into the pGEM®-T Easy Vector (Promega) (section 3.2.12). Positive colonies were selected (section 3.2.8.2), plasmid purified (section 3.2.15) and sequenced (section 3.2.11). The sequence of the sbt*Elovl5* cDNA was assembled by aligning the sequences of the RT-PCR and 3' and 5' RACE-PCR products.

3.2.18 Cloning of the 3' and 5' ends of the sbt*Δ6des* cDNA

The SMART™ RACE cDNA Amplification Kit (Clontech) and M-MLV Reverse Transcriptase RHase H Minus, Point Mutant (Promega) were used for first strand cDNA synthesis, using 1 µg total RNA, for 3' and 5' RACE of the sbt*Δ6des.* The sbt*Δ6des* 5' RACE GSP1 (Table 3.2) was used during the synthesis of 5' RACE ready cDNA, rather than the 5' CDS primer A. The 3' RACE ready cDNA was synthesised with the 3' CDS primer A. Synthesis was performed according to the manufacturer's protocol, with the exception of adding 5 µl M-MLV RT 5X reaction buffer, 20 mM dNTP (Promega), 200 U M-MLV Reverse Transcriptase RHase H Minus, Point Mutant (Promega) and 40 U RNaseOUT Ribonuclease Inhibitor, in a final reaction volume of 25 µl. RNaseOut prevents RNA degradation by RNases during cDNA synthesis. Following cDNA synthesis, any untranscribed RNA was removed by treating with RNase H. The cDNA was incubated at room temperature for 15 min with 20 µg mL^{-1} of RNase H. The cDNA was cleaned up to remove short cDNA fragments, primers, dNTPs and enzymes used during synthesis (section 3.2.8.1). The cDNA was stored at -20°C for up to 3 months.

The 3' and 5' ends of the sbt*Δ6des* cDNA were cloned using 3' and 5' RACE-PCR with the SMART™ RACE cDNA Amplification Kit and the Advantage® 2 PCR Kit (Clontech). The primers for RACE-PCR (Table 3.2, primer sets C and D) were designed based on the known sequence of the internal fragment of the sbt*Δ6des* cDNA. The first round 3' RACE was performed with 3' RACE ready cDNA and

sbt*Δ6des* 3' RACE GSP1. Second round (nested) 3' RACE was performed with a 1:50 dilution of the template produced during first round 3' RACE and sbt*Δ6des* 3' RACE GSP2. Third round (nested) 3' RACE was performed with a 1:50 dilution of the template produced during second round 3' RACE and sbt*Δ6des* 3' RACE GSP3. First round 5' RACE was performed with 5' RACE ready cDNA and sbt*Δ6des* 5' RACE GSP2. Second round (nested) 5' RACE was performed with a 1:2 dilution of the template produced during first round 5' RACE and sbt*Δ6des* 5' RACE GSP3.

RACE-PCR cycling conditions for first round PCR were 5 cycles of 94°C for 30 s and 72°C for 2 min, followed by 5 cycles of 94°C for 30 s, 70°C for 30 s and 72°C for 2 min, followed by 30 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 2 min. RACE-PCR cycling conditions for second and third round (nested) PCR were 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 2 min, followed by 72°C for 5 min.

The gel purified RACE-PCR products (section 3.2.8.1) were cloned into the pGEM®-T Easy Vector (Promega) (section 3.2.12). Positive colonies were selected (section 3.2.8.2), plasmid purified (section 3.2.15) and sequenced (section 3.2.11). The sequence of the sbt*Δ6des* cDNA was assembled by aligning the sequences of the RT-PCR and 3' and 5' RACE-PCR products.

3.2.19 Cloning of the full length sbt*Elovl5* and sbt*Δ6des* cDNAs

The full length sbt*Elovl5* and sbt*Δ6des* cDNAs were cloned from the SBT liver tissue RNA using RT-PCR. First strand cDNA was synthesised using 1 μg of total RNA with M-MLV Reverse Transcriptase RHase H Minus, Point Mutant (Promega), according to the manufacturer's protocol, with the addition of 40 U RNaseOUT Ribonuclease Inhibitor. The cDNA was stored at -20°C for up to 3 months.

PCR amplification of the full length sbt*Elovl5* and sbt*Δ6des* cDNA was performed using sbt*Elovl5* primer set C (Table 3.1) or sbt*Δ6des* primer set E (Table 3.2) and Platinum® *Taq* DNA Polymerase High Fidelity (0.025 U). An initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C (sbt*Elovl5*) or 56°C (sbt*Δ6des*) for 30 s, and extension at 68°C for

1.5 min (sbt*Elovl5*) or 2 min (sbt*Δ6des*), followed by a final extension at 68°C for 5 min was used.

The gel purified sbt*Elovl5* and sbt*Δ6des* PCR products (section 3.2.8.1) were cloned into the pGEM®-T Easy Vector (Promega) (section 3.2.12). Positive colonies were selected (section 3.2.8.2), plasmid purified (section 3.2.15) and sequenced (section 3.2.11).

3.2.20 Site directed mutagenesis of the sbt*Δ6des* translation initiation sequence

Site directed mutagenesis (SDM) was used to change two bases in the sequence preceding the start codon of the sbt*Δ6des,* termed the sbt*Δ6des* translation initiation sequence. Complementary primers with a minimum of ten base pairs either side of the introduced mutation were designed. PCR amplification was performed using the pYES2-sbt*Δ6des* construct (≈ 100 ng) with primer set G (Table 3.2) (100 ng of each) and Finnzymes Phusion® hot start high-fidelity DNA polymerase (1U) (New England BioLabs® Inc., Australia). Within the 50 µl reaction there was also 5X Phusion HF buffer, 1 µl 10 mM dNTP and 1.5 µl DMSO. An initial denaturation step at 98°C for 30 s, followed by 25 cycles of 98°C for 10 s and 72°C for 4 min, followed by 72°C for 5 min was used. The PCR product, sbt*Δ6des*SDM, was visualised by gel electrophoresis before the PCR product was cleaned up (section 3.2.8.1). The methylated pYES2-sbt*Δ6des* was incubated with DpNI (60 U) (New England BioLabs® Inc.) at 37°C for 4 h followed by 80°C for 20 min, leaving the newly synthesized unmethylated sbt*Δ6des*SDM. The PCR product was confirmed by visualisation with gel electrophoresis once more. Approximately 50 ng of sbt*Δ6des*SDM was transformed into *E. coli* DH5α (section 3.2.13). Positive colonies were selected (section 3.2.8.2), plasmid purified (section 3.2.15) and sequenced (section 3.2.11).

3.2.21 Maintaining *S. cerevisiae* INVSc1 cells

Yeast extract peptone dextrose (YPD) broth or agar (Sigma-Aldrich®) (Appendix B.2) was used to culture *S. cerevisiae* INVSc1 cells. The cells were grown in YPD medium at 30°C with shaking (250 rpm). The growth on YPD plates was observed after 2 days at 30°C.

3.2.22 Glycerol stocks of *S. cerevisiae* INVSc1 cells

Glycerol stocks of untransformed or transformed *S. cerevisiae* INVSc1 cells were prepared from cells which were streak plated on YPD or synthetic minimal defined medium for yeast without uracil (SC-U) plates (Appendix B.3), respectively, and incubated for 2 days at 30°C. The cells were scraped from the plate and resuspended in 800 µl of YPD medium or SC-U medium containing 2% (w/v) raffinose and 200 µl of autoclaved 80% (v/v) glycerol in 1.5 mL tubes. The stocks were stored at -80°C. For revival of *S. cerevisiae* INVSc1 cells, the glycerol stocks were scraped and streak plated onto a YPD or SC-U plate.

3.2.23 Preparing competent *S. cerevisiae* INVSc1 cells

The *S. c.* EasyComp™ Transformation Kit was used to prepare competent *S. cerevisiae* INVSc cells. Ten mL of YPD medium was inoculated with a single *S. cerevisiae* INVSc1 colony and grown overnight at 30°C with shaking (250 rpm). The overnight culture was used to inoculate 10 mL of fresh YPD medium to obtain a final OD_{600} of 0.3. The culture was grown at 30°C with shaking (250 rpm) until the OD_{600} was 0.6-1.0. The cells were centrifuged at 500 *g* for 5 min at room temperature and the pellet was resuspended in 10 mL of Solution I. This was followed by the cells being centrifuged at 500 *g* for 5 min at room temperature and the pellet was resuspended in 1 mL of Solution II. Competent *S. cerevisiae* INVSc1 cells were stored at -80°C.

3.2.24 Transformation of competent *S. cerevisiae* INVSc1 cells

The *S. c.* EasyComp™ Transformation Kit was used to transform 50 µl of competent *S. cerevisiae* INVSc1 cells with 1 µg of pYES2 or pYES2/CT vector DNA. The transformation reaction was vortex mixed with 500 µl of Solution III and incubated at 30°C for 1 h, mixing every 15 min. SC-U plates, containing 2% (w/v) glucose as the carbon source, were used to culture *S. cerevisiae* INVSc1 cells transformed with the pYES2 or pYES2/CT vector. Transformed colonies exhibiting uracil prototrophy grew after 2 days at 30°C.

3.2.25 Maintaining transformed *S. cerevisiae* INVSc1 cells

S. cerevisiae INVSc1 cells transformed with pYES2 or pYES2/CT were maintained in SC-U medium, containing 2% (w/v) raffinose as the carbon source. Raffinose neither represses nor activates transcription from the *GAL1* promoter.

3.2.26 Extraction of genomic DNA from *S. cerevisiae* INVSc1 cells

Genomic DNA was extracted from yeast pellets stored at -80°C. The yeast cells were lysed with either Pierce Y-PER Reagent (Thermo Scientific Inc., USA) or lysis buffer containing lyticase.

3.2.26.1 S. cerevisiae cell lysis with Y-PER Reagent

Pierce Y-PER Reagent (Thermo Scientific Inc.) (8 µl mg^{-1} pellet) was vortex mixed with the cells. The cell mixture was incubated at 65°C for 10 min followed by centrifugation at 13,000 *g* for 5 min. The supernatant was discarded and the cells were treated with 500 µl of TE buffer (100 mM Tris pH 8, 50 mM EDTA pH 8), 35 µl of 20% (w/v) SDS and 20 µl of Proteinase K (20 mg mL^{-1}). The cells were incubated for 5 min at 65°C. The lysate was treated with 130 µl of 5 M potassium acetate (Sigma-Aldrich®) and incubated on ice for 5 min. After centrifugation at 15,000 *g* for 10 min, the supernatant was transferred to a new tube and 640 µl of isopropyl alcohol (Sigma-Aldrich®) and 60 µl of 3 M sodium acetate (Sigma-

Aldrich®) were added. The samples were incubated at -20°C for 10 min followed by centrifugation at 15,000 *g* for 10 min. The pellet was washed with 300 µl of 70% (v/v) molecular grade ethanol (Sigma-Aldrich®) and centrifuged at 15,000 *g* for 5 min. The ethanol was removed and the pellet was allowed to dry. The genomic DNA pellet was resuspended in 50 µl of H_2O.

3.2.26.2 S. cerevisiae cell lysis with lyticase

One mL of freshly prepared lysis buffer, containing 1 M sorbitol pH 7.4, 0.1 M EDTA pH 7.4, 1 µl β-mercaptoethanol, and 50 U of lyticase per 10^7 cells was vortex mixed with the cells. The cell mixture was incubated at 30°C for 1 h followed by centrifugation at 13,000 *g* for 5 min. The supernatant was discarded and the cells were treated with 500 µl of TE buffer (100 mM Tris pH 8, 50 mM EDTA pH 8) and 35 µl of 20% (w/v) SDS. The cells were incubated for 30 min at 65°C. The lysis of the cells proceeds the same as outlined in section 3.2.26.1 when potassium acetate was added.

3.2.27 Extraction of RNA from *S. cerevisiae* INVSc1 cells

RNA was extracted from <5 x 10^7 fresh *S. cerevisiae* cells. The cells were harvested by centrifugation at 1,000 *g* for 5 min at 4°C and all medium was removed. The pellet was resuspended in 2 mL of freshly prepared lysis buffer as outlined in section 3.2.26.2. The cells were incubated for 15-30 min at 30°C with gentle shaking to generate spheroplasts. The spheroplasts were pelleted by centrifugation at 300 *g* for 5 min. The supernatant was discarded and the RNeasy Mini Kit (Qiagen) was used according to the manufacturer's protocol for extraction of RNA from yeast cells.

3.2.28 Extraction of total protein from *S. cerevisiae* INVSc1 cells

Total protein was extracted from yeast pellets (5 mL cultures) stored at -80°C. The buffer used for lysing the yeast cells contained 50 mM Tris base pH 8, 100 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol and 0.25 mM Triton X-100. The cell pellet was resuspended in 500 µl of lysis buffer. The addition of 1 mM of PMSF,

Benzamide, Benzamidine-HCl (protease inhibitor cockatil) and 1 mM of DTT (reducing agent) followed. Approximately 400 µl of pre-washed glass beads in lysis buffer was added and the cell suspension vortex mixed for 2 min. The cell debris and glass beads were pelleted at 16,000 *g* for 10 min at 4°C. The supernatant was loaded onto a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel for separation of total proteins or concentrated as outlined in section 3.2.29.

3.2.29 Precipitation of proteins with methanol/chloroform

Protein was extracted from yeast cells as outlined in section 3.2.28. The resulting supernatant (approximately 400 µl) was transferred to a sterile 1.5 mL tube and 400 µl of methanol was added. The sample was centrifuged briefly at 1,000 *g* for 30 s, followed by the addition of 100 µl of chloroform. Brief centrifugation followed once more at 1,000 *g* for 30 s and then 300 µl of H_2O was added. The aqueous and organic layers were separated by centrifugation at 15,000 *g* for 2 min. The aqueous layer was removed and 300 µl of methanol was added to the organic layer. The sample was vortex mixed and centrifuged for a further 2 min at 15,000 *g* to pellet the protein. The supernatant was removed and the pellet washed with 400 µl of methanol. Centrifugation at 15,000 *g* for 2 min was used to re-pellet the protein. The methanol was removed and the pellet was allowed to dry. The pellet was resuspended in SDS-PAGE sample buffer for separation by SDS-PAGE gel electrophoresis.

3.2.30 Heterologous expression of the sbt*Elovl5* and sbt*Δ6des* ORFs in *S. cerevisiae*

3.2.30.1 Cloning the sbtElovl5 and sbtΔ6des into pYES2

The sbt*Elovl5* and sbt*Δ6des* sequences were subject to *in silico* restriction enzyme (RE) digestion to determine suitable RE for cloning into the expression vector, pYES2. PCR was used to introduce an *Eco*RI restriction site upstream of the putative translation initiation codon and a *Not*I restriction site downstream of the putative translation termination codon in the sbt*Elovl5* and sbt*Δ6des* cDNAs. Two putative translational start sites were found in the sbt*Elovl5* cDNA. The second putative start

codon, 42 bases downstream of the first putative start codon, conformed to the canonical Kozak sequence (Kozak 1996), but the first did not. The sbt*Elovl5* and sbt*Elovl5-42* forward primers were designed to span each of the putative translational start sites, respectively. PCR amplification was performed using the pGEM®-T Easy Vector-sbt*Elovl5* construct and the primer sets listed in primer set D (Table 3.1) or the pGEM®-T Easy Vector-sbt*Δ6des* construct and primer set F (Table 3.2) and Platinum® *Taq* DNA Polymerase High Fidelity. An initial denaturation of 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 57°C (sbt*Elovl5*/sbt*Elovl5-42*) or 58°C (sbt*Δ6des*) for 30 s and 68°C for 1.5 min (sbt*Elovl5*/sbt*Elovl5-42*) or 2 min (sbt*Δ6des*), followed by a final extension of 68°C for 5 min was used.

3.2.30.1.1 Restriction digestion

The gel purified sbt*Elovl5*, sbt*Elovl5-42* and sbt*Δ6des* PCR products (section 3.2.8.1) and the pYES2 vector were restricted with *Eco*R1 and *Not*1, using Buffer H (Promega). A 5-fold excess of enzyme over DNA was used in each reaction. The digestion was incubated for 3 h at 37°C. The digested pYES2 vector and the PCR fragments were cleaned up (section 3.2.8.1).

3.2.30.1.2 Shrimp alkaline phosphatase treatment of digested vector

The exposed 5' phosphate on the digested pYES2 vector was removed by shrimp alkaline phosphatase (SAP) (1U μg^{-1} plasmid DNA) (Promega). This treatment prevented re-circularization of the linearized plasmid. The incubation was for 15 min at 37°C, followed by SAP inactivation for 15 min at 65°C.

Ligation of the restriction enzyme treated sbt*Elovl5*, sbt*Elovl5-42* and sbt*Δ6des* PCR products into the pYES2 vector was performed (section 3.2.12). An insert:vector ratio of 3:1 was used. The resulting constructs, pYES2-sbt*Elovl5*, pYES2-sbt*Elovl5-42* and pYES2-sbt*Δ6des*, were transformed into *E. coli* DH5α (section 3.2.13). Putative transformants were selected using 100 $\mu g\ mL^{-1}$ of ampicillin and they were confirmed using PCR screening with the pYES2 specific forward 5'-CTGGGGTAATTAATCAGCGAAGCG-3' and reverse 5'-

CGTGACATAACTAATTACATGATGC-3' primers. Confirmed positive colonies were selected (section 3.2.8.2), plasmid purified (section 3.2.15) and sequenced (section 3.2.11) using the pYES2 primers.

3.2.30.2 Cloning the sbtElovl5 and sbtΔ6des into pYES2/CT

PCR amplification was performed using the pYES2-sbt*Elovl5* construct with the forward primer from primer set D and the reverse primer E (Table 3.1), the pYES2-sbt*Δ6des* construct with the forward primer from primer set F and the reverse primer H (Table 3.2) or the pYES2-sbt*Δ6des*SDM construct with the forward primer from primer set G and the reverse primer H (Table 3.2) and Platinum® *Taq* DNA Polymerase High Fidelity. An initial denaturation of 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 56°C for 30 s and 68°C for 2 min, followed by a final extension of 68°C for 5 min was used.

The method for cloning the PCR products into pYES2/CT was the same as outlined in section 3.2.30.1 for pYES2.

3.2.30.3 Expression of recombinant protein in S. cerevisiae INVSc1 cells

Functional characterisation was done by expressing the sbt*Elovl5*, sbt*Elovl5-42*, sbt*Δ6des* and sbt*Δ6des*SDM ORFs in *S. cerevisiae.* The cultures were grown at 30°C for 2 days with shaking (200 rpm). This starter culture was used to obtain a final OD_{600} of 0.4 in 10 mL of induction medium. The required amount of starter culture was centrifuged at 1,500 *g* for 5 min at room temperature. The supernatant was decanted and the cell pellet resuspended in 5 mL of induction medium. The induction medium contained 2% (w/v) galactose as the carbon source which induced gene expression from the *GAL* promoter. The resuspended cells were used to inoculate 10 mL of induction medium. PUFA substrates were added at a concentration of 500 µM and cultures were grown at 30°C with shaking (200 rpm). Recombinant yeast were grown in SC-U medium containing raffinose and supplemented with PUFA substrates for uninduced controls. Approximately the same number of yeast cells was harvested from each culture by determining the OD_{600}. Cells with an OD_{600} of 1 (≈ 2 x 10^7 cells mL^{-1}) were harvested. The cells were pelleted by centrifugation at 1,500 *g*

for 5 min and the cell pellets were washed twice with H_2O. The cell pellets were stored at -80°C in 1 mL of H_2O until fatty acid analysis was performed.

3.2.30.4 Supplementing fatty acids to S. cerevisiae expressing sbtElovl5, sbtElovl5-42, sbtΔ6des or sbtΔ6desSDM

Recombinant *S. cerevisiae* cells transformed with sbt*Elovl5* were supplemented with 500 μM of 18:4n-3 to establish the best length of time for substrate incorporation and product conversion, for all subsequent experiments with all other PUFA substrates. Cells were harvested from induced and uninduced cultures every 12 h, over a period of 96 h in total. Subsequently, recombinant yeast containing sbt*Elovl5* or sbt*Elovl5-42* were individually supplemented with 500 μM of one of the following PUFA substrates: 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 or 22:4n-6. Multiple PUFA substrate supplementations of 500 μM in total or 500 μM of each of 18:4n-3 and 18:3n-6, 20:5n-3 and 20:4n-6 or 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6 were also supplied to *S. cerevisiae* cells transformed with sbt*Elovl5*. Cells were harvested for analysis after 24 h of fatty acid supplementation.

Recombinant *S. cerevisiae* cells transformed with sbt*Δ6des* or sbt*Δ6des*SDM were supplemented with 500 μM of 18:3n-3, 18:2n-6, 20:3n-6, 20:5n-3 or 22:5n-3.

Recombinant *S. cerevisiae* cells transformed with the pYES2 empty vector were supplemented with 500 μM of 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 or 22:4n-6.

3.2.30.5 Supplementing metal cofactors and fatty acids to S. cerevisiae expressing sbtΔ6des or sbtΔ6desSDM

Recombinant *S. cerevisiae* transformed with sbt*Δ6des* or sbt*Δ6des*SDM were supplemented with 100 μM of iron sulphate and 100 μM of copper sulphate. Recombinant yeast were cultured in the presence of these metal cofactors and 500 μM of 18:3n-3 or 20:3n-6.

3.2.31 Fatty acid analysis

3.2.31.1 Extraction of total lipid from SBT liver tissue

Extraction performed as outlined in section 2.2.8.1.

3.2.31.2 Extraction of total lipid from S. cerevisiae cells

Total lipid was extracted basically according to the method in section 2.2.8.1. Exceptions to the method for extraction from *S. cerevisiae* included using 2 mL of isopropanol instead of 3 mL of methanol and 4 mL of chloroform rather than 6 mL. After the addition of isopropanol, 80 µl of 0.4 mg mL^{-1} $C_{17:0}$ free fatty acid (FFA) internal standard (Sigma-Aldrich®) was added. After the addition of chloroform to the samples they were allowed to stand for 1 h at room temperature or overnight at 4°C.

3.2.31.3 Extraction of total lipid from SC-U medium

Total lipid was extracted from SC-U medium basically according to the method in section 2.2.8.1. Two mL of medium was added to a tube and the weight recorded. This was followed by the addition of 0.5 mL of cold saline, 2 mL of methanol and 80 µl of 0.4 mg mL^{-1} $C_{17:0}$ FFA internal standard (Sigma-Aldrich®). The only other exception to the method was the addition of 4 mL of chloroform rather than 6 mL.

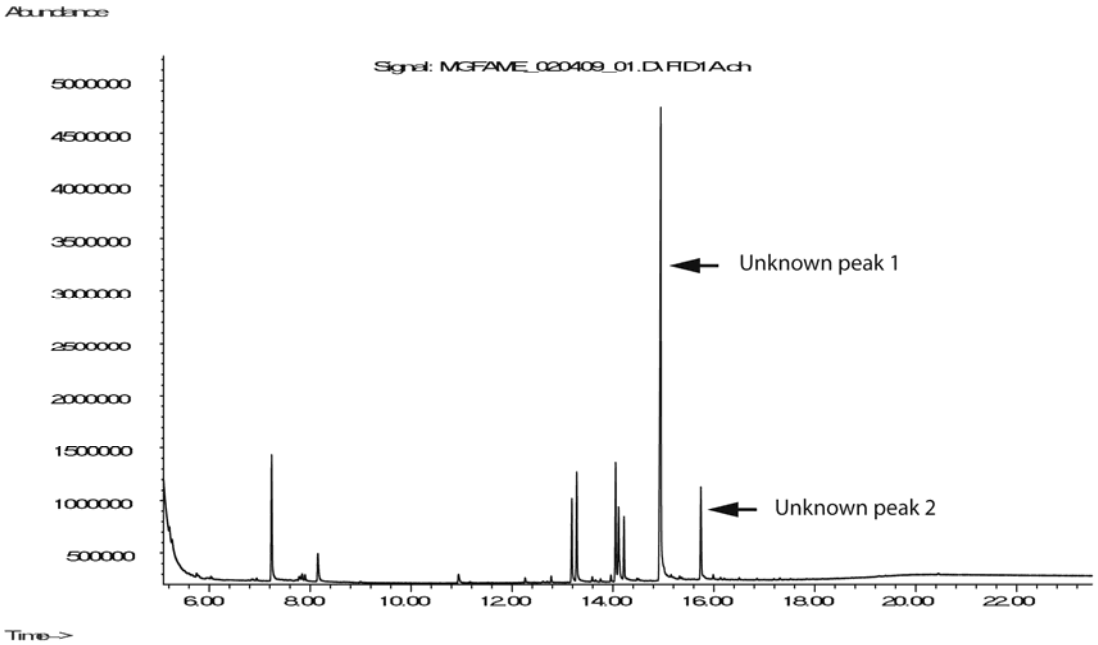
3.2.31.4 Analysis of FAME by gas chromatography

FAME were analysed by gas chromatography as outlined in section 2.2.8.4. When *S. cerevisiae* cells containing the heterologous sbt*Elovl5* were supplemented with DPA, peaks beyond the retention time of DHA were expected. The column temperature was held at 220°C for 8 min when C_{24} fatty acid products were expected. Also, the additional lipid standards 24:5n-3 and 24:6n-3 were used (Larodan Fine Chemicals, Sweden).

FAME from the *S. cerevisiae* cells supplemented with 18:4n-3 were sent to Flinders Advanced Analytical Laboratory (FAAL), Flinders University for analysis by gas chromatography-mass spectrometry (GC-MS). The mass spectra of the unknown peaks, which were identified as 20:4n-3 and 22:4n-3, can be seen in Figure 3.1.

The proportion of substrate fatty acid converted to longer chain fatty acid product was calculated as [% product/(% product + % substrate)] x 100.

A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Signal: MGFAME_020409_01.D\FID1A.ch
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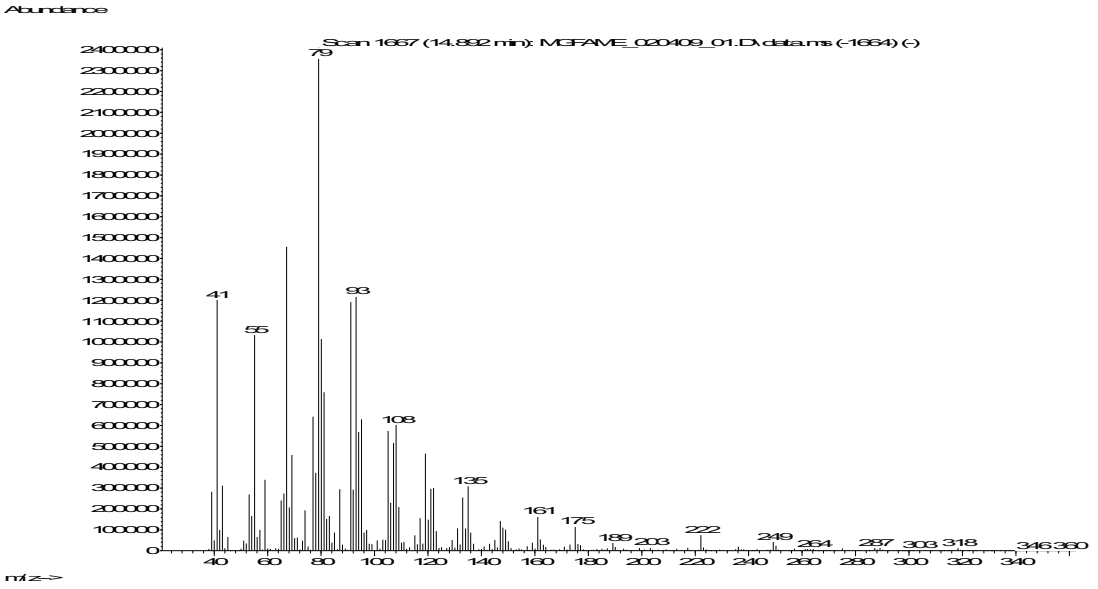
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
Unknown peak 1
Unknown peak 2
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
Time->

B


Abundance
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55
93
108
135
161
175
189
203
222
249
264
287
303
318
346
360
m/z-->

C

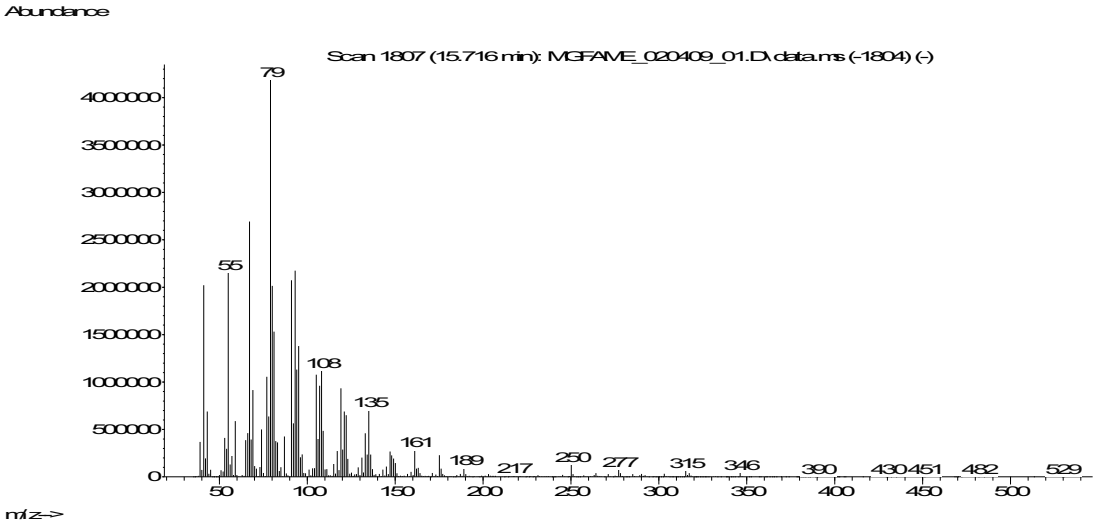


Figure 3.1 Gas chromatography-mass spectrometry analysis of unknown fatty acid methyl esters in the lipid extracted from *S. cerevisiae* cells. Cells were supplemented with 500 μM 18:4n-3. (A) Gas chromatogram of *S. cerevisiae* cell total lipid containing two unknown peaks at 14.95 min and 15.74 min respectively. (B) Mass spectrum of unknown peak 1, subsequently identified as 20:4n-3. (C) Mass spectrum of unknown peak 2, subsequently identified as 22:4n-3.

3.2.32 Western Blotting

3.2.32.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The glass plates used for pouring the SDS-PAGE gel were assembled to form a gel sandwich and secured in the pouring stand (BioRad). A 10% (v/v) resolving gel (Appendix B.5) was poured into the gel sandwich (approximately 3.5 cm from the top of the smaller glass plate) and overlaid with iso-propanol to allow the gel to polymerize. Once the gel was set, the iso-propanol was removed and the gel rinsed with distilled H_2O. A 4% (v/v) stacking gel (Appendix B.6) was poured, the comb was inserted and the gel was allowed to polymerize. Once the stacking gel was set, the comb was removed and the wells were flushed with SDS-PAGE running buffer. The gel was transferred to the SDS-PAGE tank and the tank was filled with running buffer (Appendix B.7).

3.2.32.2 Protein sample preparation for SDS-PAGE

Total protein was extracted from *S. cerevisiae* cells and precipitated as described in section 3.2.28 and section 3.2.29. Protein was extracted from the same amount of cell pellet each time. Ten µl of the total protein extract was mixed with 20 µl of SDS-PAGE sample buffer (Appendix B.8). The precipitated protein pellet (section 3.2.29) was resuspended in the necessary volume of SDS-PAGE sample buffer. The samples were incubated at 100°C for 5 min prior to loading on the SDS-PAGE gel. The SDS-PAGE gel was run at 170 Volts for approximately 1 h or until the Prestained SDS-PAGE Standards, Broad Range (BioRad) were sufficiently separated.

3.2.32.3 Protein transfer

The electrophoresed proteins were transferred from the gel onto BioTrace™ NT nitrocellulose transfer membrane (Pall Corporation, USA). The fibre pad, filter paper (3 MM chromatography paper) (Whatman International Ltd., Kent, UK), SDS-PAGE gel, nitrocellulose membrane (Pall Corporation), filter paper (Whatman International Ltd.) and fibre pad were assembled in order in the western blot transfer cage (BioRad) while submerged in western blot transfer buffer (Appendix B.9). The

western blot transfer cage was transferred to the western blot tank which was filled with transfer buffer. The transfer was conducted at 0.2 Amps for 1 h.

3.2.32.4 Blotting and immunodetection

All washes and incubations were done at room temperature on an orbital shaker. The membrane was washed twice for 10 min each with TBS buffer (Appendix B.10), followed by an incubation at 1 h in blocking buffer (Appendix B.11). The membrane was then washed twice for 10 min each with TBS-Tween/Triton (Appendix B.12), followed by one wash for 10 min with TBS. The primary antibody incubation was performed overnight. The rabbit-anti-6X His tag (Rockland Immunochemicals, Inc., PA, USA) and rabbit-anti-V5 epitope tag (Rockland Immunochemicals, Inc.) were diluted 1:1000 and 1:2500, respectively, in blocking buffer. The following day the membrane was washed twice for 10 min each with TBS-Tween/Triton, followed by one wash for 10 min with TBS. The secondary antibody incubation was for 1 h. The membranes which were incubated with the His and V5 primary antibodies were both incubated with goat-HRP-anti-rabbit IgG (Rockland Immunochemicals, Inc.) diluted 1:2000 or 1:5000, respectively, in TBS buffer supplemented with 10% (w/v) non-fat dried milk powder. The membrane was then washed four times for 10 min each with TBS-Tween/Triton.

Western blots were developed using the SuperSignal® West Pico Chemiluminescence Substrate kit (Thermo Scientific Inc., USA). The membrane was incubated for 5 min on an orbital shaker with 1 mL of both the SuperSignal® West Pico Luminol Enhancer and Stable Peroxide solution, covered with foil. The membrane was then placed in a cassette containing a sheet of X-OMAT film (Kodak®, Australia) for an exposure time of 10 min or 2 h. The film was then developed using an X-OMAT 1000 Processor (Kodak®) with fixer and developer reagents (Kodak®).

3.2.33 Statistical analysis

When required, one-way ANOVA was performed according to section 2.2.11.

Table 3.1 Primers used for amplifying the sbt*Elovl5* cDNA and ORF

Primer Set	Primer Name	Sequence (5' → 3')
A	sbt*Elovl5* F	GGT CTA CAA TCT GGG CCT C
	sbt*Elovl5* R	CCA CCA TAG ATA GGG CCG
B	sbt*Elovl5* 3' RACE	CAC ATC TAC CAC CAC GCT AGC ATG C
	sbt*Elovl5* 5' RACE	CTT CCA CCA AAG ATA CGG CCG CAT G
C	sbt*Elovl5* Full Length F	GGT TAC ACA GCC GCG TTC TCC G
	sbt*Elovl5* Full Length R	GGA TAT ATG GGG CTA TGG CTT ATT TCA
D	sbt*Elovl5 Eco*R1 F	GCG *GAA TTC* AGG TGA CAA <u>ATG</u> GAG ACT TTC AAT
	sbt*Elovl5-42 Eco*R1 F	GCG *GAA TTC* CTT AGA AAC <u>ATG</u> GAT GGG TCC CAG
	sbt*Elovl5 Not*1 R	GAC T*GC GGC CGC* TCT CAA ATG <u>TCA</u> ATC CAC CCG CAG
E	sbt*Elovl5 Not*1 pYES2CT R	TC TC*G CGG CCG C*CC ATC CAC CCG CAG TTT CTT ATG

Restriction enzyme sites are indicated by italics and the start or stop codons are underlined.

Table 3.2 Primers used for amplifying the sbt*Δ6des* cDNA and ORF

Primer Set	Primer Name	Sequence (5' → 3')
A	sbt*Δ6des* F1	TAC ACC TGG GAG GAG GTG CAG
	sbt*Δ6des* R1	GTG TTG GTG ATG GTA GGG C
B	sbt*Δ6des* F2	TTC CAG CAT CAC GCT AAA CCC
	sbt*Δ6des* R2	GGA GAT AAG CAT CCA GCC A
C	sbt*Δ6des* 3' RACE GSP1	CAT GCA GAT CGA CTA TGA GAA GCA CCA GG
	sbt*Δ6des* 3' RACE GSP2	CCT TCA ACG ACT GGT TCA GCG GAC AC
	sbt*Δ6des* 3' RACE GSP3	CCT CAA CTT CCA GAT CGA GCA CCA CCT C
D	sbt*Δ6des* 5' RACE GSP1	GCT CTG TTG CTG CCA GCT CTC CAA TCA G
	sbt*Δ6des* 5' RACE GSP2	GCG TAG TGG CTG ATG ACC CGA AAC CC
	sbt*Δ6des* 5' RACE GSP3	ACC TTC CGA TCG ATG ACC AAC CAC TG
E	sbt*Δ6des* Full Length F	GTG TTA AAG TTT GTG GCT GAC TGG C
	sbt*Δ6des* Full Length R	AAA ATC CTG TGA GCA CTA TAA AAA TCC TG
F	sbt*Δ6des Eco*R1 F	GCG *GAA TTC* ACA GTG AGG ATG GGT GGT GGA
	sbt*Δ6des Not*1 R	GA CT*G CGG CC*G CGA TCA TTT ATG AAG ATA TGC A
G	sbt*Δ6des*SDM F	CTG G*AA TTC* ACA GTG AAA ATG GGT GGT GGA GGC C
	sbt*Δ6des*SDM R	G GCC TCC ACC ACC CAT TTT CAC TGT *GAA TT*C CAG
H	sbt*Δ6des Not*1 pYES2CT R	TC TC*G CGG CCG C*CC TTT ATG AAG ATA TGC ATC AAG

Restriction enzyme sites are indicated by italics and the start or stop codons are underlined.

3.3 Results

3.3.1 SBT liver fatty acid profile

The fatty acid profile of the SBT liver tissue, from which the fatty acyl desaturase and elongase cDNAs were subsequently cloned, was determined (Table 3.3). The major fatty acids were 16:0, 16:1n-7, 18:0, 18:1n-7, 18:1n-9, 20:5n-3, 22:5n-3 and 22:6n-3. These fatty acid components accounted for more than 85% of the total fatty acids in the SBT liver. The profile was comprised of 36.5% n-3 PUFA, 33.6% saturated fatty acids, 25.6% monounsaturated fatty acids and 4% n-6 PUFA. The most abundant individual fatty acid was DHA, at 18.3% of the total fatty acids. Notably, the liver tissue contained 0.6% ALA, 10.9% EPA and 18.3% DHA and a DHA/EPA ratio of 1.7.

3.3.2 Sequence analyses of the SBT fatty acyl elongase

The work described in this paragraph was performed by Valene H.L. See. Overlapping fragments of a putative fatty acyl elongase cDNA were amplified from SBT liver RNA using a combination of RT-PCR with 3'- and 5'-RACE-PCR. The fragments were assembled to give a sequence of 1120 base pairs (bp) which was deposited in the GenBank database as accession number FJ156735. The 1120 bp sequence included an ORF of 885 bp plus a 42 bp 5'-untranslated region (UTR) and a 193 bp 3'-UTR.

All of the remaining work was performed by Melissa K. Gregory. Subsequently, the ORF was amplified as one fragment with flanking 5'- and 3'-UTR (1056 bp amplified in total) (Figure 3.2) and the 885 bp ORF was deposited in the GenBank database as accession number GQ204105. The ORF encoded a predicted protein of 294 amino acids (Figure 3.3). The predicted protein sequence included all of the characteristic features of microsomal fatty acyl elongases, including the conserved motifs KxxExxDT (Box 1), QxxFLHxYHH containing the single histidine box redox centre motif HxxHH (Box 2), NxxxHxxMYxYY (Box 3) and TxxQxxQ (Box 4) and lysine or arginine residues close to the C terminus which may function as an ER retention signal (Figure 3.3) (Agaba *et*

al. 2005; Jakobsson *et al.* 2006). Fatty acyl elongases contain multiple transmembrane regions and the SBT fatty acyl elongase was predicted to contain four transmembrane regions (SOSUI software, bp.nuap.nagoya-u.ac.jp/sosui/).

There are seven different human fatty acyl elongases (ELOVL1-ELOVL7) in the GenBank database, of which only ELOVL2 and ELOVL5 elongate PUFA substrates. Phylogenetic analysis revealed that the SBT fatty acyl elongase was most similar to the human ELOVL5, followed by the human ELOVL2 (Figure 3.4). Pairwise comparisons of the deduced amino acid sequences of fish and human fatty acyl elongases revealed that the SBT fatty acyl elongase sequence was most similar to other fatty acyl elongase sequences from gilthead sea bream (AAT81404), cobia (ACJ65150), turbot (AAL69984) and Nile tilapia (AAO13174), with percentage identities of 92%, 92%, 87% and 84%, respectively (Table 3.4). The SBT fatty acyl elongase was most similar to human ELOVL5 with a percentage identity of 71%, compared to human ELOVL2 with a percentage identity of 55% (Table 3.4). Phylogenetic analysis revealed that the SBT fatty acyl elongase formed a clade with the gilthead sea bream, cobia, turbot and Nile tilapia sequences, which was reasonably well supported by a bootstrap value of 89 (Figure 3.5). The anadromous salmonids (cherry salmon, rainbow trout and Atlantic salmon) formed another clade which was strongly supported by a bootstrap value of 100. All of these fish fatty acyl elongases are designated as Elovl5. Thus, the SBT sequence was designated sbt*Elovl5*.

Table 3.3 SBT liver fatty acid profile

Fatty Acid	Composition (% of total fatty acids)
14:0	1.6
15:0	0.4
16:0	17.2
18:0	13.1
Total Saturated	33.6
16:1n-7	2.1
18:1n-9	14.7
18:1n-7	4.7
20:1n-9	1.5
22:1n-11	1.1
24:1n-9	0.6
Total Monounsaturated	25.6
18:2n-6	1.4
20:2n-6	0.3
20:4n-6	1.6
22:5n-6	0
Total n-6	4.0
18:3n-3	0.6
18:4n-3	0.8
20:5n-3	10.9
22:5n-3	5.3
22:6n-3	18.3
Total n-3	36.5
n-3/n-6	9.1
DHA/EPA	1.7

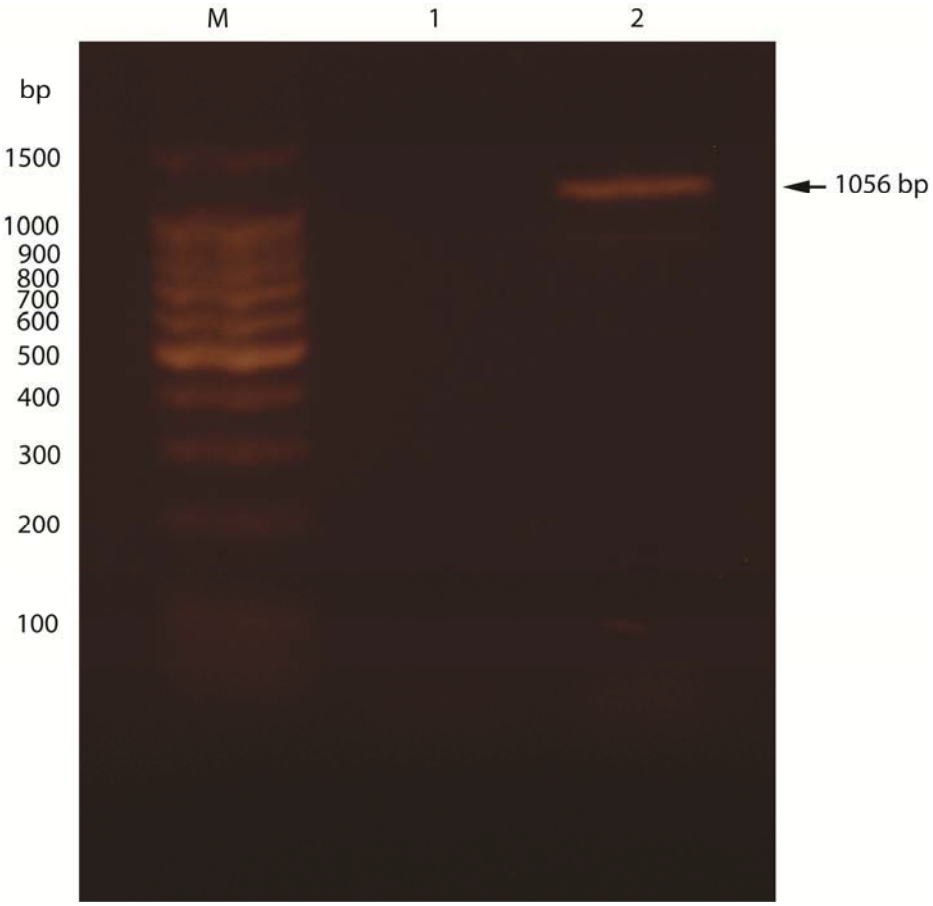


Figure 3.2 Visualisation of a SBT fatty acyl elongase cDNA PCR product.
PCR was conducted as described in section 3.2.19 with the sbt*Elovl5* primer set C (Table 3.1) to amplify a 1056 bp product.

Lane 1 – negative control (no template)
Lane 2 – SBT fatty acyl elongase cDNA

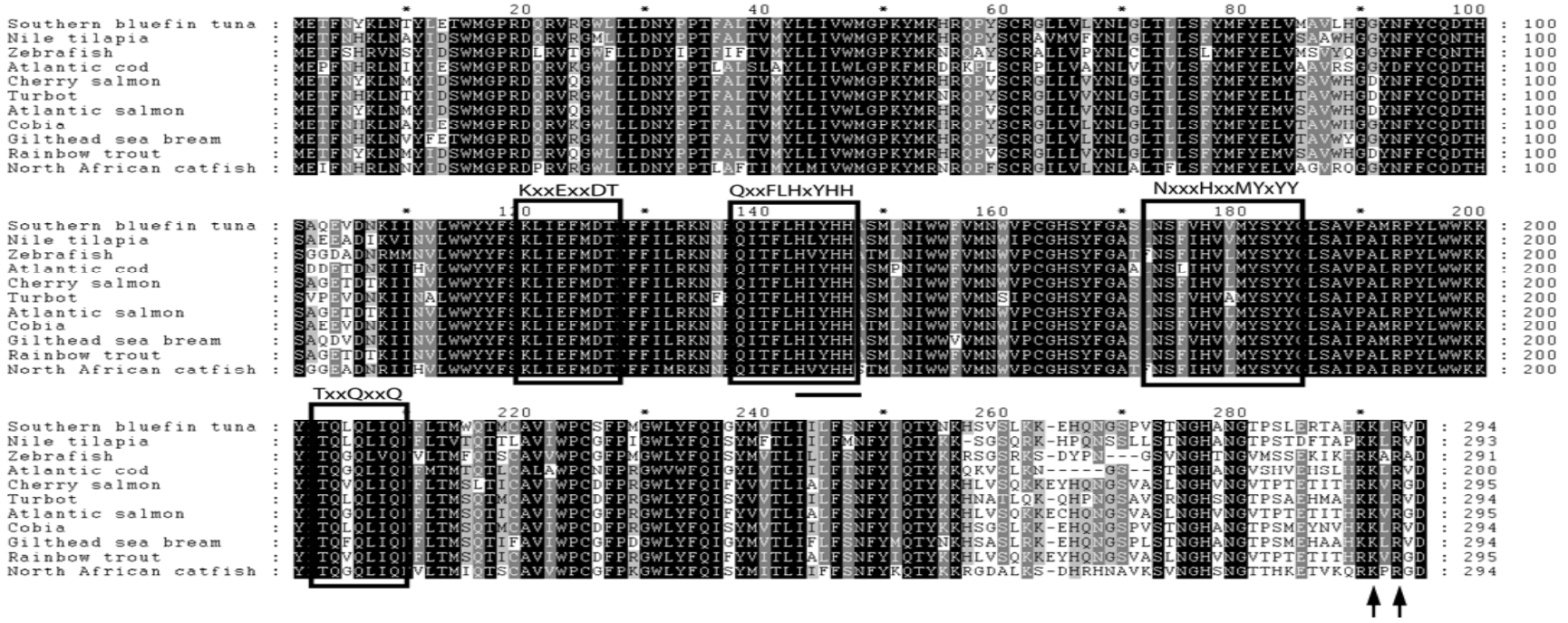


Figure 3.3 An alignment of fatty acyl elongase deduced amino acid sequences. The sbtElovl5 from southern bluefin tuna was compared with fatty acyl elongases from Nile tilapia, zebrafish, Atlantic cod, cherry salmon, turbot, Atlantic salmon, cobia, gilthead sea bream, rainbow trout and North African catfish. Identity/similarity shading was based on the Gonnet series matrix produced by ClustalX where primary black shading indicates identical residues and secondary and tertiary grey shading indicates similar residues with an 80% and 60% cut off, respectively. Highly conserved motifs are boxed, the histidine box is underlined and the ER retention signal is indicated by arrows.

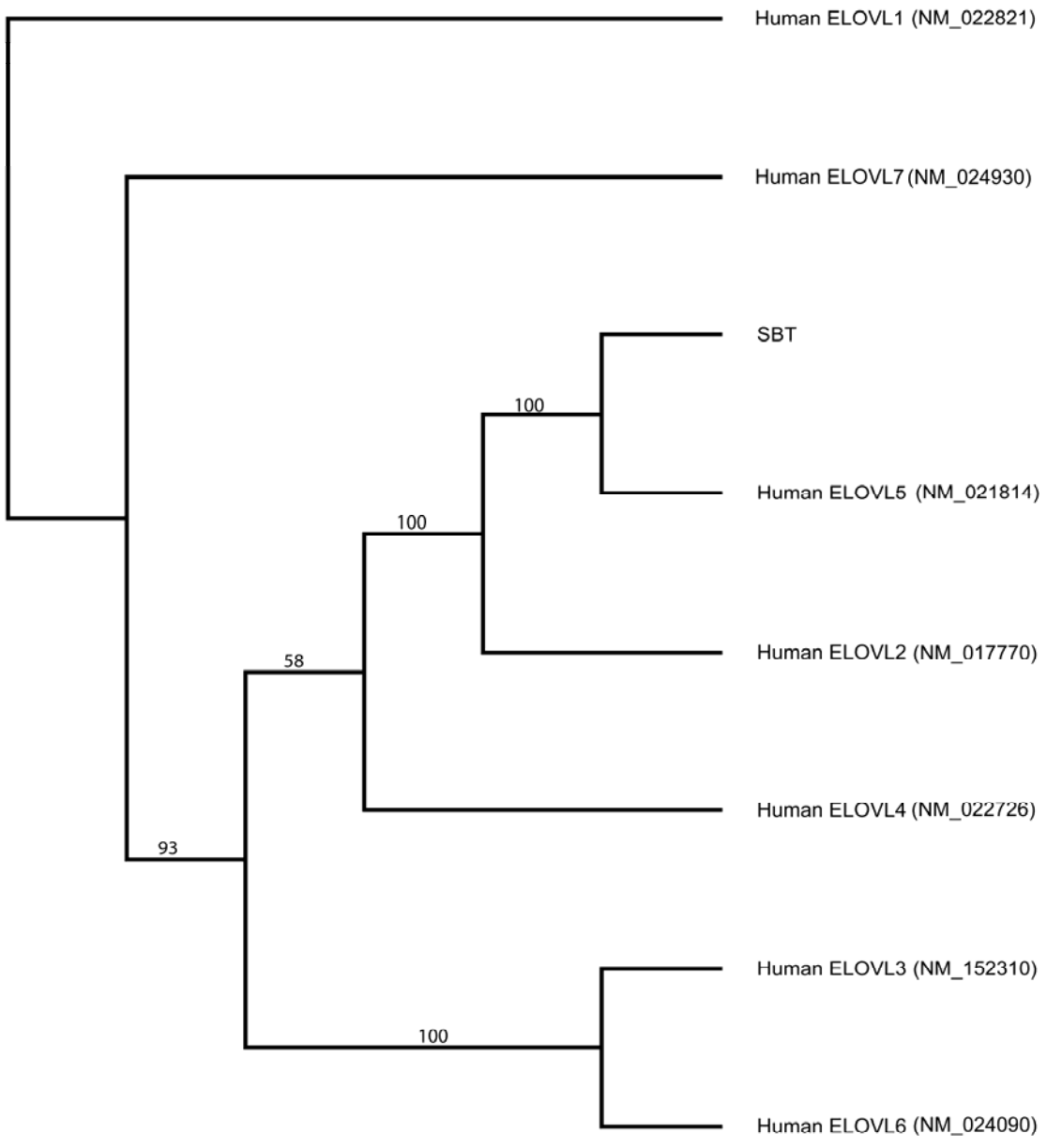


Figure 3.4 A phylogenetic tree comparing deduced amino acid sequences of fatty acyl elongases from southern bluefin tuna and the human elongases (ELOVL1-7). The accession numbers are from the GenBank database. The tree was constructed using maximum parsimony analysis with ClustalX and PAUP* (Swofford 2003). The numbers on the branches represent the frequencies with which the tree topology presented was replicated after 1,000 bootstrap iterations.

Table 3.4 Identity matrix showing the results of a pairwise comparison between the identities of the deduced amino acid sequences of fish fatty acyl elongases and the human ELOVL5 and ELOVL2.

	Gilthead sea bream	Cobia	Turbot	Nile tilapia	Atlantic salmon	Cherry salmon	Rainbow trout	Atlantic cod	Zebrafish	Catfish	Human ELOVL5	Human ELOVL2
Southern bluefin tuna	92	92	87	84	83	83	83	78	75	74	71	55
Gilthead sea bream		91	86	84	81	81	81	75	74	74	70	54
Cobia			89	86	83	83	83	78	76	76	69	55
Turbot				82	82	82	82	74	73	74	67	54
Nile tilapia					79	78	78	72	74	73	67	55
Atlantic salmon						97	97	76	75	75	71	53
Cherry salmon							99	75	74	75	71	52
Rainbow trout								75	74	76	71	52
Atlantic cod									69	72	65	48
Zebrafish										78	69	55
North African catfish											69	53

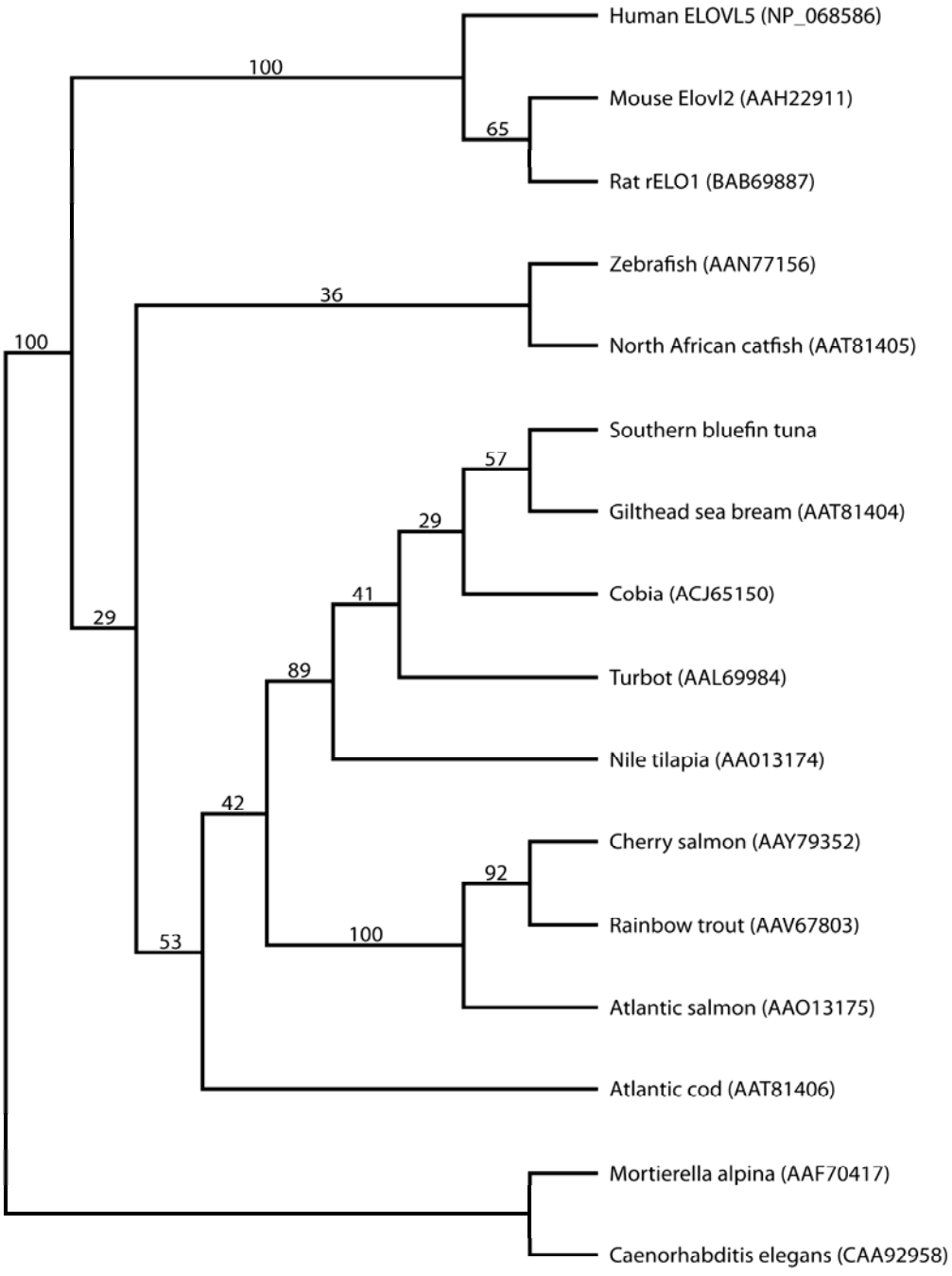


Figure 3.5 A phylogenetic tree comparing deduced amino acid sequences of fatty acyl elongases from southern bluefin tuna, other fish species and mammals. The accession numbers are from the GenBank database. The tree was constructed using maximum parsimony analysis with ClustalX and PAUP* (Swofford 2003). The numbers on the branches represent the frequencies with which the tree topology presented was replicated after 1,000 bootstrap iterations. *M. alpina* and *C. elegans* were chosen as the out groups.

3.3.3 Cloning the SBT *Δ6desaturase* cDNA

RT-PCR produced two internal fragments of the putative SBT Δ6desaturase which spanned 1267 bp. The products from primer sets A and B (Table 3.2) were approximately 729 bp and 676 bp, respectively (Figure 3.6), with an overlap of 137 bp. The putative SBT Δ6desaturase sequence information was used to design 3' and 5' RACE-PCR primers (Table 3.2). First round 3' RACE-PCR produced two distinct bands of approximately 600 and 850 bp, while 5' RACE-PCR produced a faint band at approximately 450 bp (Figure 3.7A). Subsequent second round nested 3′ RACE-PCR significantly intensified the larger band, which made size determination difficult but it appeared to be approximately 700 bp whilst a smaller band remained at 600 bp (Figure 3.7B). Second round nested 5' RACE-PCR produced a band with less definition than the 3' RACE-PCR of approximately 350 bp (Figure 3.7B). All of the template dilutions used in the second round nested 3' and 5' RACE-PCR produced bands of similar intensity (Figure 3.7B). A final third round nested 3' RACE-PCR produced an intense band at 850 bp and two other bands at 650 bp and 750 bp (Figure 3.7C).

3.3.4 Sequence analyses of the SBT *Δ6desaturase*

Overlapping fragments of a putative SBT Δ6desaturase cDNA were amplified using a combination of RT-PCR with 3'- and 5'-RACE-PCR as described above. The fragments were assembled to give a sequence of 2048 bp. The sequence included an ORF of 1338 bp plus a 218 bp 5'-UTR and a 492 bp 3'-UTR. Subsequently, the ORF was amplified as one fragment with flanking 3'- and 5'- UTR (1601 bp amplified in total) (Figure 3.8) and the ORF was deposited in the GenBank database as accession number HM032095. The predicted protein of 445 amino acids included all of the characteristic features of microsomal fatty acyl desaturases, including two transmembrane regions, the three histidine boxes HXXXH, HXXHH and QIEHH, and an N-terminal cytochrome b_5-like domain containing the heme-binding motif (Figure 3.9) (Knutzon *et al.* 1998; Sperling and Heinz 2001). Thus, the SBT sequence was designated sbt*Δ6des.* The salmon and trout sequences have an additional ten amino acid residues at the N-terminal end of the sequence (Figure 3.9).

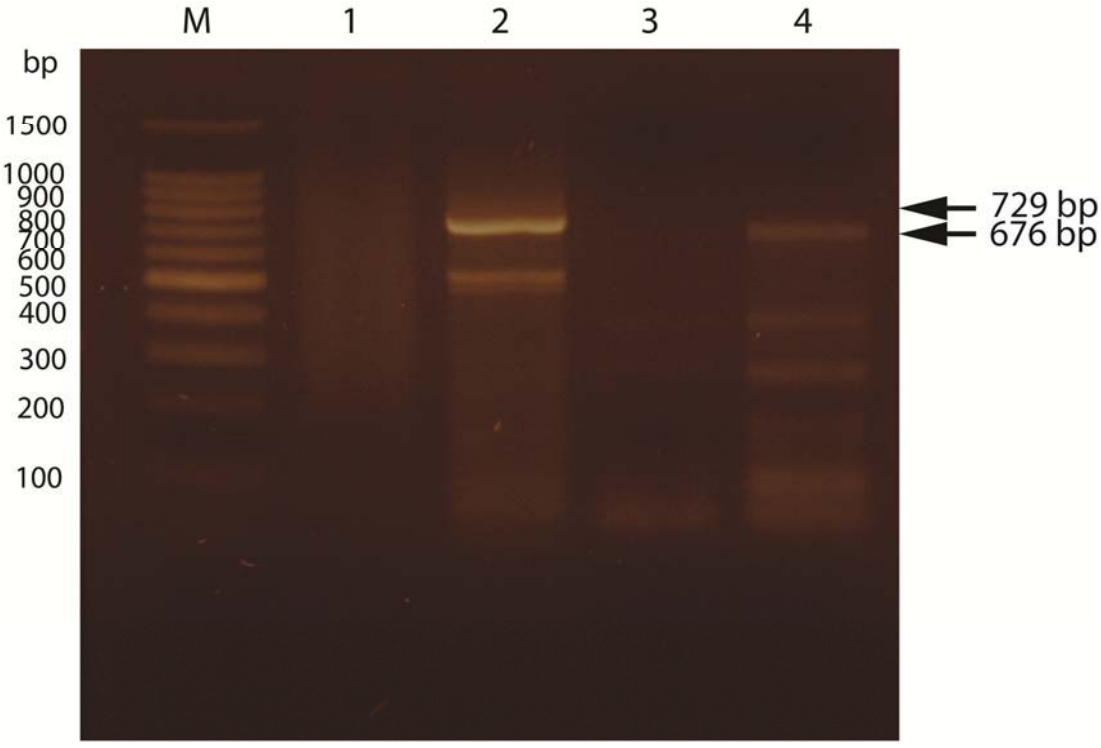


Figure 3.6 Visualisation of SBT *Δ6desaturase* cDNA PCR products.

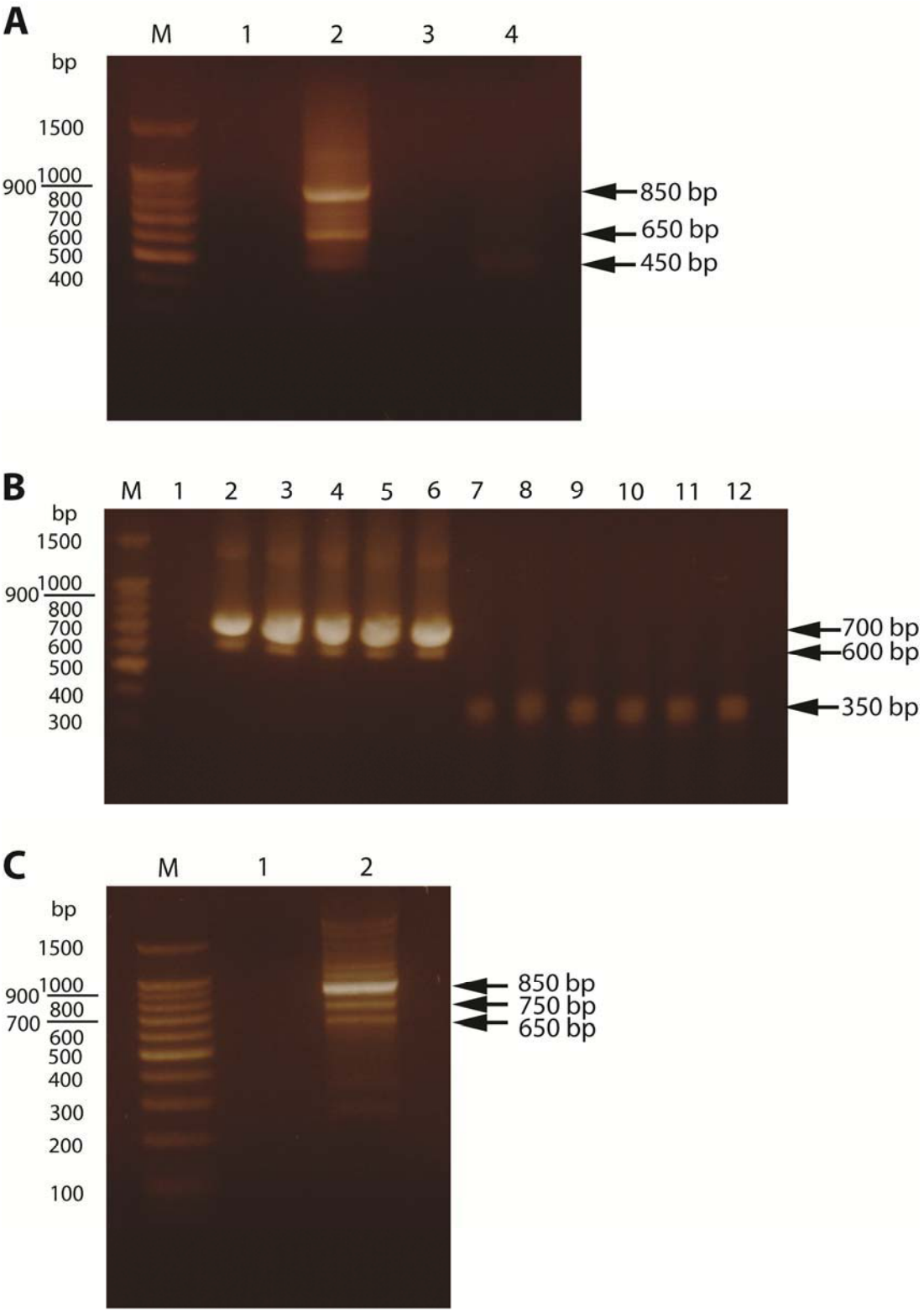
PCR was conducted as described in section 3.2.16 with the sbt*Δ6des* primer sets A and B (Table 3.2) to obtain 729 and 676 bp products.

Lane 1 – negative control (no template) primer set A

Lane 2 – SBT *Δ6desaturase* internal fragment using primer set A

Lane 3 – negative control (no template) primer set B

Lane 4 – SBT *Δ6desaturase* internal fragment using primer set B


A
M
1
2
3
4
bp
1500
1000
900
800
700
600
500
400
850 bp
650 bp
450 bp
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M
1
2
3
4
5
6
7
8
9
10
11
12
bp
1500
1000
900
800
700
600
500
400
300
700 bp
600 bp
350 bp
C
M
1
2
bp
1500
1000
900
800
700
600
500
400
300
200
100
850 bp
750 bp
650 bp

Figure 3.7 Visualisation of the amplification of the 3' and 5' ends of the SBT *Δ6desaturase* cDNA.

(A) 1st round 3' and 5' RACE-PCR products amplified using primers sets C and D (Table 3.2) as described in section 3.2.18.

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Lane 1 – negative control (no template) 3' RACE-PCR (1st round)
Lane 2 – 3' RACE-PCR products (1st round)
Lane 3 – negative control (no template) 5' RACE-PCR (1st round)
Lane 4 – 5' RACE-PCR product (1st round)

(B) 2nd round (nested) 3' and 5' RACE-PCR products amplified using primers sets C and D (Table 3.2) as described in section 3.2.18.

Lane 1 – negative control (no template) 3' RACE-PCR (2nd round)
Lane 2 – 3' RACE-PCR using a 1:10 dilution of 1st round product (2nd round)
Lane 3 – 3' RACE-PCR using a 1:25 dilution of 1st round product (2nd round)
Lane 4 – 3' RACE-PCR using a 1:50 dilution of 1st round product (2nd round)
Lane 5 – 3' RACE-PCR using a 1:100 dilution of 1st round product (2nd round)
Lane 6 – 3' RACE-PCR using a 1:1000 dilution of 1st round product (2nd round)
Lane 7 – negative control (no template) 5' RACE-PCR (2nd round)
Lane 8 – 5' RACE-PCR using a 1:2 dilution of 1st round product (2nd round)
Lane 9 – 5' RACE-PCR using a 1:10 dilution of 1st round product (2nd round)
Lane 10 – 5' RACE-PCR using a 1:25 dilution of 1st round product (2nd round)
Lane 11 – 5' RACE-PCR using a 1:50 dilution of 1st round product (2nd round)
Lane 12 – 5' RACE-PCR using a 1:100 dilution of 1st round product (2nd round)

(C) 3rd round (nested) 3' RACE-PCR product amplified using primers set C (Table 3.2) as described in section 3.2.18.

Lane 1 – negative control (no template) 3' RACE-PCR (3rd round)
Lane 2 – 3' RACE-PCR using a 1:50 dilution of 2nd round product (3rd round)

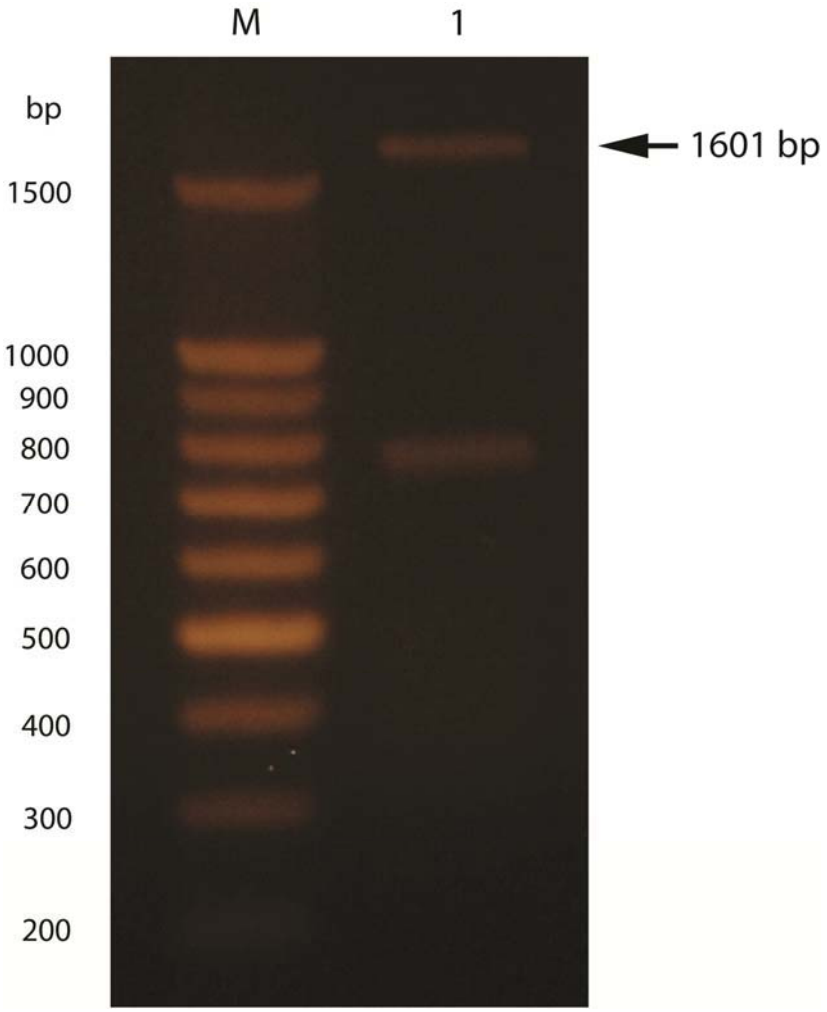

Figure 3.8 Visualisation of the SBT *Δ6desaturase* ORF PCR product. PCR was conducted as described in section 3.2.19 with primer set E (Table 3.2) to obtain a 1601 bp product, including 5'- and 3'-UTR.

Lane 1 – full length SBT *Δ6desaturase* ORF using primer set E

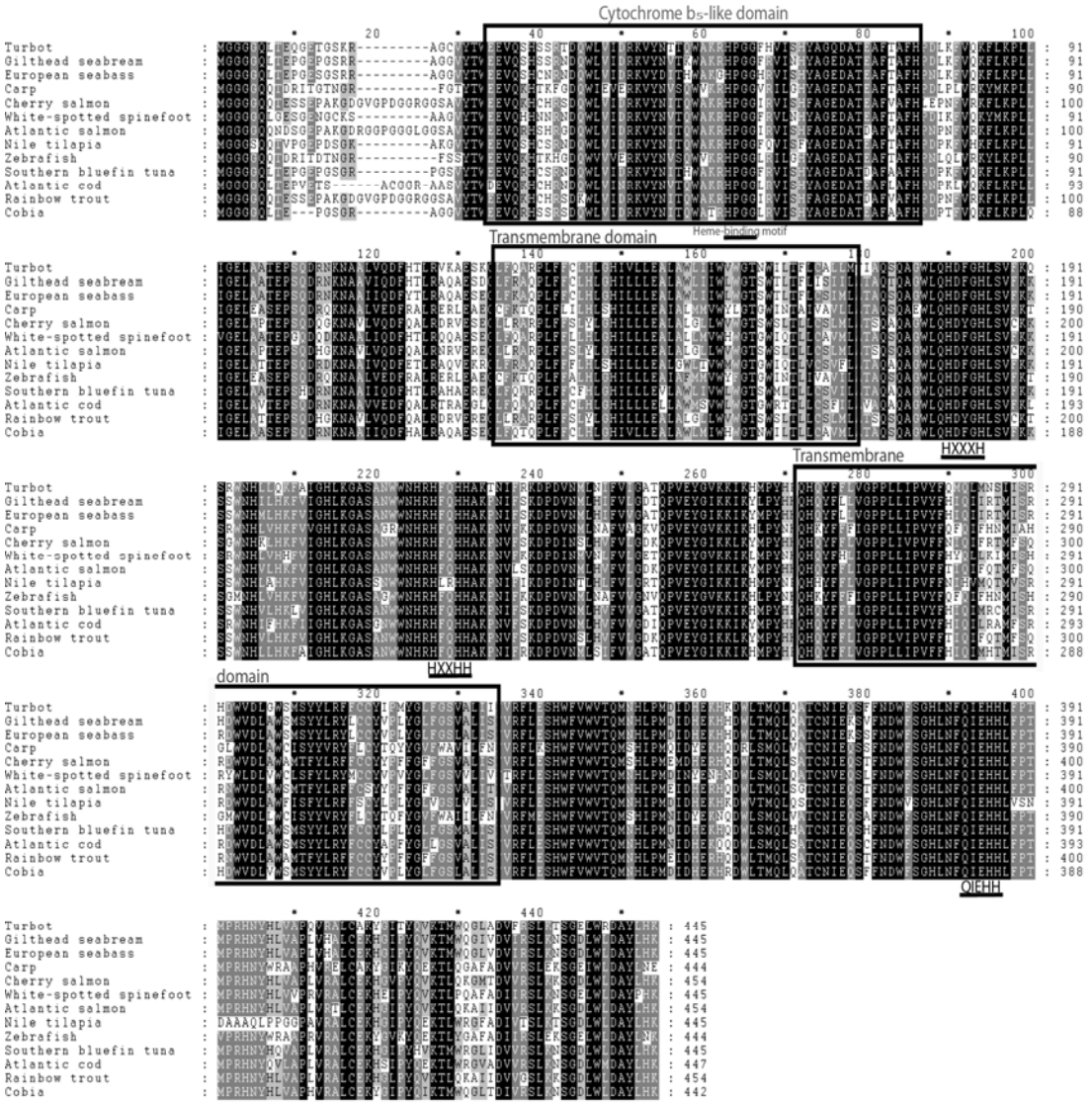

Figure 3.9 An alignment of fatty acyl Δ6desaturase deduced amino acid sequences. The sbtΔ6des from southern bluefin tuna was compared with fatty acyl desaturases from turbot, gilthead sea bream, European seabass, carp, Cherry salmon, white-spotted spinefoot, Atlantic salmon, Nile tilapia, zebrafish, Atlantic cod, rainbow trout and cobia. Identity/similarity shading was based on the Gonnet series matrix produced by ClustalX where primary black shading indicates identical residues and secondary and tertiary grey shading indicates similar residues with an 80% and 60% cut off, respectively. The cytochrome b_5-like domain and two transmembrane domains are boxed, and the heme-binding motif and three histidine boxes are underlined.

Pairwise comparisons of the deduced amino acid sequences of fish and human fatty acyl Δ6desaturases revealed that the putative sbt*Δ6des* sequence was most similar to the sequences from European sea bass (ACD10793), gilthead sea bream (AAL17639), cobia (ACJ65149) turbot (AAS49163) and Atlantic cod (AAY46796), with percentage identities of 88%, 87%, 87%, 82% and 82%, respectively (Table 3.5). The Δ6desaturase sequence from thirteen fish species compared to the human Δ6desaturase sequence ranged from 61-65% identity (Table 3.5). The putative sbt*Δ6des* shared 65% identity with the human Δ6desaturase (Table 3.5). Phylogenetic analysis revealed that the sbt*Δ6des* formed a clade with the cobia, turbot, European seabass and gilthead sea bream Δ6desaturase sequences, although this was poorly supported by a bootstrap value of 39 (Figure 3.10). The anadromous salmonids (cherry salmon, rainbow trout and Atlantic salmon) and freshwater omnivores (carp and zebrafish) formed separate clades which were strongly supported by bootstrap values of 100 (Figure 3.10).

The functionally characterized Δ5desaturase and Δ6desaturase amino acid sequences contain similar conserved regions. Thus, the putative sbt*Δ6des* was also compared with functionally characterized Δ5desaturase amino acid sequences in order to assess their similarity (Figure 3.11). The characteristic microsomal fatty acyl desaturase features were all present in the Δ5desaturase proteins (Figure 3.11). As seen with the Δ6desaturase, the cherry salmon and Atlantic salmon Δ5desaturase sequences have an additional ten amino acid residues at the N-terminal end of the sequence (Figure 3.11). Pairwise comparisons with the deduced amino acid sequences of cherry salmon, Atlantic salmon, zebrafish, rat, mouse and human fatty acyl Δ5desaturases revealed that the putative sbt*Δ6des* sequence was most similar to cherry salmon with a percentage identity of 78% (Table 3.6). The sbt*Δ6des* had higher identity to the cherry salmon Δ5desaturase than the cherry salmon Δ6desaturase, while the identity to the Atlantic salmon Δ5desaturase and Δ6desaturase and zebrafish Δ5/6desaturase was the same. The putative sbt*Δ6des* shared 57% identity with the human Δ5desaturase compared to 65% identity to the human Δ6desaturase (Table 3.6).

Table 3.5 Identity matrix showing the results of a pairwise comparison between the identities of the deduced amino acid sequences of fish Δ6desaturase and the human Δ6desaturase.

	European sea bass	Gilthead sea bream	Cobia	Turbot	Atlantic cod	Nile tilapia	Rainbow trout	Atlantic salmon	White-spotted spinefoot	Cherry salmon	Carp	Zebrafish	Human
Southern bluefin tuna	88	87	87	82	82	77	77	77	77	76	66	68	65
European sea bass		94	87	83	80	76	75	76	78	76	66	68	64
Gilthead sea bream			86	84	81	76	75	76	78	76	66	68	64
Cobia				85	78	77	75	75	78	75	68	70	65
Turbot					76	73	71	72	76	71	66	67	62
Atlantic cod						72	76	75	73	77	66	69	64
Nile tilapia							70	69	72	70	62	64	61
Rainbow trout								94	71	94	64	65	64
Atlantic salmon									71	91	64	65	64
White-spotted spinefoot										70	68	70	63
Cherry salmon											63	64	62
Carp												89	62
Zebrafish													64

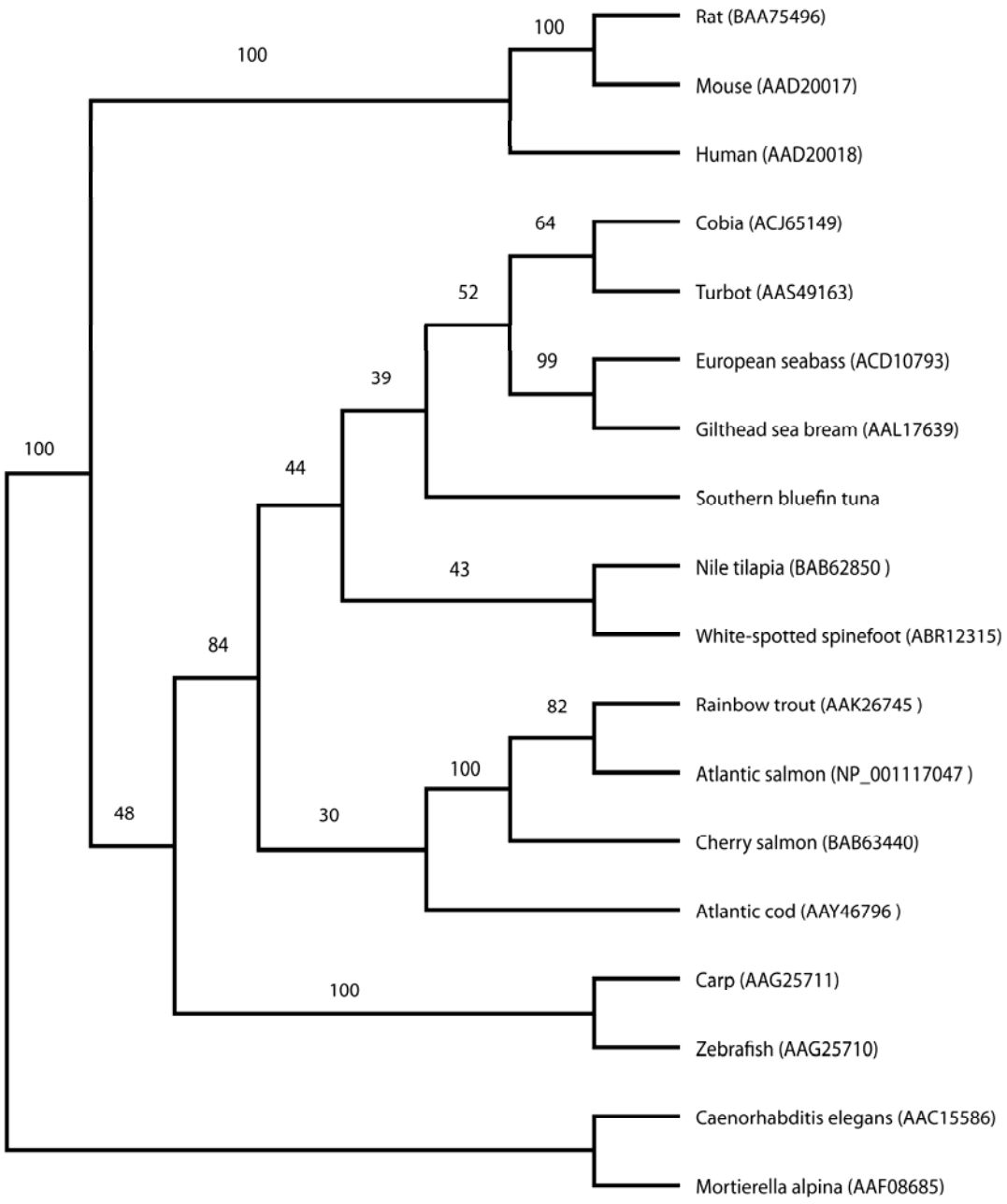

Figure 3.10 A phylogenetic tree comparing deduced amino acid sequences of fatty acyl desaturases from southern bluefin tuna, other fish species and mammals. The accession numbers are from the GenBank database. The tree was constructed using maximum parsimony analysis with ClustalX and PAUP* (Swofford, 2003). The numbers on the branches represent the frequencies with which the tree topology presented was replicated after 1,000 bootstrap iterations. *M. alpina* and *C. elegans* were chosen as the out groups.

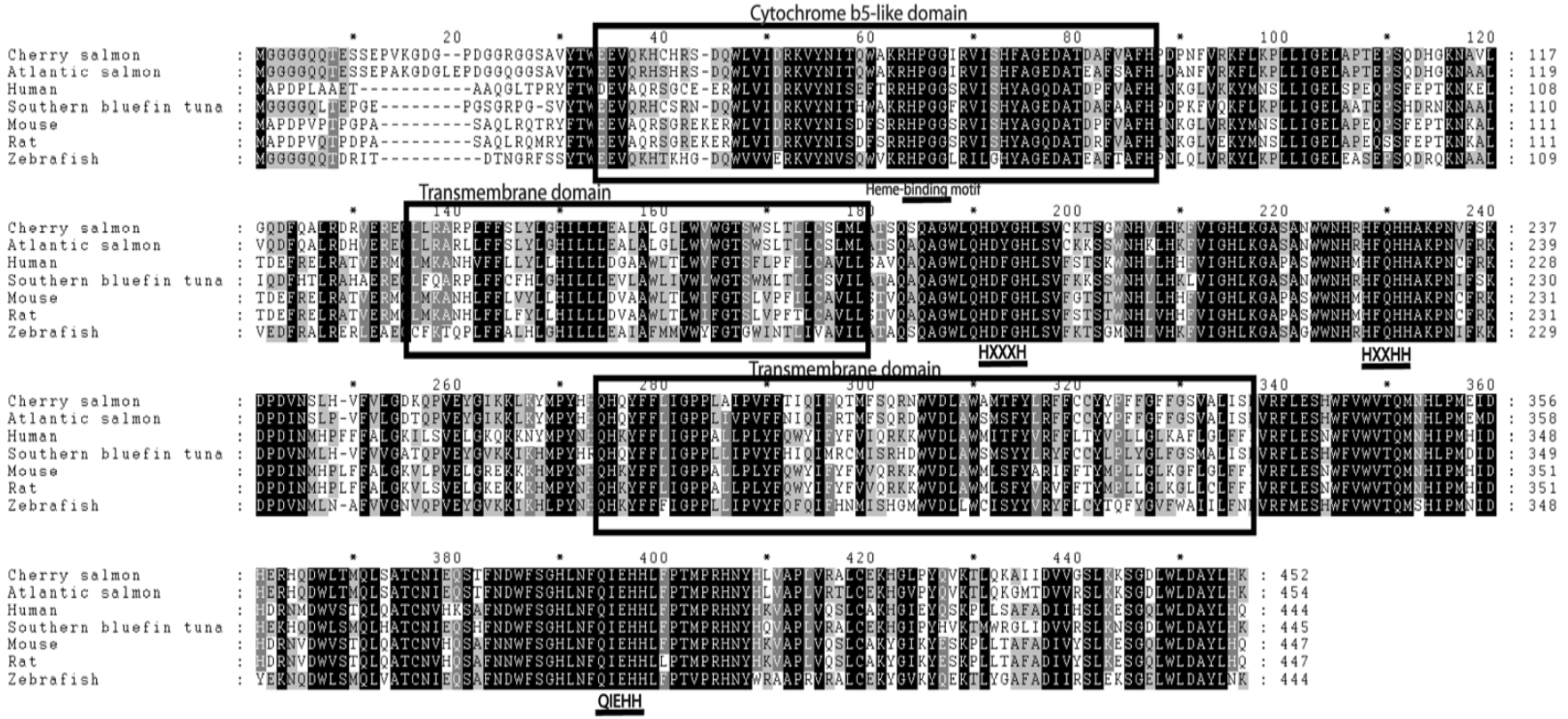

Figure 3.11 An alignment of fatty acyl Δ5desaturase amino acid sequences. The sbtΔ6des from southern bluefin tuna was compared with Δ5desaturases from Cherry salmon, Atlantic salmon, zebrafish, mouse, rat and human. Identity/similarity shading was based on the Gonnet series matrix produced by ClustalX where primary black shading indicates identical residues and secondary and tertiary grey shading indicates similar residues with an 80% and 60% cut off, respectively. The cytochrome b_5-like domain and two transmembrane domains are boxed, and the heme-binding motif and three histidine boxes are underlined.

Table 3.6 Identity matrix showing the results of a pairwise comparison between the identities of the deduced Δ5desaturase amino acid sequences from fish and mammals and the SBT Δ6desaturase.

	Cherry salmon	Atlantic salmon	Zebrafish	Mouse	Rat	Human
Southern bluefin tuna	78	77	68	59	58	57
Cherry salmon		92	65	57	57	57
Atlantic salmon			64	58	57	57
Zebrafish				57	56	56
Mouse					96	87
Rat						87

3.3.5 Functional characterization of the sbt*Elovl5* ORF

Two different expression constructs were produced to test the two possible translation start sites in the sbt*Elovl5* sequence. The pYES2-sbt*Elovl5* construct contained the sbt*Elovl5* 885 bp ORF. A second putative start codon was identified in the sbt*Elovl5* ORF, which was 42 bp downstream. A construct was produced which contained the shorter sbt*Elovl5* 843 bp ORF and this construct was referred to as the pYES2-sbt*Elovl5-42.* The pYES2-sbt*Elovl5* and pYES2-sbt*Elovl5-42* constructs were expressed in *S. cerevisiae* to investigate their functionality. The sbtElovl5-42 did not elongate the PUFA substrates 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 or 22:4n-6, whereas the sbtElovl5 did. The sbtElovl5 was used for all further work. All individual PUFA substrate supplementations were replicated with two colonies containing the sbt*Elovl5* ORF. They are referred to as colonies A and B.

The main endogenous fatty acids in the yeast cells were 16:0, 16:1n-7, 18:0 and 18:1n-9. The fatty acid profile of induced *S. cerevisiae* cultures containing the empty pYES2 vector included the four endogenous fatty acids and the PUFA substrate supplied, consistent with the lack of PUFA elongase activity in *S. cerevisiae* (Agaba *et al.* 2004) (Appendix C.1). Similar profiles were seen for the uninduced *S. cerevisiae* cultures containing the pYES2-sbtElovl5 construct (Appendix C.2). The sbtElovl5 converted the endogenous 16:0, 16:1n-7 and 18:1n-9 fatty acids to 18:0, 18:1n-7 and 20:1n-9, respectively (Appendix C.2).

In a preliminary experiment, induced cells containing sbtElovl5 were supplemented with 18:4n-3 to establish the best length of time for substrate incorporation and product conversion, for all subsequent experiments with all other PUFA substrates. The time-course experiment showed that 18:4n-3 was incorporated into the cells within 12 h and that it was progressively elongated to 20:4n-3 followed by 22:4n-3 over a period of 60-96 h (Figure 3.12). The time-course was performed with two colonies and a similar trend of incorporation and 18:4n-3 elongation was seen (Figure 3.12 and Figure 3.13). There was some 'leaky' sbtElovl5 expression with detectable 20:4n-3 and after 84 h detectable 22:4n-3 in the uninduced yeast cells (Figure 3.12 and Figure 3.13). However, the level of 20:4n-3 remained constant

while the level of 18:4n-3 decreased with time (Figure 3.12 and Figure 3.13). Since the most dramatic changes occurred within the first 24 h, this time-point was chosen for harvesting the recombinant *S. cerevisiae* cells for all subsequent experiments.

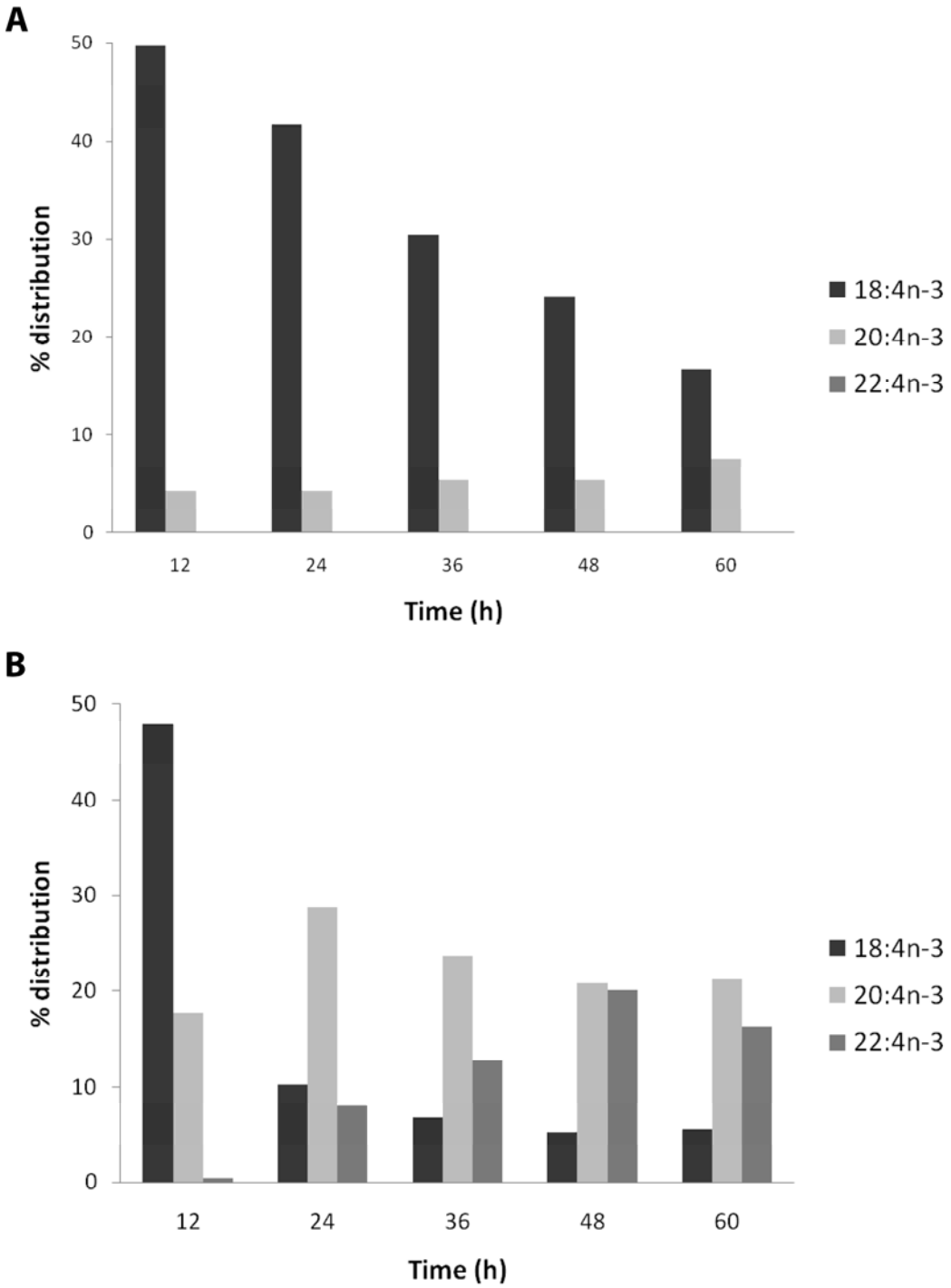

Figure 3.12 Time-course for the elongation of 18:4n-3 by recombinant *S. cerevisiae* induced to express the sbt*Elovl5* ORF, colony A. Fatty acids were extracted from *S. cerevisiae* transformed with the pYES2 vector containing the sbt*Elovl5* ORF and cultured in the induction medium containing galactose as the carbon source. The amount of each fatty acid was expressed as a percentage of the total amount of all fatty acids. This was done by expressing the peak area for an individual fatty acid as a percentage of the total peak area for all fatty acids.

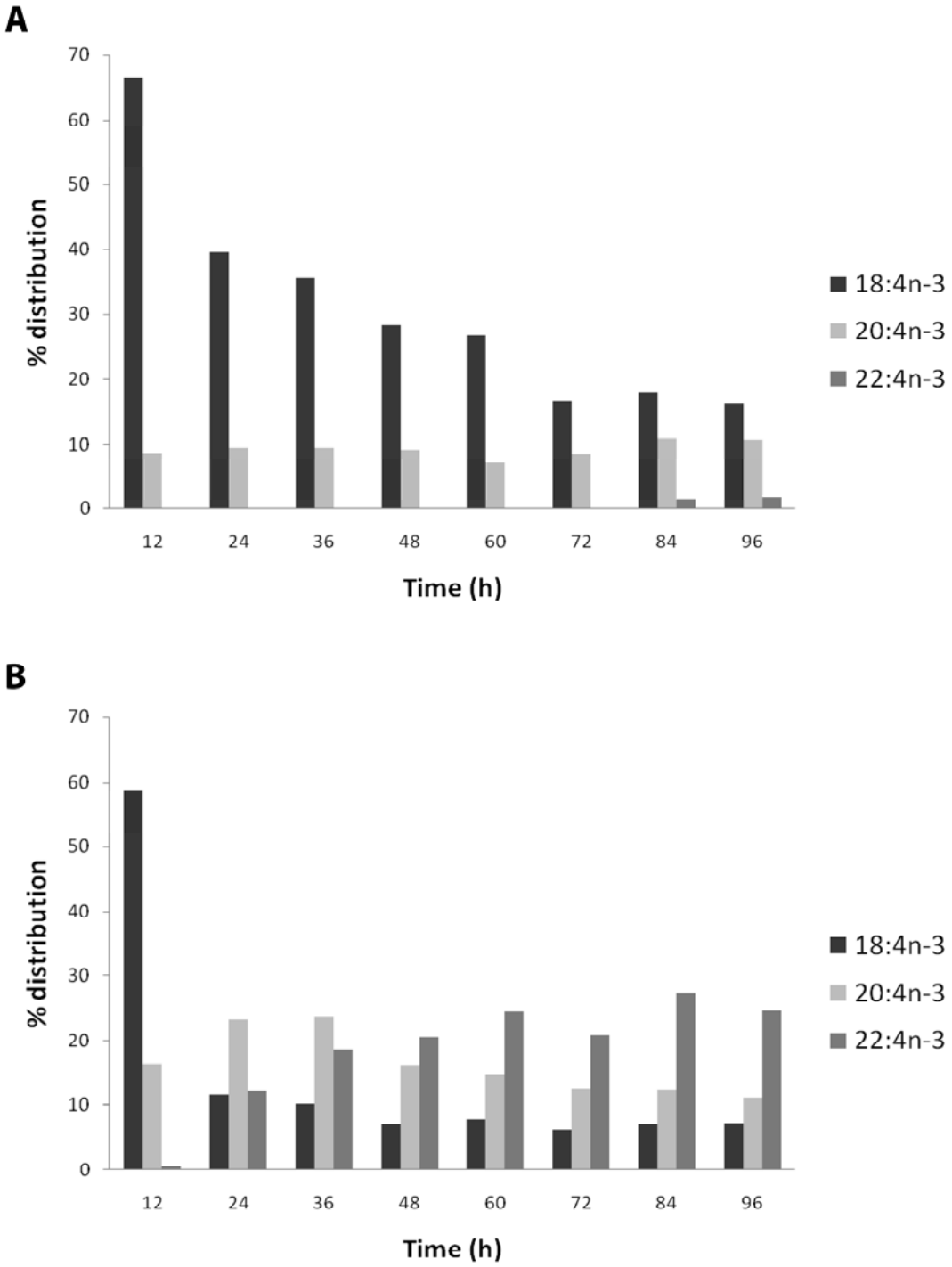

Figure 3.13 Time-course for the elongation of 18:4n-3 by recombinant *S. cerevisiae* induced to express the sbt*Elovl5* ORF, colony B. Fatty acids were extracted from *S. cerevisiae* transformed with the pYES2 vector containing the sbt*Elovl5* ORF and cultured in the induction medium containing galactose as the carbon source. The amount of each fatty acid was expressed as a percentage of the total amount of all fatty acids. This was done by expressing the peak area for an individual fatty acid as a percentage of the total peak area for all fatty acids.

Recombinant *S. cerevisiae* cells were cultured in the presence of various C_{18}, C_{20} and C_{22} PUFA substrates to determine the sbtElovl5 enzyme's substrate specificity (Figure 3.14). The activity seen using sbt*Elovl5* colony A will be described in detail but the trend was similar for colony B (Figure 3.15). All of the substrates were incorporated into the cells. The uninduced cells showed low levels of elongation products from the C_{18} and C_{20} PUFA substrates, suggesting there was some 'leaky' sbtElovl5 expression, in the absence of the inducer, galactose. When the cells were induced in the presence of a PUFA substrate, sbtElovl5 showed high $C_{18\text{-}20}$ elongase activity, converting 83.5% of 18:4n-3 to 20:4n-3 and 60.9% of 18:3n-6 to 20:3n-6 (Figure 3.14C and D). The sbtElovl5 also showed high $C_{20\text{-}22}$ elongase activity, converting 76.8% of 20:5n-3 to 22:5n-3 and 59.7% of 20:4n-6 to 22:4n-6 (Figure 3.14E and F). In contrast, the $C_{22\text{-}24}$ elongase activity of sbtElovl5 was much lower with only 1.7% of 22:5n-3 converted to 24:5n-3 and no detectable conversion of 22:4n-6 to 24:4n-6 observed (Figure 3.14G and H). The cells supplied with the C_{18} substrates also showed detectable levels of the expected C_{22} products. This provided evidence that further elongation of the C_{20} intermediates was occurring. The percentage conversion data for colonies A and B are summarised in Tables 3.7 and 3.8, respectively. It is clear that the sbtElovl5 exhibited a preference for n-3 over n-6 PUFA substrates. However, appreciable activity was still exhibited with the n-6 PUFA substrates. Although not immediate substrates, the sbtElovl5 could also elongate 18:3n-3 to 20:3n-3 and 18:2n-6 to 20:2n-6 with 15.7% and 10.8% conversion, respectively (Tables 3.7 and 3.8).

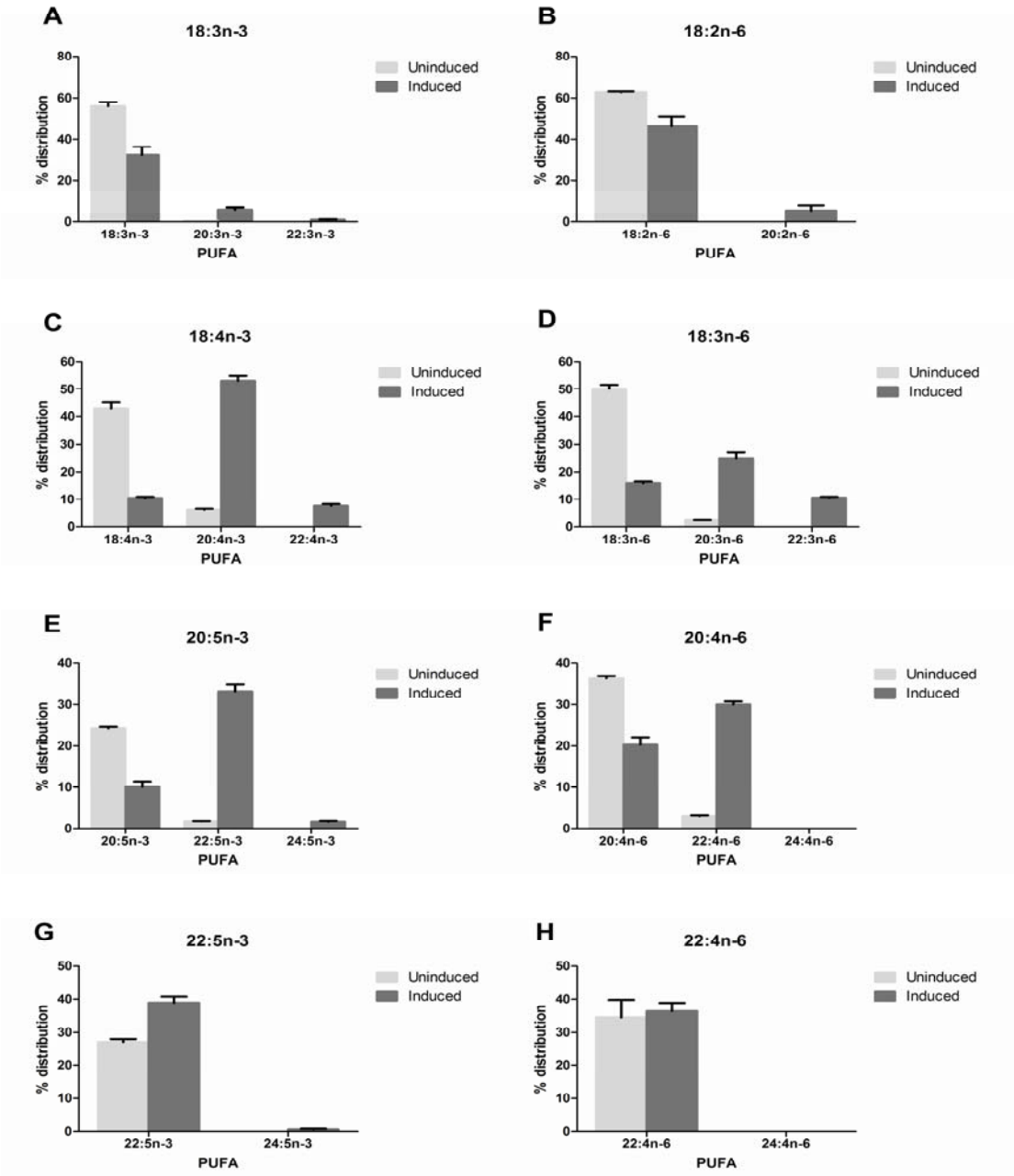

Figure 3.14 Functional characterisation of the sbt*Elovl5* ORF, colony A, in recombinant *S. cerevisiae* grown in the presence of 18:3n-3 (A)**, 18:2n-6** (B)**, 18:4n-3** (C)**, 18:3n-6** (D)**, 20:5n-3** (E)**, 20:4n-6** (F)**, 22:5n-3** (G) **and 22:4n-6** (H) **fatty acids.** The yeast were cultured in either non-inducing medium containing raffinose or inducing medium containing galactose. Fatty acids were extracted from yeast transformed with the pYES2 vector containing the sbt*Elovl5* ORF. The amount of each fatty acid was expressed as a percentage of the total amount of all fatty acids. This was done by expressing the peak area for an individual fatty acid as a percentage of the total peak area for all fatty acids. The data are the mean ± S.D. (n=3).

Table 3.7 Functional characterization of the sbt*Elovl5* ORF, colony A, with individual substrate supplementations

Substrate	Product	Conversion (%)	Substrate	Product	Conversion (%)
18:3n-3	20:3n-3	15.7 ± 1.0	18:2n-6	20:2n-6	10.8 ± 5.2
	22:3n-3	13.6 ± 3.1		22:2n-6	N.D.
	Total	29.3		Total	10.8
18:4n-3	20:4n-3	83.5 ± 0.8	18:3n-6	20:3n-6	60.9 ± 1.5
	22:4n-3	12.7 ± 1.2		22:3n-6	30.1 ± 2.7
	Total	96.2		Total	91
20:5n-3	22:5n-3	76.8 ± 1.9	20:4n-6	22:4n-6	59.7 ± 1.5
	24:5n-3	4.8 ± 0.8		24:4n-6	N.D.
	Total	81.6		Total	59.7
22:5n-3	24:5n-3	1.7 ± 0.4	22:4n-6	24:4n-6	N.D.

The results are expressed as the percentage of fatty acid substrate converted to elongated fatty acid product. The calculation used was [% product/(% product + % substrate)] x 100. Percentage conversion into intermediary products along the elongation pathway is also shown. The data are the mean ± S.D. (n=3). N.D., not detected.

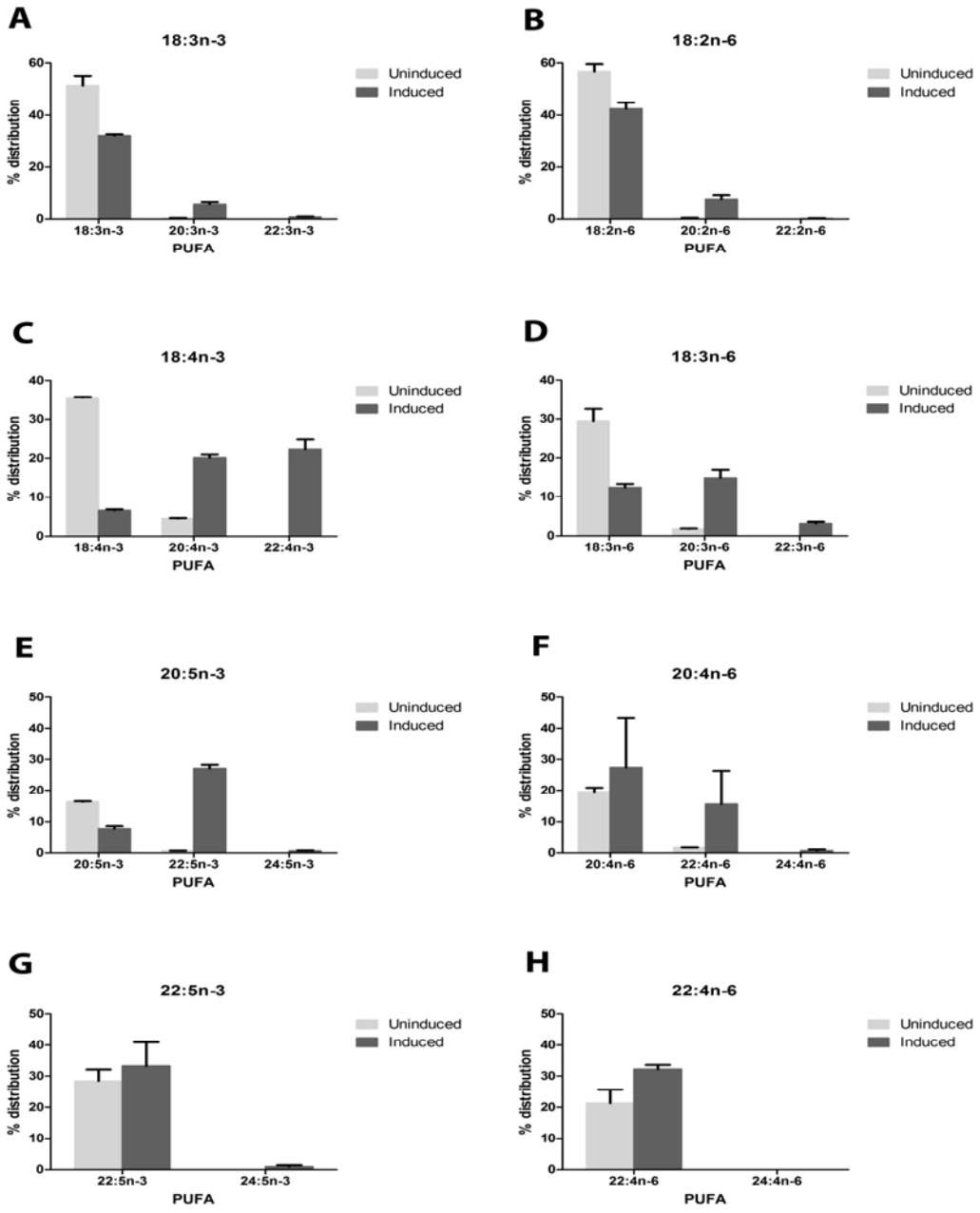

Figure 3.15 Functional characterisation of the sbt*Elovl5* ORF, colony B, in recombinant *S. cerevisiae* grown in the presence of 18:3n-3 (A)**, 18:2n-6** (B)**, 18:4n-3** (C)**, 18:3n-6** (D)**, 20:5n-3** (E)**, 20:4n-6** (F)**, 22:5n-3** (G) **and 22:4n-6** (H) **fatty acids.** The yeast were cultured in either non-inducing medium containing raffinose or inducing medium containing galactose. Fatty acids were extracted from yeast transformed with the pYES2 vector containing the sbt*Elovl5* ORF. The amount of each fatty acid was expressed as a percentage of the total amount of all fatty acids. This was done by expressing the peak area for an individual fatty acid as a percentage of the total peak area for all fatty acids. The data are the mean ± S.D. (n=3).

Table 3.8 Functional characterization of the sbt*Elovl5* ORF, colony B, with individual substrate supplementations

Substrate	Product	Conversion (%)	Substrate	Product	Conversion (%)
18:3n-3	20:3n-3	14.6 ± 1.7	18:2n-6	20:2n-6	15.6 ± 1.8
	22:3n-3	12.1 ± 0.4		22:2n-6	1.2 ± 2.1
	Total	26.7		Total	16.9
18:4n-3	20:4n-3	75.2 ± 1.5	18:3n-6	20:3n-6	54.3 ± 2.0
	22:4n-3	52.5 ± 2.0		22:3n-6	17.4 ± 0.8
	Total	127.6		Total	71.7
20:5n-3	22:5n-3	77.6 ± 1.4	20:4n-6	22:4n-6	37.9 ± 26.2
	24:5n-3	7.4 ± 1.3		24:4n-6	5.5 ± 4.0
	Total	85.0		Total	43.4
22:5n-3	24:5n-3	3.0 ± 2.3	22:4n-6	24:4n-6	N.D.

The results are expressed as the percentage of fatty acid substrate converted to elongated fatty acid product. The calculation used was [% product/(% product + % substrate)] x 100. Percentage conversion into intermediary products along the elongation pathway is also shown. The data are the mean ± S.D. (n=3). N.D., not detected.

Supplementations with more than one potential substrate were performed with either a total of 500 μM of PUFA or 500 μM of each PUFA (Appendix C.4 and C.5). When a total of 500 μM of PUFA substrate was supplied approximately the same amount of each of the substrates was incorporated into the cells (Figure 3.16 A1, B1 and C1). Supplementations with 500 μM in total of 18:4n-3 and 18:3n-6 or 20:5n-3 and 20:4n-6 confirmed that the sbtElovl5 had high C_{18-20} and C_{20-22} elongase activity. The sbtElovl5 converted 58.9% of 18:4n-3 to 20:4n-3 and 35.8% of 18:3n-6 to 20:3n-6 (Figure 3.16 A2). Similar to the individual supplementation with C_{18} substrates a detectable level of the 22:4n-3 was seen, with 16.7% of 20:4n-3 being further elongated to 22:4n-3, but there was no detectable 22:3n-6. The sbtElovl5 converted 50.4% of 20:5n-3 to 22:5n-3 and 40.8% of 20:4n-6 to 22:4n-6 (Figure 3.16 B2). No detectable levels of C_{24} elongation products were produced from either the C_{18} or C_{20} substrates. Supplementations with 500 μM in total of 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6 showed that 18:4n-3 and 18:3n-6 as substrates were preferentially elongated to 20:4n-3 and 20:3n-6 compared to 20:5n-3 and 20:4n-6 as substrates elongated to 22:5n-3 and 22:4n-6 (Figure 3.16 C2). Under these conditions the sbtElovl5 converted 60.4% of 18:4n-3 to 20:4n-3 and 31.4% of 18:3n-6 to 20:3n-6, whilst the conversion of 20:5n-3 to 22:5n-3 was not detectable and 9.8% of 20:4n-6 was converted to 22:4n-6. In all supplementation experiments the conversion of the n-3 PUFA substrates was greater than the conversion of the n-6 PUFA substrates (Table 3.9). This indicated that the sbtElovl5 exhibited a preference for n-3 over n-6 PUFA substrates. However, appreciable activity was still exhibited with the n-6 PUFA substrates.

When 500 μM of each PUFA substrate was supplied it appeared as if substrate competition for the sbtElovl5 increased. The sbtElovl5 could elongate less C_{18} and C_{20} substrates when higher concentrations were supplied (Figure 3.17). When 18:4n-3 and 18:3n-6 were supplied as substrates the elongation of n-3 and n-6 PUFA was 9.3% and 8.1% lower, respectively, when 500 μM of each substrate was supplied. More notable, when 20:5n-3 and 20:4n-6 were supplied as substrates the elongation of n-3 and n-6 PUFA was 15% and 19.9% lower, respectively, when 500 μM of each substrate was supplied. Also, when 500 μM of 18:4n-3, 18:2n-6, 20:5n-3 and 20:4n-6 were each supplied only 18:4n-3 and 18:3n-6 were elongated (Table 3.10).

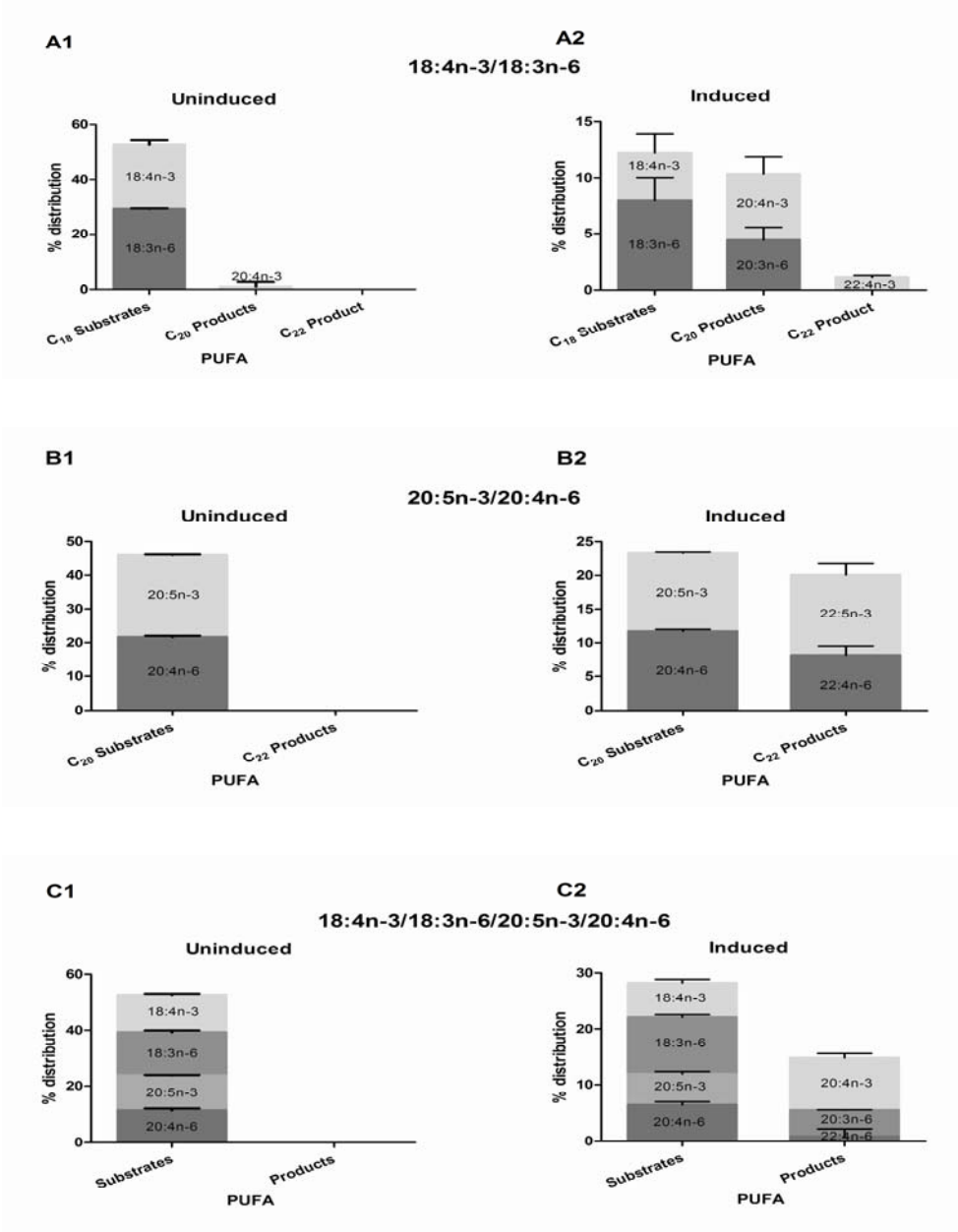

Figure 3.16 Functional characterisation of the sbt*Elovl5* ORF, colony A, in recombinant *S. cerevisiae* grown in the presence of 500 µM in total of 18:4n-3 and 18:3n-6, uninduced (A1) **or induced** (A2)**, 20:5n-3 and 20:4n-6, uninduced** (B1) **or induced** (B2), **or 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6, uninduced** (C1) **or induced** (C2)**.** The yeast were cultured in non-inducing medium containing raffinose or inducing medium containing galactose. Fatty acids were extracted from yeast transformed with the pYES2 vector containing the sbt*Elovl5* ORF. The amount of each fatty acid was expressed as a percentage of the total amount of all fatty acids. This was done by expressing the peak area for an individual fatty acid as a percentage of the total peak area for all fatty acids. The data are the mean ± S.D. (n=3).

Table 3.9 Functional characterization of the sbt*Elovl5* ORF, colony A, using multiple substrate supplementations with 500 µM in total of PUFA

Substrates	Products	Conversion (%)	Pathway
18:4n-3 + 18:3n-6	20:4n-3	58.9 ± 3.4	18:4n-3 → 20:4n-3
	20:3n-6	35.8 ± 1.0	18:3n-6 → 20:3n-6
	22:4n-3	16.7 ± 1.4	18:4n-3 → 20:4n-3 → 22:4n-3
	Total n-3	75.6	
	Total n-6	35.8	
20:5n-3 + 20:4n-6	22:5n-3	50.4 ± 3.6	20:5n-3 → 22:5n-3
	22:4n-6	40.8 ± 3.6	20:4n-6 → 22:4n-6
	Total n-3	50.4	
	Total n-6	40.8	
18:4n-3 + 18:3n-6 +	20:4n-3	60.4 ± 0.5	18:4n-3 → 20:4n-3
20:5n-3 + 20:4n-6	20:3n-6	31.4 ± 1.4	18:3n-6 → 20:3n-6
	22:4n-6	9.8 ± 17.0	20:4n-6 → 22:4n-6
	Total C_{18-20}	60.4	
	Total C_{20-22}	41.2	

The results are expressed as the percentage of fatty acid substrate converted to elongated fatty acid product. The calculation used was [% product/(% product + % substrate)] x 100. Percentage conversion into intermediary products along the elongation pathway is also shown. The data are the mean ± S.D. (n=3). N.D., not detected.

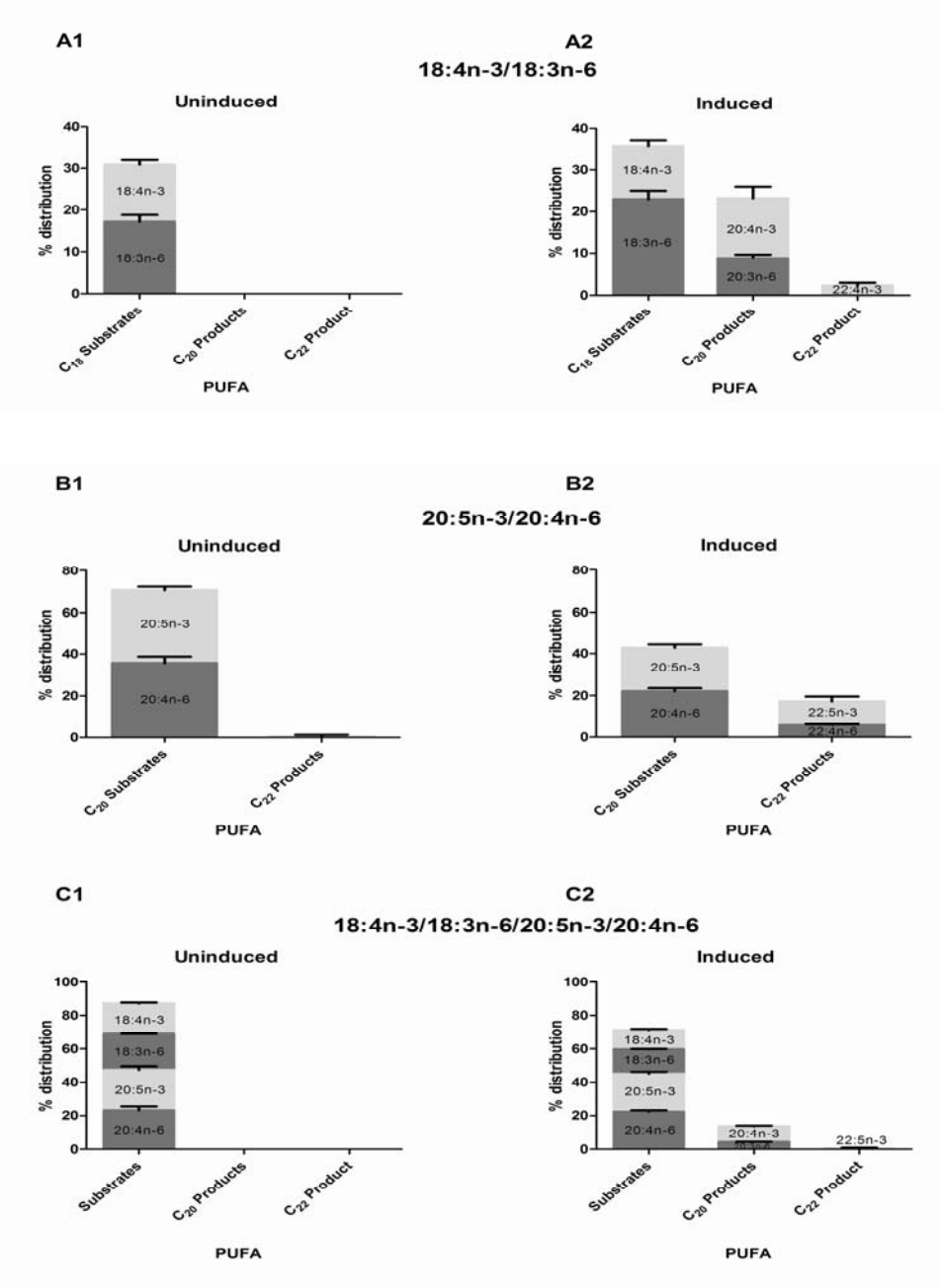

Figure 3.17 Functional characterisation of the sbt*Elovl5* ORF, colony A, in recombinant *S. cerevisiae* grown in the presence of 500 µM of each 18:4n-3 and 18:3n-6, uninduced (A1) **or induced** (A2)**, 20:5n-3 and 20:4n-6, uninduced** (B1) **or induced** (B2)**, or 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6, uninduced** (C1) **or induced** (C2)**.** The yeast were cultured in inducing medium containing galactose. Fatty acids were extracted from yeast transformed with the pYES2 vector containing the sbt*Elovl5* ORF. The amount of each fatty acid was expressed as a percentage of the total amount of all fatty acids. This was done by expressing the peak area for an individual fatty acid as a percentage of the total peak area for all fatty acids. The data are the mean ± S.D. (n=3).

Table 3.10 Functional characterization of the sbt*Elovl5* ORF, colony A, using multiple substrate supplementations with 500 µM of each PUFA

Substrates	Products	Conversion (%)	Pathway
18:4n-3 + 18:3n-6	20:4n-3	52.7 ± 2.3	18:4n-3 → 20:4n-3
	20:3n-6	27.7 ± 1.4	18:3n-6 → 20:3n-6
	22:4n-3	13.6 ± 1.7	18:4n-3 → 20:4n-3 → 22:4n-3
	Total n-3	66.3	
	Total n-6	27.7	
20:5n-3 + 20:4n-6	22:5n-3	35.4 ± 6.5	20:5n-3 → 22:5n-3
	22:4n-6	20.9 ± 1.6	20:4n-6 → 22:4n-6
	Total n-3	35.4	
	Total n-6	20.9	
18:4n-3 + 18:3n-6 +	20:4n-3	44.5 ± 1.9	18:4n-3 → 20:4n-3
20:5n-3 + 20:4n-6	20:3n-6	22.7 ± 0.3	18:3n-6 → 20:3n-6
	Total C_{18-20}	67.2	
	Total C_{20-22}	0	

The results are expressed as the percentage of fatty acid substrate converted to elongated fatty acid product. The calculation used was [% product/(% product + % substrate)] x 100. Percentage conversion into intermediary products along the elongation pathway is also shown. The data are the mean ± S.D. (n=3). N.D., not detected.

The relative amount of PUFA being supplemented to the media and then incorporated into the *S. cerevisiae* cells expressing sbtElovl5 was measured. At the start (T_0) of an individual 500 μM substrate supplementation with 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 or 22:4n-6, the amount of PUFA in the uninducing medium appeared to be higher than in the inducing medium (Figure 3.18). However, the uninducing medium was only found to contain significantly more fatty acid than the inducing medium when supplemented with 18:3n-3, 22:5n-3 or 22:4n-6 (Figure 3.18). The amount of 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3 or 20:4n-6 in the medium after the recombinant *S. cerevisiae* containing sbtElovl5 were incubated for 24 h was not detectable or very minimal (Figure 3.18). Conversely, after the recombinant *S. cerevisiae* containing sbtElovl5 were incubated with 22:5n-3 or 22:4n-6, approximately 10% and 37%, respectively, of each of these substrates remained in the medium after 24 h (Figure 3.18). Elongation products were not released from the yeast cells into the medium.

Approximately the same amount of total PUFA was incorporated into the *S. cerevisiae* cells regardless of the growth medium (Figure 3.19). The induced cultures incorporated approximately the same amount of total PUFA, measured as the accumulation of the supplemented fatty acid and the subsequent elongation products, compared to the uninduced cultures which incorporated the supplemented fatty acid only (Figure 3.19).

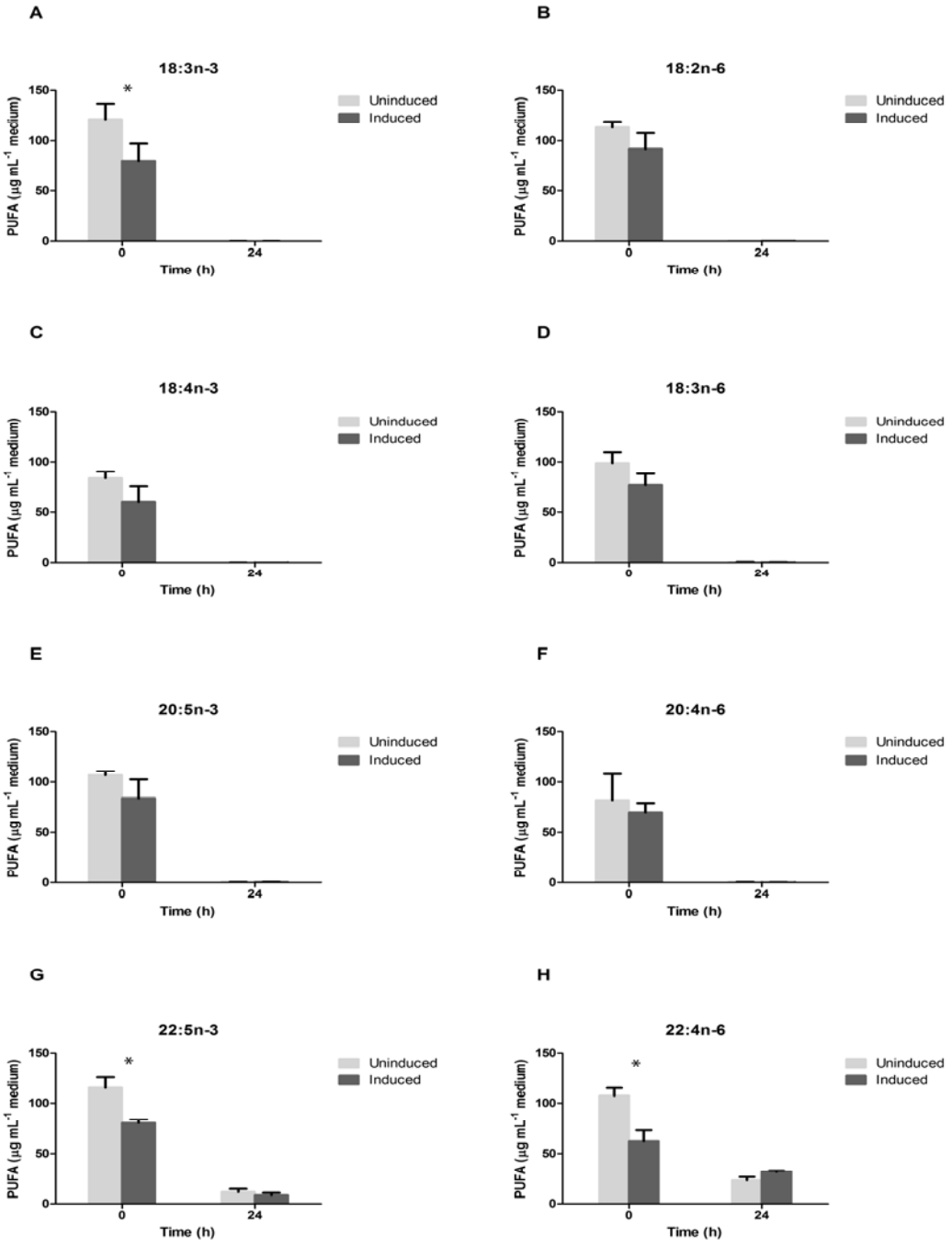

Figure 3.18 Characterisation of the media used in the recombinant *S. cerevisiae* expression system containing the sbt*Elovl5* ORF, colony A. Fatty acids were extracted from the media at T_{0h} and T_{24h} after supplementation with 18:3n-3 (A), 18:2n-6 (B), 18:4n-3 (C), 18:3n-6 (D), 20:5n-3 (E), 20:4n-6 (F), 22:5n-3 (G) and 22:4n-6 (H). The yeast were cultured in either non-inducing medium containing raffinose or inducing medium containing galactose. The amount of each fatty acid was expressed in µg per mL of medium. The data are the mean ± S.D. (n=3). Within each time point the mean values bearing an asterisk are significantly different.

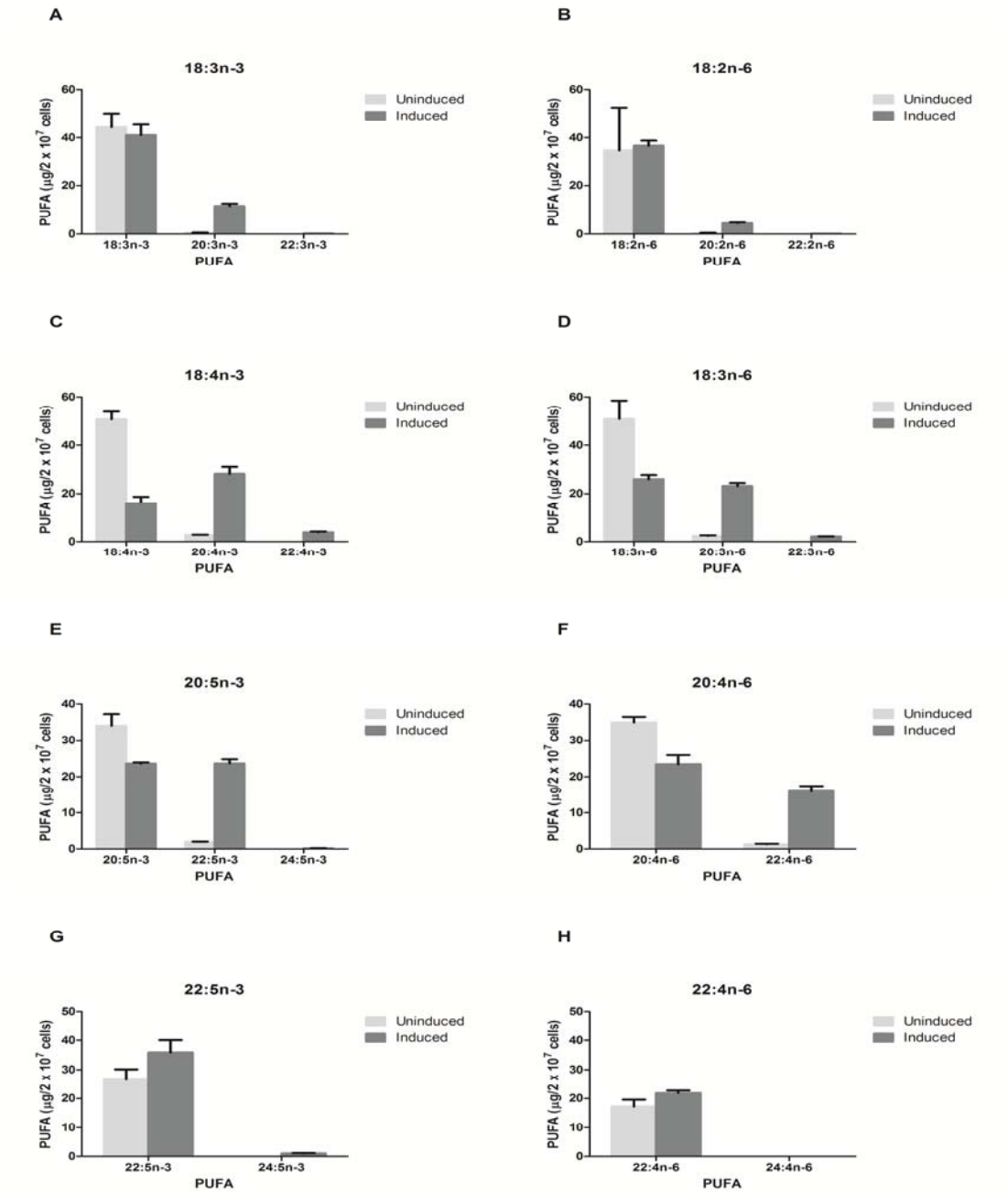

Figure 3.19 Characterisation of the cells in the recombinant *S. cerevisiae* expression system containing the sbt*Elovl5* ORF, colony A. The recombinant *S. cerevisiae* were grown in the presence of 18:3n-3 (A), 18:2n-6 (B), 18:4n-3 (C), 18:3n-6 (D), 20:5n-3 (E), 20:4n-6 (F), 22:5n-3 (G) and 22:4n-6 (H) fatty acids. The yeast were cultured in either non-inducing medium containing raffinose or inducing medium containing galactose. Fatty acids were extracted from yeast transformed with the pYES2 vector containing the sbt*Elovl5* ORF after 24 h. The amount of each fatty acid was expressed in µg per 2 x 10^7 cells (cells from an OD_{600}=1). The data are the mean ± S.D. (n=3).

3.3.6 Functional characterization of the sbt*Δ6des* ORF

The sbt*Δ6des* ORF was expressed in *S. cerevisiae* to investigate its functionality. Recombinant *S. cerevisiae* cells were cultured in the presence of the Δ6desaturase substrates 18:3n-3 or 18:2n-6 to determine the sbtΔ6des enzyme's substrate specificity. The expected desaturation products 18:4n-3 and 18:3n-6 were not detected. Recombinant *S. cerevisiae* cells cultured in the presence of a Δ5desaturase substrate 20:3n-6 did not produce 20:4n-6. Furthermore, other PUFA substrates including 20:5n-3, 22:5n-3 or 22:4n-6 were supplied but no desaturation products were detected. All of the substrates were incorporated into the cells but they were apparently not desaturated (Appendix C.3). Six individual transformed yeast colonies containing the sbtΔ6des were screened and the result was the same.

The translation initiation sequence preceding the start codon of the sbt*Δ6des* was modified using SDM to enable the *S. cerevisiae* to scan the translation initiation region and locate the start codon, without interference from secondary structures. This form of the sbt*Δ6des* sequence was referred to as sbt*Δ6des*SDM. A nucleotide alignment of the sbt*Δ6des* and sbt*Δ6des*SDM sequences shows the two bases which were modified using site directed mutagenesis (Figure 3.20). The sbt*Δ6des*SDM ORF was expressed in *S. cerevisiae* to investigate if the modified translation initiation sequence enabled sbtΔ6des functionality. Recombinant *S. cerevisiae* cells cultured in the presence of 18:3n-3 or 20:3n-6 did not produce any desaturation products. The substrates were incorporated into the cells but were once more apparently not desaturated. Six individual colonies containing the sbtΔ6desSDM were screened and the result was the same.

Heterologous expression in yeast can be enhanced by the addition of trace metals. Iron sulphate and copper sulphate were added as metal cofactors to the *S. cerevisiae* cultures expressing sbt*Δ6des* or sbt*Δ6des*SDM. Recombinant *S. cerevisiae* cells cultured in the presence of metal cofactors and 18:3n-3 or 20:3n-6 did not produce any desaturation products. The substrates were incorporated into the cells but the metal cofactors did not enhance the sbt*Δ6des* or sbt*Δ6des*SDM functionality.

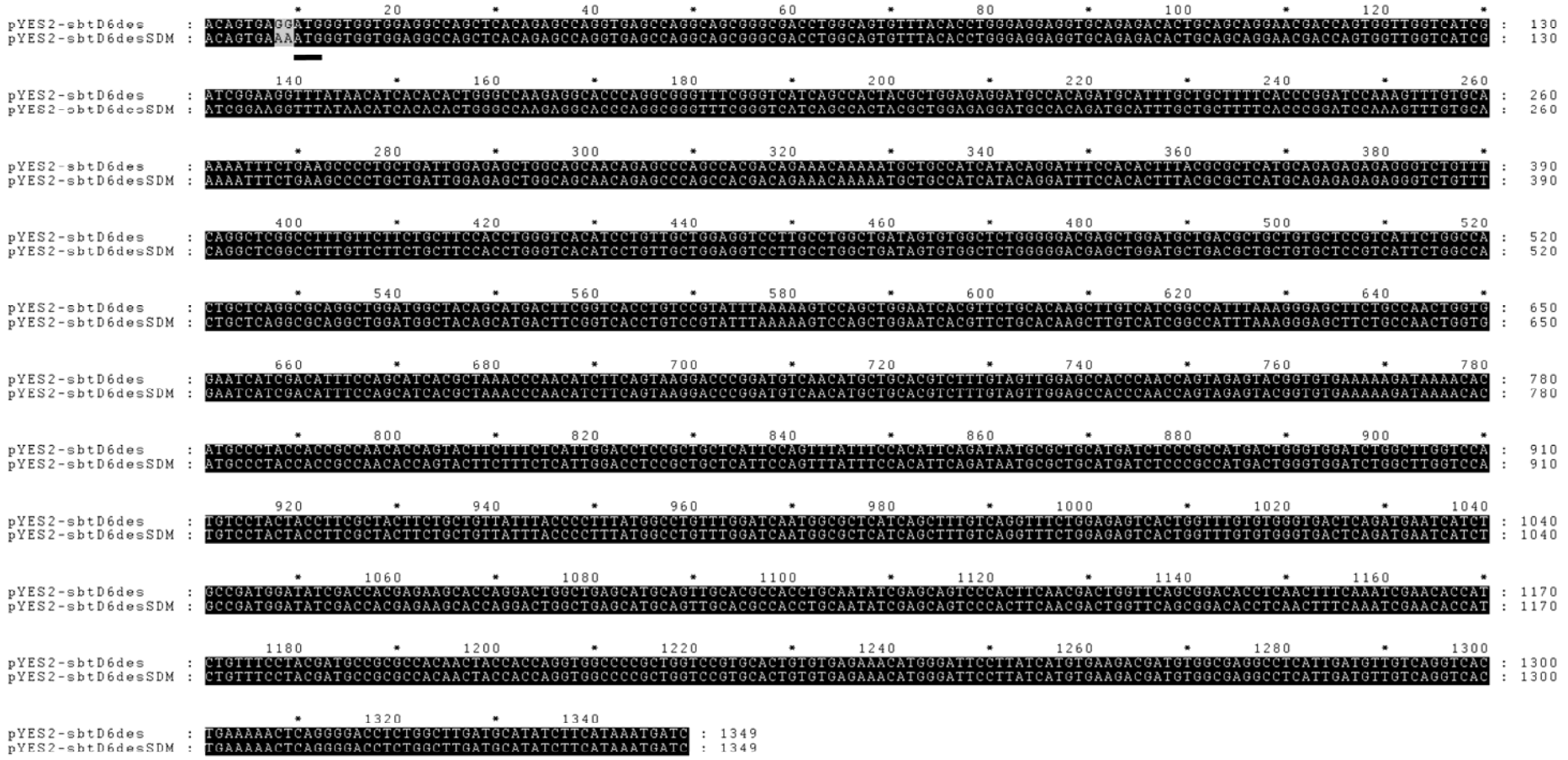

Figure 3.20 Pairwise alignment of the pYES2-sbt*Δ6des* and pYES2-sbt*Δ6des*SDM nucleotide sequences which were expressed in *S. cerevisiae*. The underlined bases are the start codon.

3.3.7 Confirmation of the presence of the sbt*Δ6des* and sbt*Δ6des*SDM constructs within the *S. cerevisiae* cells

The presence within the recombinant *S. cerevisiae* cells of the sbt*Δ6des* and sbt*Δ6des*SDM constructs was confirmed by PCR of genomic DNA and subsequent sequence analysis. Amplification of genomic DNA from six sbt*Δ6des* colonies with the pYES2 specific primer set produced a band of approximately 1400 bp (Figure 3.21A). These findings were confirmed by PCR amplification from the same six colonies using the sbt*Δ6des* specific primer set F, which produced a band of approximately 1700 bp and a smaller product of 350 bp (Figure 3.21B). Amplification of genomic DNA from two sbt*Δ6des*SDM colonies using sbt*Δ6des*SDM specific primers produced a 1400 bp band (Figure 3.21C). These findings indicated that the sbt*Δ6des* and sbt*Δ6des*SDM constructs had been successfully transformed into the *S. cerevisiae* cells. For final confirmation, sequencing was done. Sequencing revealed that the sbt*Δ6des* products were identical to the original full length sbt*Δ6des* product amplified except for two base differences (Figure 3.22). Despite these two base changes, when the deduced protein sequences were compared, there were no amino acid changes (Figure 3.23). Sequencing confirmed the sbt*Δ6des*SDM construct expressed in *S. cerevisiae* included the two modified bases prior to the start codon, but no other base changes (Figure 3.20).

3.3.8 Confirmation of the transcription of the sbt*Δ6des* and sbt*Δ6des*SDM constructs within the *S. cerevisiae* cells

The cDNAs from uninduced and induced *S. cerevisiae* cultures containing sbt*Elovl5* (positive control), sbt*Δ6des* and sbt*Δ6des*SDM constructs were successfully amplified. The sbt*Δ6des* and sbt*Δ6des*SDM products were approximately 1400 bp (Figure 3.24). The cDNAs from uninduced and induced cultures for each colony were amplified at relatively similar intensities (Figure 3.24). This confirmed that the sbt*Δ6des* and sbt*Δ6des*SDM constructs were being transcribed in the *S. cerevisiae* cells.

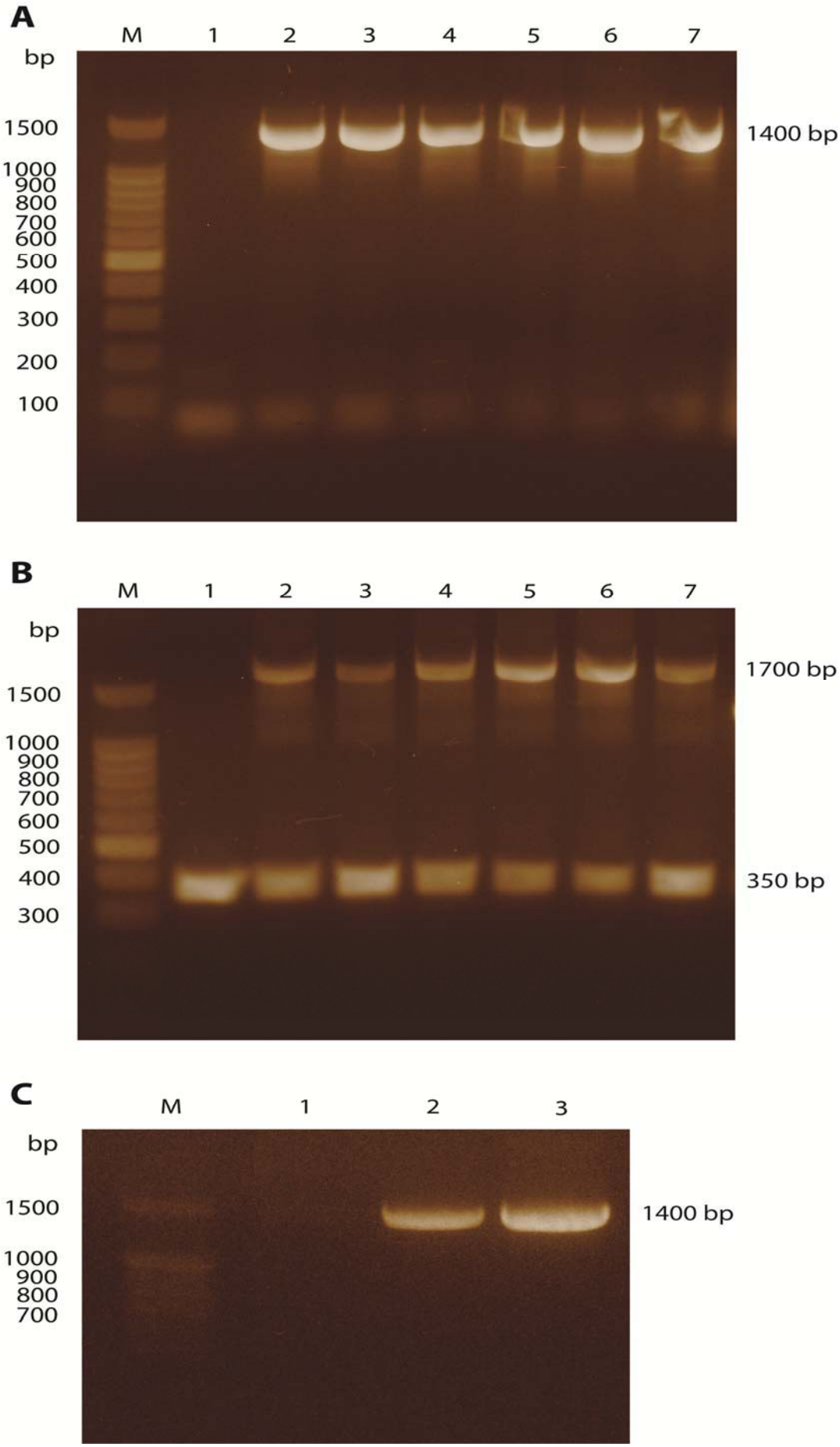
A
M 1 2 3 4 5 6 7
bp
1500
1000
900
800
700
600
500
400
300
200
100
1400 bp
B
M 1 2 3 4 5 6 7
bp
1500
1000
900
800
700
600
500
400
300
1700 bp
350 bp
C
M 1 2 3
bp
1500
1000
900
800
700
1400 bp

Figure 3.21 Visualisation of sbt*Δ6des* or sbt*Δ6des*SDM PCR products amplified from *S. cerevisiae* genomic DNA, as described in section 3.2.26.

(A) sbt*Δ6des* PCR products amplified using the pYES2 specific primer set.

Lane 1 – negative control (no template) pYES2 specific primer set
Lane 2 – colony A - *S. cerevisiae* containing sbt*Δ6des*
Lane 3 – colony B - *S. cerevisiae* containing sbt*Δ6des*
Lane 4 – colony C - *S. cerevisiae* containing sbt*Δ6des*
Lane 5 – colony D - *S. cerevisiae* containing sbt*Δ6des*
Lane 6 – colony E - *S. cerevisiae* containing sbt*Δ6des*
Lane 7 – colony F - *S. cerevisiae* containing sbt*Δ6des*

(B) sbt*Δ6des* PCR products amplified using the sbt*Δ6des* primer set F (Table 3.2). The gel lanes contain the same samples as Gel A, except amplification was performed with sbt*Δ6des* primer set F rather than the pYES2 specific primer set.

(C) sbt*Δ6des*SDM PCR products amplified using sbt*Δ6des*SDM F and sbt*Δ6des* *Not*1 R (Table 3.2)

Lane 1 – negative control (no template) sbt*Δ6des*SDM F and sbt*Δ6des* *Not*1 R primers
Lane 2 – colony A - *S. cerevisiae* containing sbt*Δ6des*SDM
Lane 3 – colony B - *S. cerevisiae* containing sbt*Δ6des*SDM

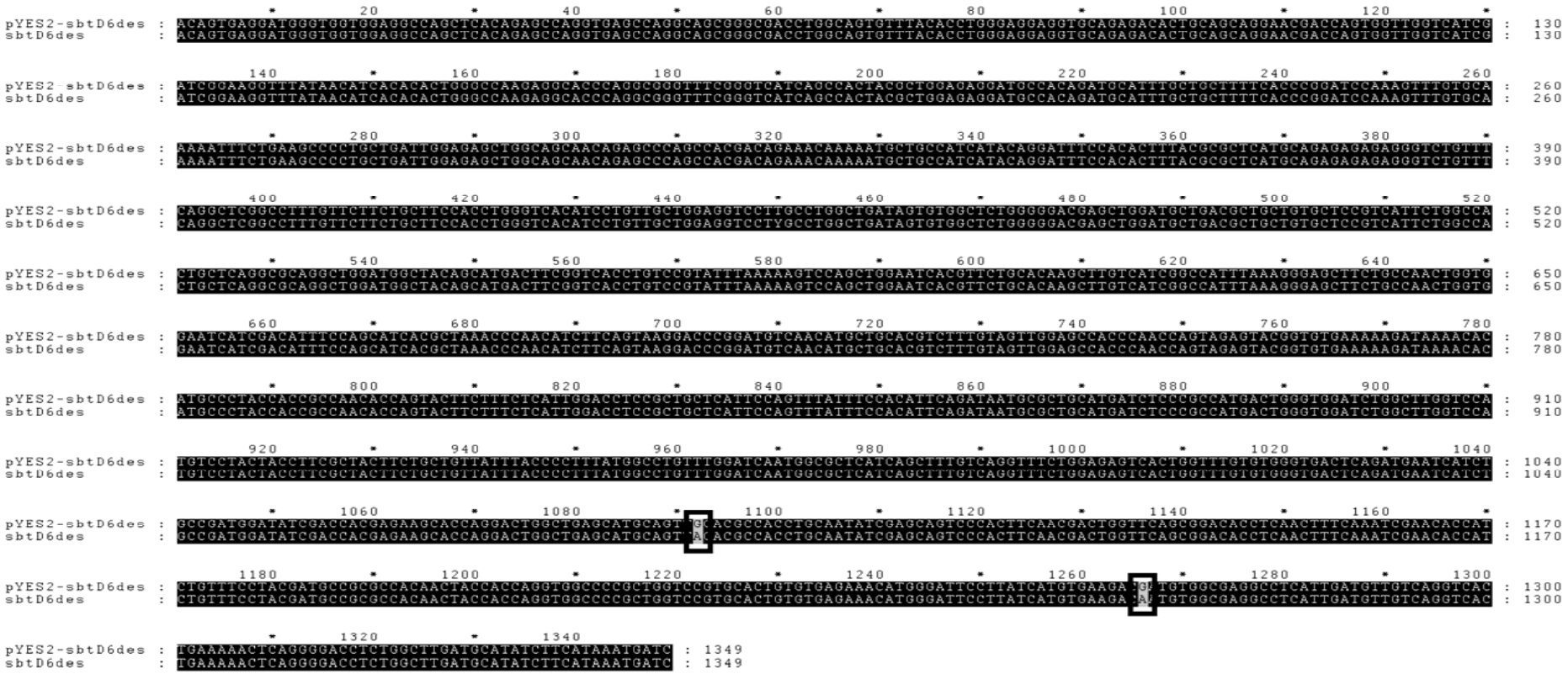

Figure 3.22 Pairwise alignment of the sbt*Δ6des* and pYES2-sbt*Δ6des* nucleotide sequences. The sbt*Δ6des* was amplified as one fragment, sequenced and cloned into pYES2. The pYES2-sbt*Δ6des* was subsequently expressed in *S. cerevisiae*. The boxes highlight the base differences.

Figure 3.23 Pairwise alignment of the sbtΔ6des and pYES2-sbtΔ6des predicted protein sequences. The sbt*Δ6des* was amplified as one fragment, sequenced and cloned into pYES2. The pYES2-sbt*Δ6des* was subsequently expressed in *S. cerevisiae*. There are no amino acid residue differences between the sequences.

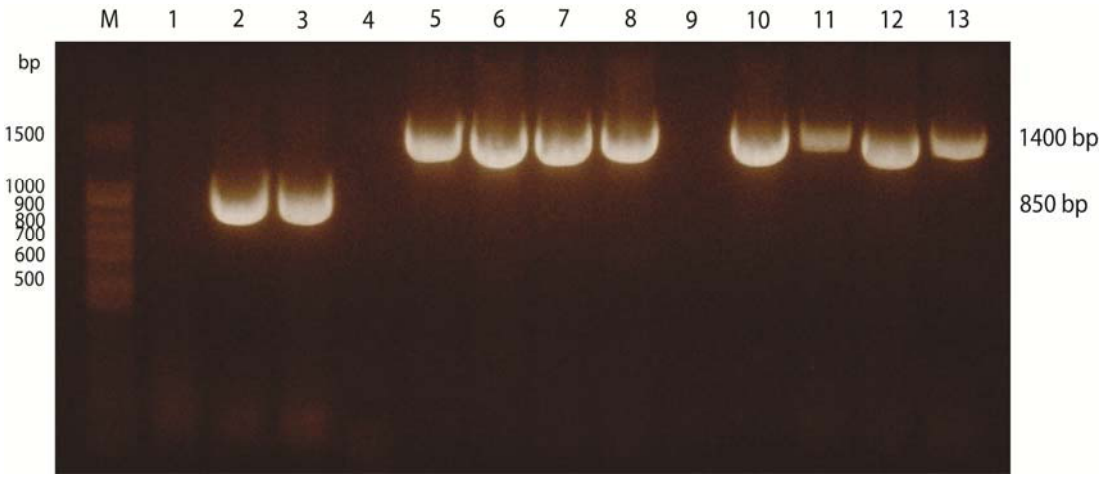

Figure 3.24 Visualisation of PCR products amplified from RNA extracted from *S. cerevisiae* containing sbt*Elovl5*, sbt*Δ6des* or sbt*Δ6des*SDM and synthesized into cDNA, as described in section 3.2.27. PCR amplification of sbt*Elovl5* used the sbt*Elovl5* primer set D, sbtΔ6des used the sbtΔ6des primer set F and sbt*Δ6des*SDM used the sbt*Δ6des*SDM F and sbt*Δ6des Not*1 R primers (Table 3.1 and Table 3.2). The sbt*Elovl5* product can be seen at 850 bp, while the sbt*Δ6des* and sbt*Δ6des*SDM products can be seen at 1400 bp.

Lane 1 – negative control (no template) sbt*Elovl5* primer set D
Lane 2 – uninduced *S. cerevisiae* containing sbt*Elovl5* – colony A
Lane 3 – induced *S. cerevisiae* containing sbt*Elovl5* – colony A
Lane 4 – negative control (no template) sbtΔ6des primer set F
Lane 5 – uninduced *S. cerevisiae* containing sbt*Δ6des* – colony A
Lane 6 – induced *S. cerevisiae* containing sbtΔ6des – colony A
Lane 7 – uninduced *S. cerevisiae* containing sbt*Δ6des* – colony B
Lane 8 – induced *S. cerevisiae* containing sbt*Δ6des* – colony B
Lane 9 – negative control (no template) sbt*Δ6des*SDM F and sbt*Δ6des Not*1 R primers
Lane 10 – uninduced *S. cerevisiae* containing sbt*Δ6des*SDM – colony A
Lane 11 – induced *S. cerevisiae* containing sbt*Δ6des*SDM – colony A
Lane 12 – uninduced *S. cerevisiae* containing sbt*Δ6des*SDM – colony B
Lane 13 – induced *S. cerevisiae* containing sbt*Δ6des*SDM – colony B

3.3.9 Analysis of the relative protein levels of the sbtElovl5, sbtΔ6des and sbtΔ6desSDM in *S. cerevisiae*

The sbtElovl5 enzyme expressed in *S. cerevisiae* elongated various PUFA substrates when they were supplied to the cultures. In contrast, the sbtΔ6des or sbtΔ6desSDM enzymes expressed in *S. cerevisiae* did not appear to desaturate PUFA substrates when they were supplied to the cultures. Thus, the sbtElovl5 enzyme was active, while no detectable activity was seen for the sbtΔ6des or sbtΔ6desSDM enzymes. It was previously shown that transcription of sbtElovl5, sbtΔ6des and sbtΔ6desSDM in *S. cerevisiae* appeared to be occurring. To investigate the apparent lack of activity of sbtΔ6des and sbtΔ6desSDM, despite the detectable transcript levels, immunoblot analysis was done on the total protein extracted from *S. cerevisiae* cells expressing sbtElovl5, sbtΔ6des or sbtΔ6desSDM. For detection of the recombinant proteins, the sbt*Elovl5*, sbt*Δ6des* and sbt*Δ6des*SDM were cloned into the pYES2/CT vector which contained 6X His and V5 epitope tags. The total protein from *S. cerevisiae* cells which contained the empty pYES2/CT vector or pYES2/CT-sbtElovl5, pYES2/CT-sbtΔ6des or pYES2/CT-sbtΔ6desSDM constructs was extracted. Concentrating of the total protein extracts was performed and an equal amount of both samples was loaded onto a SDS-PAGE gel. The V5 antibody was found to be more specific than the His antibody, so it was selected for further use.

The immunoblot confirmed the observations seen for the sbtElovl5 transcript levels and activity. The expression of sbtElovl5 was detected in the total protein extract from the *S. cerevisiae* cells, at approximately 35 kDa, as expected (www.expasy.ch/tools/pi_tool.html) (Figure 3.25, lane 3). In contrast, the expression of sbtΔ6des or sbtΔ6desSDM was below the detection level in the total protein extract from the *S. cerevisiae* cells (Figure 3.25, lanes 5 and 7). The total protein extracted was concentrated to increase the amount of sbtΔ6des or sbtΔ6desSDM protein for detection. Degradation of the sbtElovl5, sbtΔ6des and sbtΔ6desSDM proteins occurred after concentrating the proteins. This can be seen most clearly with sbtElovl5 which apparently degraded into two smaller fragments of approximately 30 and 5 kDa (Figure 3.25, lane 4). The sbtΔ6des protein was expected to be approximately 52 kDa (www.expasy.ch/tools/pi_tool.html). The sbtΔ6des protein could only be detected in an apparently degraded form of the protein at

approximately 6 kDa, while the apparently degraded forms of the sbtΔ6desSDM protein were detected at approximately 35 and 15 kDa (Figure 3.25). Intact sbtΔ6des or sbtΔ6desSDM proteins were not detected but a similar pattern of degradation was seen for the sbtElovl5 and sbtΔ6desSDM (Figure 3.25).

The relative abundances of the sbtElovl5, sbtΔ6des and sbtΔ6desSDM proteins after concentrating the samples suggest that sbtElovl5 was more highly expressed in *S. cerevisiae* than sbtΔ6des or sbtΔ6desSDM (Figure 3.25). The sbtΔ6desSDM protein appears to be more highly expressed than sbtΔ6des because very little sbtΔ6des protein could be detected although all sbtΔ6desSDM and sbtΔ6des proteins detected were apparently in a degraded form (Figure 3.25). This may suggest that the modified translation initiation sequence increased translation of the sbtΔ6des protein in *S. cerevisiae*.

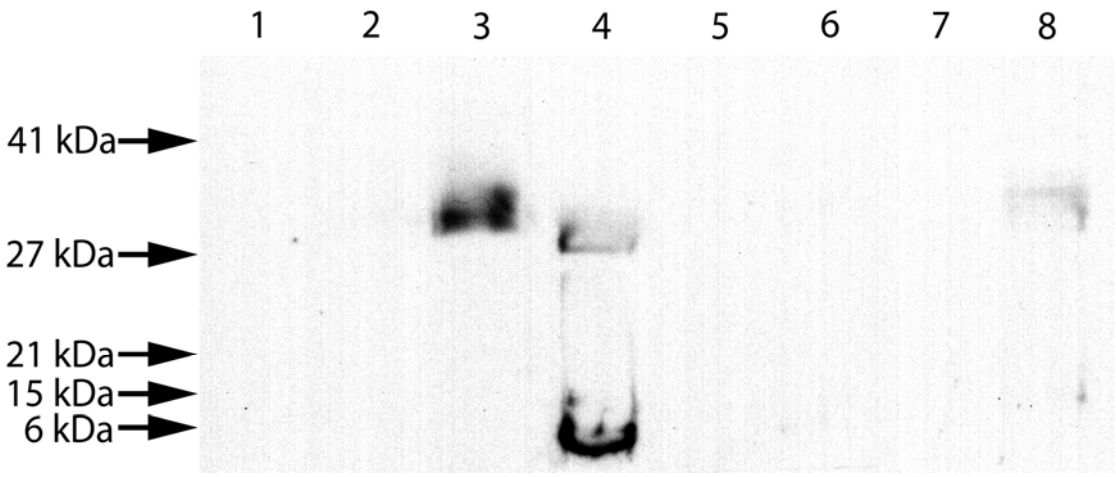

Figure 3.25 Western blot analysis of the relative protein levels of the sbtElovl5, sbtΔ6des and sbtΔ6desSDM in *S. cerevisiae*. Protein levels of the sbtElovl5, sbtΔ6des and sbtΔ6desSDM in *S. cerevisiae* after 24 h induction. Protein was detected using the V5 antibody after a 2 h exposure.

Lane 1 – empty pYES2/CT vector – total protein extract
Lane 2 – empty pYES2/CT vector – concentrated total protein
Lane 3 – sbtElovl5 – total protein extract
Lane 4 – sbtElovl5 – concentrated total protein
Lane 5 – sbtΔ6des – total protein extract
Lane 6 – sbtΔ6des – concentrated total protein
Lane 7 – sbtΔ6desSDM – total protein extract
Lane 8 – sbtΔ6desSDM – concentrated total protein

3.3.10 qRT-PCR analysis of SBT tissues

3.3.10.1 Amplification efficiency

The amplification efficiency of the *Δ6desaturase* and *Elovl5* primer pairs was assessed by measuring the C_T value with various template dilutions (plasmid containing gene of interest). A standard curve was produced and the reaction efficiency for *Δ6desaturase* and *Elovl5* were 0.95 and 0.99, respectively.

3.3.10.2 Amplification of Δ6desaturase and Elovl5 PCR products

The *Δ6desaturase* and *Elovl5* PCR products were analysed by gel electrophoresis to test for the presence of any contaminating genomic DNA or primer-dimer formation. The melt curve analysis at the end of the qRT-PCR cycling also checked for these contaminants. Agarose gel electrophoresis and melt curve analysis of *Δ6desaturase* and *Elovl5* PCR products were performed. A typical melt curve obtained after amplification of *Δ6desaturase* and *Elovl5* can be seen in Appendices D.1 and D.2, respectively. The findings showed the primers were specific, there was no contaminating genomic DNA as the expected product sizes were seen and there was no visible primer-dimer formation.

3.3.10.3 Δ6desaturase and Elovl5 mRNA abundance

The *Elovl5* transcripts were three times more abundant in the liver and gonad tissues than they were in the spleen and kidney tissues (Figure 3.26). The *Δ6desaturase* transcript levels from the liver, gonad and kidney tissues were very low and detection only occurred after 30 cycles of qRT-PCR. Only one sample for each tissue type was available. Therefore, the tissue samples were pseudoreplicates, as separate cDNA synthesis reactions were performed from total RNA extracted from one sample.

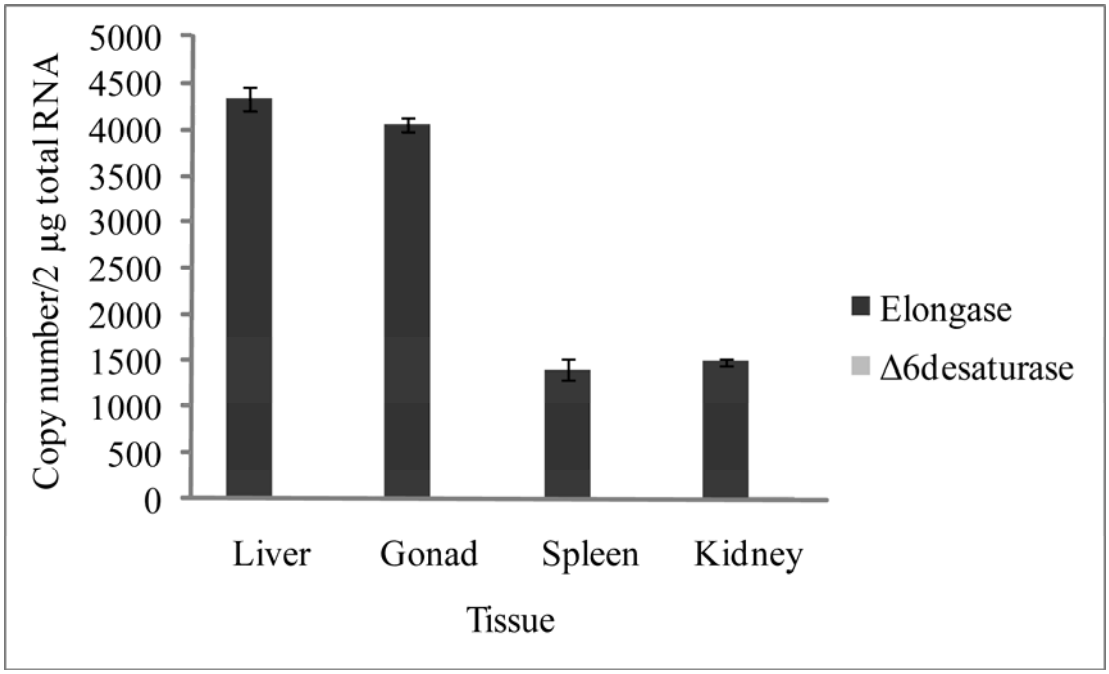

Figure 3.26 Tissue distribution of the expression of the SBT fatty acyl elongase and Δ6desaturase genes. Transcript (mRNA) copy number was determined by qRT-PCR as described in section 3.2.9. Data are expressed as the copy number in 2 µg of total RNA. Data are the mean ± SE (n = 3). Replicates are all from the same tissue source.

3.4 Discussion

A fatty acyl elongase and a fatty acyl desaturase cDNA were cloned and characterized from the important southern Australian aquaculture fish species SBT. The fatty acyl elongase cDNA encoded a predicted protein which had the main structural characteristic features of microsomal fatty acyl elongases from mammals and other fish (Agaba *et al.* 2005; Jakobsson *et al.* 2006). The structural features included the four conserved motifs, a histidine box (HxxHH), C-terminal ER retention signal and multiple transmembrane regions. Sequence comparisons and phylogenetic analyses revealed that the sbtElovl5 predicted protein was most closely related to previously described fatty acyl elongases from the marine fish gilthead sea bream, cobia and turbot. The phylogenetic analysis of fatty acyl elongases revealed four clusters of fish which follow the evolution of the modern teleosts. The clusters were the zebrafish and North African catfish (Ostariophysi; cypriniformes and siluriformes), southern bluefin tuna, gilthead sea bream, cobia, turbot and Nile tilapia (Acanthopterygii; perciformes and pleuronectiformes), cherry salmon, rainbow trout and Atlantic salmon (Protacanthopterygii; salmoniformes) and Atlantic cod (Paracanthopterygii; gadiformes) (Nelson 1994). In a comparison with the human fatty acyl elongases, the sbtElovl5 more closely resembled the human ELOVL5 than the ELOVL2.

The fatty acyl desaturase cDNA encoded a predicted protein which had the main structural characteristic features of microsomal fatty acyl desaturases, including two transmembrane regions, three histidine boxes HXXXH, HXXHH and QIEHH, and an N-terminal cytochrome b_5-like domain containing the heme-binding motif (Knutzon *et al.* 1998; Sperling and Heinz 2001). Sequence comparison and phylogenetic analysis revealed that the sbtΔ6des predicted protein was most closely related to previously described fatty acyl desaturases from the marine fish European sea bass, gilthead sea bream, cobia and turbot. Similar to the phylogenetic analysis of the fatty acyl elongases, the desaturases revealed the same clusters when a similar range of teleosts was analysed in addition to the European sea bass and white-spotted spinefoot (Acanthopterygii; perciformes), and carp (Ostariophysi; cypriniformes).

Functional characterisation of the sbtElovl5 and sbtΔ6des was done in *S. cerevisiae* as it does not contain Δ5-, Δ6-, Δ12- or Δ15-desaturases or the elongases required to elongate PUFA (Knutzon *et al.* 1998; Leonard *et al.* 2000). This renders *S. cerevisiae* incapable of producing LCPUFA and lends itself to be a suitable host for heterologous expression of elongase or Δ6desaturase genes (Knutzon *et al.* 1998; de Antueno *et al.* 2001). Yeast episomal plasmid (YEp) vectors transform *S. cerevisiae* very efficiently (5,000-20,000 recombinants per μg DNA) and replicate autonomously (Strathern *et al.* 1982; Grivell 1992). Two *S. cerevisiae* colonies containing the exogenous gene were screened to ensure the integrity of the over-expression system. Heterologous expression of the sbtElovl5 ORF resulted in reproducible levels of enzyme activity in the two colonies screened.

Functional characterization of Elovl5-like fatty acyl elongases from fish species has been reported for zebrafish (Agaba *et al.* 2004), rainbow trout (Meyer *et al.* 2004), Nile tilapia, North African catfish, Atlantic cod, gilthead sea bream, turbot (Agaba *et al.* 2005), Atlantic salmon (Hastings *et al.* 2005), cherry salmon (Alimuddin *et al.* 2008) and cobia (Zheng *et al.* 2009). All of these fatty acyl elongases preferred C_{18} and C_{20} PUFA substrates over C_{22} PUFA substrates. The catfish, sea bream, zebrafish and Atlantic salmon elongases showed more C_{18-20} than C_{20-22} PUFA conversion (Agaba *et al.* 2004; Agaba *et al.* 2005; Hastings *et al.* 2005) whereas the tilapia, turbot and cobia fatty acyl elongases showed approximately equal C_{18-20} and C_{20-22} PUFA conversion (Agaba *et al.* 2005; Zheng *et al.* 2009). The sbtElovl5 was more like the second group showing approximately equal 18:4n-3 and 20:5n-3 conversion, with percentage conversion values of 83.5% and 76.8%, respectively. The sbtElovl5 was able to elongate both the C_{20} and C_{22} n-3 PUFA substrates through to C_{24} PUFA products. The sbtElovl5 exhibited higher activity towards the n-3 substrates than the n-6 substrates, regardless of the chain length of the PUFA substrate. This finding has also been reported for other fish species such as Atlantic salmon, zebrafish, catfish and tilapia (Agaba *et al.* 2005). The freshwater and salmonid species are generally more active towards the n-3 substrates than the marine species (Zheng *et al.* 2009). This could be a result of evolutionary selective pressure in freshwater and salmonid species for highly active LCPUFA synthesis enzymes (Zheng *et al.* 2009).

Broad substrate specificity is a notable feature of vertebrate fatty acyl elongases compared to the lower eukaryote fatty acyl elongases from *M. alpina* and *C. elegans* (Beaudion *et al.* 2000; Parker-Barnes *et al.* 2000). The lower eukaryote enzymes are highly specific for C_{18} PUFA substrates. The human elongase ELOVL5 and the rat elongase rELO1, were both found to elongate C_{18} and C_{20} PUFA substrates, but not C_{22} PUFA substrates (Leonard *et al.* 2000; Inagaki *et al.* 2002). The mouse Elovl2 was shown to be active in elongating C_{20} and C_{22} PUFA substrates, whilst only a small amount of conversion of C_{18} PUFA substrates occurred and no saturated or monounsaturated fatty acids were elongated (Leonard *et al.* 2002). The human ELOVL2 was only active in elongating C_{20} and C_{22} PUFA substrates, with no conversion of C_{18} PUFA or saturated and monounsaturated fatty acids (Leonard *et al.* 2002). The sbtElovl5 substrate preference suggests that it is most similar to the mammalian ELOVL5 and rELO1 enzymes (Leonard *et al.* 2000; Inagaki *et al.* 2002).

The recombinant sbtElovl5 enzyme was very efficient at elongating the immediate precursors 18:4n-3 and 18:3n-6 but it was also capable of elongating 18:3n-3 and 18:2n-6, albeit with much lower efficiencies. The 18:3n-3 and 18:2n-6 substrates are of interest because they are abundant in vegetable oils which are increasingly replacing fish oils in the diets of farmed fish (Whelan and Rust 2006). The sbtElovl5 converted 15.7% of 18:3n-3 to 20:3n-3 and 10.8% of 18:2n-6 to 20:2n-6, respectively. Interestingly, ELOVL5, rELO1 and the *C. elegans* fatty acyl elongase have all been shown to elongate 18:3n-3 and 18:2n-6 at a lower efficiency than 18:4n-3 and 18:3n-6 (Beaudion *et al.* 2000; Leonard *et al.* 2000; Inagaki *et al.* 2002). Human ELOVL5 elongated 30.4% of 18:3n-3 compared to 79.2% of 18:4n-3, whilst 18:2n-6 as a substrate has not been tested (Leonard *et al.* 2000). The rELO1 elongated 33% of 18:3n-3 compared to 96.8% of 18:4n-3 and 13% of 18:2n-6 compared to 82.4% of 18:3n-6 (Inagaki *et al.* 2002). The enzyme-substrate interaction is dependent on weak hydrophobic interactions which can result in lower substrate specificity (Sargent *et al.* 2002). 20:3n-3 and 20:2n-6 are not considered intermediates of the accepted metabolic pathway for the synthesis of LCPUFA from C_{18} PUFA (Figure 1.1). An alternative pathway involving Elovl5 followed by Δ8desaturase (18:3n-3/18:2n-6 → 20:3n-3/20:2n-6 → 20:4n-3/20:3n-6) rather than the traditional Δ6desaturase followed by Elovl5 (18:3n-3/18:2n-6 → 18:4n-3/18:3n-6 → 20:4n-3/20:3n-6) would synthesize 20:4n-3 and 20:3n-6 for further

Δ5desaturation and continuation through the pathway. Park *et al.* (2009) have shown that the baboon FADS2 has both Δ6desaturation and Δ8desaturation activity when the substrates 18:3n-3 and 20:3n-3 or 18:2n-6 and 20:2n-6 were available. The introduction of vegetable oils into the SBT diet will increase the abundance of 18:3n-3 and 18:2n-6. The high enzyme activity of sbtElovl5 towards C_{18} PUFA substrates may enable an alternative Elovl5-Δ8desaturase pathway to be used.

The LCPUFA synthesis pathway may use the same rate-limiting enzymes in both the n-3 and n-6 pathway, so it is important to understand the sbtElovl5 activity when more than one PUFA substrate is present. In the LCPUFA synthesis pathway 18:4n-3 and 18:3n-6 are in direct competition for elongation by the sbtElovl5 (Figure 1.1). Similarly, after Δ5desaturation, 20:5n-3 and 20:4n-6 are competing for elongation by the sbtElovl5 (Figure 1.1). This direct competition showed that the sbtElovl5 strongly preferred n-3 over n-6 PUFA substrates. The multiple substrate supplementation experiments uniquely addressed the issue of substrate competition which is found in nature. The four immediate substrates of sbtElovl5, 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6 all compete for elongation at the same step in the LCPUFA synthesis pathway (Figure 1.1). When equal amounts of all of the potential sbtElovl5 substrates were available, the sbtElovl5 preferentially utilized 18:4n-3 and 18:3n-6 over 20:5n-3 and 20:4n-6. Interestingly, the effect of increasing the total concentration of direct substrates increased the competition for elongation by sbtElovl5 and subsequently sbtElovl5 was less efficient at converting C_{18} and C_{20} PUFA. When 500 μM of each of 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6 were supplied, there was an abundance of C_{18} PUFA which was preferentially elongated, resulting in the C_{20} PUFA substrates not being accepted. In nature the availability of 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6 may not be in equal proportions and so the competition at the sbtElovl5 position of the LCPUFA synthesis pathway would be intensified. For example, a high intake of dietary vegetable oil would make more 18:4n-3 and 18:3n-6 available and the efficiency of the sbtElovl5 to elongate 20:5n-3 and 20:4n-6 may be compromised. Future work could include supplementing with various ratios of $C_{18/20}$ PUFA and n-3/n-6 PUFA to understand the impact a natural versus a formulated pellet diet would have on the SBT lipid metabolism.

The sbtElovl5 was also active towards the endogenous yeast saturated and monounsaturated fatty acids. Elongation of the endogenous saturated fatty acids was made apparent by an increased 18:0/16:0 ratio for all of the n-3 and n-6 PUFA substrates. These data are consistent with the zebrafish fatty acyl elongase activity towards saturated fatty acids (Agaba *et al.* 2004). In contrast no such activity was observed with the Atlantic salmon, North African catfish, turbot, Nile tilapia or gilthead sea bream fatty acyl elongases (Hastings *et al.* 2004; Agaba *et al.* 2005). The elongation products of the endogenous monounsaturated fatty acids 16:1n-7 and 18:1n-9 were 18:1n-7 and 20:1n-9, respectively. Zebrafish, Atlantic salmon and gilthead sea bream fatty acyl elongases also elongated 16:1n-7 and 18:1n-9, whilst North African catfish, Nile tilapia and turbot only elongated 16:1n-7 (Agaba *et al.* 2004; Hastings *et al.* 2004; Agaba *et al.* 2005). Thus, it appears that the sbtElovl5 was not only active with PUFA substrates, but also with saturated and monounsaturated fatty acid substrates. The enzyme's catalytic domain responsible for substrate specificity needs to be investigated further to elucidate the mechanism of substrate recognition.

The first putative start codon of the sbt*Elovl5* does not conform to the canonical Kozak sequence, having a C three nucleotides upstream of the ATG translation initiation codon. The nucleotides which have the strongest effect in the motif are the G four bases downstream of the ATG codon and the A three bases upstream of the ATG codon (Kozak 1996). A strong context for vertebrate initiation of translation is considered to be RNNatgG, while an adequate context for initiation of translation is considered to be RNNatgY or YNNatgG (Kozak 1996). Turbot, Atlantic salmon and cherry salmon fatty acyl elongases also have an atypical Kozak sequence but have been shown by heterologous expression to be functional (Agaba *et al.* 2005, Hastings *et al.* 2005, Alimuddin *et al.* 2008). There was a second putative start codon 42 bp downstream of the first start codon in the sbt*Elovl5*. This ORF was referred to as the sbt*Elovl5-42* and the start codon conformed to the Kozak sequence. For this reason, the sbt*Elovl5-42* was investigated in the *S. cerevisiae* expression system. However, heterologous expression of sbtElovl5-42 suggested the protein was not active because the PUFA substrates were not elongated. Therefore, the SBT fatty acyl elongase was only functional when translation initiated from the first start codon. Although the first start codon did not conform to the Kozak sequence, expression

may occur in *S. cerevisiae* due to the different translation initiation control. The translational initiation region in *S. cerevisiae* is rich in A nucleotides, lacks G:C stem loop structures, does not have a Shine-Dalgarno type ribosomal binding site and the first AUG codon closest to the 5' end of the sequence is used as the initiation of translation (Yoon and Donahue 1992). The ribosome binds near the 5' end of the sequence and scans the translation initiation region for the first start codon (Yoon and Donahue 1992). The preferred sequence around the yeast AUG start codon is 5'-(A/Y)A(A/U)AAUGUCU-3', with the most crucial positions being at -3 and +4 (Grivell 1992; Yoon and Donahue 1992). If secondary structures are formed, the ribosome may not be able to bind to the 5' end of the sequence or it may be prevented from locating the first AUG codon at the 5' end of the sequence by scanning (Grivell 1992; Yoon and Donahue 1992).

The transport of free fatty acids into *S. cerevisiae* cell membranes proceeds via adsorption, transmembrane movement and desorption (van Roermund *et al.* 2003). The incorporation of 22:5n-3 and 22:4n-6 into the yeast cells was less efficient than 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3 or 20:4n-6. The properties of fatty acids, including low solubility and high hydrophobicity, mean that they bind quickly to membranes, move across the membrane and then slowly desorb depending on chain length and unsaturation (van Roermund *et al.* 2003). In their work, de Antueno *et al.* (2001) found that the level of uptake by *S. cerevisiae* of various fatty acids was not the same. They found that 14% of radioactively labelled 24:4n-6 or 24:5n-3, 40% of radioactively labelled 20:4n-6 and 79% of radioactively labelled 18:2n-6 or 18:3n-6 was detected in yeast cells at the same incubation time (de Antueno *et al.* 2001). Consequently in our study after 24 h approximately 10% and 37% of the supplemented 22:5n-3 and 22:4n-6, respectively, remained in the medium. Lower uptake efficiency resulted in the sbtElovl5 having less 22:5n-3 or 22:4n-6 in the cells for elongation. Therefore, the elongation of 22:5n-3 or 22:4n-6 may be underestimated in this system.

A loss of PUFA from the *S. cerevisiae* system occurred in all experiments. This loss was most notable during the time-course using 18:4n-3 as a substrate and when various PUFA substrates were measured in the medium and cells after 24 h. Elongation products were not released extracellularly and the incorporation of C_{18}

and C_{20} into the cells after 24 h was complete. Therefore, we can be sure that the PUFA loss occurring from the system was not accounted for by PUFA in the medium in these supplementations. *S. cerevisiae* can utilize saturated and unsaturated fatty acids including 18:4n-3 as sole carbon sources (van Roermund *et al.* 2003). Therefore, β-oxidation may have caused the steady decrease of 18:4n-3 in the *S. cerevisiae* cells when maintained in uninducing medium for 60-96 h. β-oxidation of PUFA in *S. cerevisiae* occurs in the peroxisome, without the involvement of the mitochondria (van Roermund *et al.* 2003). This is in contrast to mammals where β-oxidation is incomplete in the peroxisomes and therefore requires the mitochondria, with the cytoplasm functioning as the intermediate (van Roermund *et al.* 2003). There have been few studies on the β-oxidation of fatty acids in fish tissue. However, Mourente *et al.* (2005) found that European sea bass had a higher proportion of fatty acids in tissues being β-oxidized compared to desaturated.

It appeared that the non-inducing medium was supplemented with more PUFA substrate than the inducing medium. However, the same amount of PUFA was added to both media types and the medium at T_0 was not inoculated with *S. cerevisiae* cells. PUFA incorporation into the cells or utilization by the cells was not occurring at this point. The only difference in the two media was the carbon source, either raffinose or galactose. The fatty acid extraction method may have extracted fatty acids from the medium containing raffinose more effectively than the medium containing galactose. The reason for this is currently still unknown. As a result of the fatty acid extraction process, the fatty acids in the inducing media may be underestimated. The fatty acid extraction protocol for cells grown in both types of media appeared to be consistent based on the amount of substrate in the uninduced cells compared to the substrate and product(s) in the induced cells. We assumed an equal rate of β-oxidation in both non-inducing and inducing cultures. However, this assumption remains to be tested.

Heterologous expression of sbtΔ6des in *S. cerevisisae* suggested that the enzyme had low activity, which was below detectable levels, or no activity. There were no detectable desaturation products when the cultures were supplemented with various n-3 and n-6 PUFA, both direct and indirect substrates. The putative start codon was preceded by the nucleotides ACC, which are native to the sbtΔ6des. Expression in *S. cerevisisae* may have been compromised by the GC rich region and site directed

mutagenesis was used to modify the translation initiation sequence to AAA (Grivell 1992; Yoon and Donahue 1992). Agaba *et al.* (2005) used a similar method to increase the turbot elongase activity in heterologous *S. cerevisisae* expression. The turbot elongase start codon was preceded by CAA which was modified to AAA (Agaba *et al.* 2005). The turbot elongase activity increased with the conversion of 18:4n-3 to 20:4n-3 rising from 35% to 49% and the conversion of 20:5n-3 to 22:5n-3 rising from 30% to 39% (Agaba *et al.* 2005). However, the modification in the sbt*Δ6des* translation initiation sequence did not enhance activity to detectable levels.

Iron acts as an enzyme cofactor and structural component for proteins (Shi *et al.* 2003). Desaturases contain a multi histidine-coordinated di-iron catalytic core (Los and Murata 1998; Buist 2004). If the iron levels in the *S. cerevisisae* induction medium were low, the sbtΔ6des activity may have been reduced to non-detectable levels (Los and Murata 1998). In addition, iron is required for *GAL* gene induction in *S. cerevisisae* (Shi *et al.* 2003). Iron uptake in *S. cerevisisae* is dependent on Cu^{2+}, so iron and copper were supplemented to the cultures expressing sbtΔ6des (Shi *et al.* 2003; Serrano *et al.* 2004). However, supplementation with metal cofactors and PUFA substrates did not enhance sbtΔ6des activity to detectable levels, as desaturation products remained undetected.

The sbtΔ6des was supplied a Δ5desaturase substrate to determine if there was Δ5desaturase activity in the expression system. Marine fish may not have a Δ5desaturase gene and therefore it was highly unlikely that we were able to amplify a Δ5desaturase cDNA (Zheng *et al.* 2009). Attempts to amplify a Δ5desaturase from cobia and Atlantic cod have been unsuccessful (Tocher *et al.* 2006; Zheng *et al.* 2009). The sequenced genomes from the marine fish *Takifugu rupripes, Tetraodon nigroviridis* and *Oryzais latipes* have one fatty acyl desaturase gene in their genome (Zheng *et al.* 2009). Desaturation of the Δ5desaturase substrate 20:3n-6 was not detected following supplementation to the *S. cerevisiae* cells expressing the sbtΔ6des. Thus, it appears the sbtΔ6des does not exhibit Δ5desaturase activity.

Many fish fatty acyl desaturases have previously been functionally characterised in the *S. cerevisisae* expression system, including the marine fish gilthead sea bream, cobia, turbot and Atlantic cod (Zheng *et al.* 2004; Tocher *et al.* 2006; Zheng *et al.*

2009). The lack of sbtΔ6des activity in *S. cerevisisae* suggests that it may not be functional in the *S. cerevisisae* expression system. The expression vector containing the sbtΔ6des was shown to be successfully transformed into and propagated and transcribed by *S. cerevisisae*. This was done by extracting genomic DNA and RNA from *S. cerevisisae*, followed by successful PCR amplification of the sbtΔ6des from genomic DNA and cDNA, respectively. Subsequently, translation of the sbtΔ6des in the *S. cerevisisae* expression system was determined by immunoblot detection. There were some interesting findings when the translation of the sbtΔ6des and the sbtElovl5 were compared. The expression of sbtΔ6des and sbtΔ6desSDM was below the detectable protein level when total protein was extracted from the *S. cerevisiae* cells. Concentrating the total protein extract enabled detection of faint sbtΔ6des and sbtΔ6desSDM protein bands. Interestingly, Elovl5 could be detected without concentrating the total protein extract. The modified translation initiation sequence in the sbtΔ6desSDM protein may have increased translation in *S. cerevisiae* as more sbtΔ6desSDM protein was detected than sbtΔ6des protein. Although translation of sbtΔ6desSDM may have been greater than sbtΔ6des, this increase was not sufficient to enhance the enzymatic conversion of the supplemented PUFA to desaturation products for detection. The relative abundance of the sbtElovl5, sbtΔ6des and sbtΔ6desSDM proteins after concentrating the samples suggest that sbtElovl5 was more highly expressed in *S. cerevisiae* than sbtΔ6des or sbtΔ6desSDM. This finding correlates with the enzyme activities when supplemented with PUFA substrates. However, this comparison was conducted with apparently degraded proteins, as sbtΔ6des and sbtΔ6desSDM were only detected in an apparently degraded form. Although a similar pattern of sbtElovl5, sbtΔ6des and sbtΔ6desSDM protein degradation occurred while concentrating the total protein extracts, care must be taken in interpreting these results. Our results suggest that the sbtΔ6des/sbtΔ6desSDM was expressed in the *S. cerevisiae* expression system, albeit with lower expression than sbtElovl5, but functionality was not observed.

We have found that care must be taken when comparing the enzymatic activities of the other functionally characterised fish desaturases and elongases. The same *S. cerevisiae* expression system and expression vector were used, yet we have shown that translation of the sbtΔ6des can occur without detectable enzymatic activity. A heterologous *S. cerevisiae* expression system cannot be truly compared to the

expression occurring in fish. For example, 18:3n-3 and 18:2n-6 may appear to be good substrates for Δ6desaturase in the *S. cerevisiae* expression system, however, in a marine fish, they may not be such good substrates. In *S. cerevisiae* the Atlantic salmon Δ6desaturase converted over 60% of 18:3n-3 to 18:4n-3, compared to Atlantic cod and European sea bass which converted 33% and 14.5% of 18:3n-3, respectively (Zheng *et al.* 2005; Tocher *et al.* 2006; González-Rovira *et al.* 2009). The trend observed in this example is consistent with Atlantic salmon being more capable of converting PUFA to LCPUFA than other marine species. However, in nature the Atlantic cod and European sea bass Δ6desaturases may experience greater substrate competition which may reduce desaturation of C_{18} substrates.

This study was the first to investigate the expression of *Δ6desaturase* and *Elovl5* in SBT tissues. We found that the *Elovl5* transcripts were three times more abundant in the liver and gonad tissues than they were in the spleen and kidney tissues. However, the copy numbers were low in all four tissues examined compared to Atlantic cod and European sea bass (Tocher *et al.* 2006; González-Rovira *et al.* 2009). The transcript copy number of *Δ6desaturase* could not accurately be detected in the tissues examined. Our findings in SBT tissues suggest that the *Elovl5* transcript copy number was higher than the *Δ6desaturase* which contradicts the expression data from the two species with a full suite of LCPUFA synthesis enzymes, Atlantic salmon and zebrafish (Zheng *et al.* 2005; Monroig *et al.* 2009). Interestingly, in marine fish such as cobia, Atlantic cod and barramundi the abundance of *Δ6desaturase* transcripts in various tissues tends not to be consistently higher than *Elovl5* (Tocher *et al.* 2006; Mohd-Yusof *et al.* 2010; Zheng *et al.* 2009). Generally fish tissues with the highest *Δ6desaturase* and *Elovl5* expression are the intestine, liver and brain, while transcripts have been detected in other tissues such as white muscle, red muscle, kidney, spleen, heart, gill, adipose tissue and ovary (Seiliez *et al.* 2001; Zheng *et al.* 2005; Tocher *et al.* 2006; González-Rovira *et al.* 2009; Mohd-Yusof *et al.* 2010; Monroig *et al.* 2009; Morais *et al.* 2009; Zheng *et al.* 2009). The tissues with the highest *Δ6desaturase* and *Elovl5* expression are not surprising. The liver and intestine are the major sites of lipid synthesis and distribution in freshwater and salmonid fish species in particular (Jump *et al.* 1999; Monroig *et al.* 2009). However, in SBT the liver is also the site of lipid synthesis but it is not considered to store large quantities of lipid like European sea bass, for example (Mourente *et al.* 2002;

Mourente *et al.* 2005). We have found that the SBT liver, as well as the gonad, kidney and spleen, may not synthesize lipid efficiently with relatively low expression of *Elovl5* and no detectable expression of *Δ6desaturase*. The low abundance of the *Δ6desaturase* transcript, together with the difficulties encountered when cloning the sbt*Δ6des* and undetectable enzyme activity in the *S. cerevisiae* expression system, compared to the sbt*Elovl5*, all suggest that the sbt*Δ6des* may have reduced functionality in the whole organism. Unfortunately, for investigation of sbt*Δ6des* and sbt*Elovl5* expression we only had access to a few tissue types from one fish. Future research would benefit from an increase in the number of replicates. Nevertheless, the present research has shown for the first time that at least one fatty acyl elongase gene is expressed in a range of SBT tissues, while expression of sbt*Δ6des* appears to be limited.

Production of C_{24} PUFA, in particular 24:5n-3, is paramount because it is the precursor to DHA, which is widely accepted to be vital for neural tissue development and function and various human health benefits (Tocher 2003; Agaba *et al.* 2004). In this study we saw very low production of C_{24} PUFA from shorter chain precursors. It is believed that an enzyme deficiency in the LCPUFA synthesis pathway limits the production of DHA at a physiologically significant rate in marine fish such as sea bream and turbot (Ghioni *et al.* 1999; Tocher and Ghioni 1999). We have shown in SBT that the fatty acyl elongase has high enzymatic activity but a Δ6desaturase deficiency may be the limiting factor in the production of DHA from ALA. The Δ6desaturase protein sequence has areas of high conservation with other functional Δ5desaturase and Δ6desaturase sequences from fish. However, as yet unidentified differences in the protein sequence may affect the substrate specificities and activities of the enzymes (Hastings *et al.* 2005).

SBT are recognised for their ability to accumulate DHA but the mechanism is not understood. The liver is the site of lipid synthesis in tuna and it is not considered to store large quantities of lipid like other fish species (Mourente *et al.* 2002). This is evident by a lower DHA content and DHA/EPA ratio in the SBT liver than the flesh (Nichols *et al.* 1998). However, the fatty acid profile of the SBT liver tissue was particularly rich in n-3 PUFA, with an n-3/n-6 ratio of 9. As seen with ALA, EPA and DHA, the n-3 PUFA content increased as the chain length of the n-3 PUFA

increased. Due to the structure of the marine food chain, marine fish species such as SBT are expected to have a lesser capability to synthesize C_{22} and C_{24} PUFA. Phytoplankton and zooplankton at the base of the food chain are considered to be the origin of all lipids in marine fish (Tocher 2003). Although SBT are an aquaculture species, their on-farm diet consists of small fish like pilchards and mackerel, which maintain the lipid rich diet they encounter in the wild. The sbtΔ6des may no longer have a role in LCPUFA synthesis, but it may still regulate DHA content in a variety of tissues. Similarly, SBT may display a reduced capacity to elongate C_{22} PUFA because they have adapted to diets containing sufficient levels of C_{24} PUFA. Therefore, the accumulation and retention of DHA from the diet and possibly the selective use of saturated and monounsaturated fatty acids for oxidation for energy, highlights the uniqueness of SBT as a marine fish species (Saito *et al.* 1997).

As discussed above, the sbtElovl5 substrate preference was most similar to the vertebrate elongases from human (ELOVL5), rat (rELO1) and Nile tilapia. This finding is significant because the freshwater fish species Nile tilapia can survive on diets containing vegetable oils which are rich in C_{18} PUFA but low in n-3 LCPUFA (Agaba *et al.* 2005). A shift in the SBT aquaculture industry to a sustainable vegetable oil-based pellet diet will require the SBT to utilise the abundance of C_{18} PUFA to produce LCPUFA. Previous work has shown that Atlantic salmon can survive and grow on a vegetable oil-based diet but the concentration of EPA and DHA in their flesh is lowered, thus lowering the human health value (Tocher *et al.* 2003). To enable freshwater fish and salmonids to synthesize DHA from ALA all of the enzymes in the LCPUFA synthesis pathway must be active (Tocher 2003). Atlantic salmon and zebrafish are the only non-mammalian vertebrates with a functionally characterised Elovl2 (Morais *et al.* 2009; Monroig *et al.* 2009). On the contrary, the marine species SBT may not have another fatty acyl elongase which elongates C_{22} PUFA substrates. Searches of the ENSEMBL genomes suggest that other carnivorous fish species *Tetraodon nigroviridis* and *Gasterosteus aculeatus* and even omnivorous fish species *Takifugu rubripes* and *Oryzais latipes* do not contain *Elovl2* homologs (Morais *et al.* 2009). It may be possible that all fish of the order Acanthopterygii, which includes SBT, may not have a human ELOVL2 homolog (Morais *et al.* 2009). A single Δ6desaturase, an Elovl5 capable of accepting C_{18} and C_{20} substrates and a deficiency in Δ5desaturase and Elovl2 are becoming

characteristic of the superorder Acanthopterygii. Morais *et al.* (2009) speculate that during evolution *Elovl2* and *Δ5desaturase* genes may have been lost from members of the Acanthopterygii but retained in members of the Ostariophysi and Protacanthopterygii. The Acanthopterygii consists of 77% marine species which have LCPUFA rich diets. This is in comparison to the members of the Ostariophysi, where 98% live in freshwater, and the Protacanthopterygii, which are freshwater or anadromous, and are considered to have LCPUFA deficient diets (Morais *et al.* 2009, Nelson 1994). We support this theory that the selective pressure for active LCPUFA synthesis pathway enzymes in members of the Ostariophysi and Protacanthopterygii would be high. In members of the Acanthopterygii these selective pressures would be minimal and could result in a loss of genes over time. Our findings suggest that over time the SBT may have lost Δ6desaturase function but the gene remains in the genome. Evolution may have resulted in a progressive shift from Δ6desaturase primarily synthesizing PUFA, to more of a role in regulating or enhancing DHA content (Zheng *et al.* 2009). Further investigation may reveal that the Δ5desaturase and Elovl2 have not played a significant role in the LCPUFA synthesis pathway for longer than the Δ6desaturase and are subsequently no longer in the SBT genome. A complete loss of the sbt*Δ6des* may occur, as seems to have happened with these other genes in marine fish (Zheng *et al.* 2009). The zebrafish genome is the only genome which contains a functionally characterized bi-functional Δ5/Δ6desaturase (Hastings *et al.* 2001). The evolutionary pathway of Δ5desaturase and Δ6desaturase is unclear. It is proposed that the gene-duplication and divergence of single Δ5desaturase and Δ6desaturase genes may not have occurred in zebrafish like it appears to have done in other teleosts (Napier *et al.* 2003). The evolution of these genes is hard to trace with only one bi-functional Δ5/Δ6desaturase (Napier *et al.* 2003).

Research into closing the life cycle of SBT and routinely breeding them in captivity is currently taking place (Clean Seas Tuna, www.cleanseas.com.au). Future research could include genetically screening for fish with superior LCPUFA synthesis enzymatic capabilities or introducing foreign fatty acyl desaturase and elongase into SBT. For example, Alimuddin *et al.* (2008) have over-expressed a cherry salmon fatty acyl elongase in zebrafish to improve the synthesis of EPA and DHA by 1.3-fold each. Atlantic salmon genes must be considered as an option for transgenic modification of marine species, which are otherwise incapable of full LCPUFA

biosynthesis. Atlantic salmon are the only fish species with the full suite of four characterised LCPUFA synthesis genes (Morais *et al.* 2009). However, zebrafish only require three genes, *Δ5/Δ6desaturase*, *Elovl5* and *Elovl2* for full LCPUFA synthesis (Monroig *et al.* 2009). If SBT have a reduced capability to perform Δ6desaturation and the elongation of C_{22} PUFA to C_{24} PUFA, introducing a foreign *Δ6desaturase* and *Elovl2*, perhaps from a freshwater fish species which is capable of performing these conversions, would enable SBT to convert C_{18} PUFA to C_{24} LCPUFA. Introducing foreign genes may enable sufficient conversion through the LCPUFA pathway. This will be particularly important if the SBT bred and raised in captivity consume more C_{18} PUFA due to a dietary shift from pelagic fish to vegetable oil-based pelletized feeds.