Analysis of Δ12-fatty acid desaturase function revealed that two distinct pathways are active for the synthesis of PUFAs in *T. aureum* ATCC 34304

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Abstract Thraustochytrids are known to synthesize PUFAs such as docosahexaenoic acid (DHA). Accumulating evidence suggests the presence of two synthetic pathways of PUFAs in thraustochytrids: the polyketide synthase-like (PUFA synthase) and desaturase/elongase (standard) pathways. It remains unclear whether the latter pathway functions in thraustochytrids. In this study, we report that the standard pathway produces PUFA in *Thraustochytrium aureum* ATCC 34304. We isolated a gene encoding a putative Δ12-fatty acid desaturase (TauΔ12des) from *T. aureum*. Yeasts transformed with the *tauΔ12des* converted endogenous oleic acid (OA) into linoleic acid (LA). The disruption of the *tauΔ12des* in *T. aureum* by homologous recombination resulted in the accumulation of OA and a decrease in the levels of LA and its downstream PUFAs. However, the DHA content was increased slightly in *tauΔ12des*-disruption mutants, suggesting that DHA is primarily produced in *T. aureum* via the PUFA synthase pathway. The transformation of the *tauΔ12des*-disruption mutants with a *tauΔ12des* expression cassette restored the wild-type fatty acid profiles. These data clearly indicate that TauΔ12des functions as Δ12-fatty acid desaturase in the standard pathway of *T. aureum* and demonstrate that this thraustochytrid produces PUFAs via both the PUFA synthase and the standard pathways.—Matsuda, T., K. Sakaguchi, R. Hamaguchi, T. Kobayashi, E. Abe, Y. Hama, M. Hayashi, D. Honda, Y. Okita, S. Sugimoto, N. Okino, and M. Ito. **Analysis of Δ12-fatty acid desaturase function revealed that two distinct pathways are active for the synthesis of PUFAs in *T. aureum* ATCC 34304. *J. Lipid Res.* 2012.** 53: **1210–1222.**

Supplementary key words polyunsaturated fatty acid • polyketide synthase-like (PUFA synthase) pathway • desaturase/elongase (standard) pathway • homologous recombination • targeted gene disruption

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n-3 PUFAs, such as eicosapentaenoic acid (EPA; C20:5 n-3, C20:5$^{\Delta5,\ 8,\ 11,\ 14,\ 17}$) and docosahexaenoic acid (DHA; C22:6 n-3, C22:6$^{\Delta4,\ 7,\ 10,\ 13,\ 16,\ 19}$), and their metabolites have attracted increasing attention for the development of medicines and nutritional supplements based on their serological, cardiovascular, and antiinflammatory benefits (1–3). For example, EPA is used to treat hyperlipidemia and arteriosclerotic diseases. Furthermore, DHA plays important roles in the development of the retina and the brain in infants and possibly in the maintenance of normal brain function in adults (4, 5). Fish oils, such as sardine and tuna oils, are the major commercial sources of EPA and DHA. However, there is a concern that fish oils will be insufficient to meet the increasing global demand for these PUFAs in the future. Thus, several microorganisms and plants have been explored as alternative sources of PUFAs (6).

Thraustochytrids are eukaryotic marine protists, including the typical genera *Thraustochytrium* and *Aurantiochytrium* (formerly *Schizochytrium*), which belong to the Stramenopiles, class *Labyrinthulomycetes*, family *Thraustochytriaceae*. These organisms are commonly found in marine and estuarine environments and play important roles in the degradation and mineralization of organic materials in marine ecosystems. Thraustochytrids have recently received increasing attention for their ability to produce and accumulate high amounts of n-3 PUFAs in cellular lipid

Abbreviations: ASW, artificial sea water; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME(s), fatty acid methyl ester(s); LA, linoleic acid; OA, oleic acid; ORF, open reading frame; PDA, potato dextrose agar

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droplets; thus, they are under consideration as an alternative industrial source of n-3 PUFAs.

Two distinct pathways for the production of PUFAs have been proposed in thraustochytrids: the polyketide synthase-like (PUFA synthase) pathway, which occurs in several marine bacteria (7), and the desaturase/elongase (standard) pathway, which occurs widely in eukaryotes (6, 8). Gene clusters in the PUFA synthase pathway have been isolated from *Schizochytrium* (now reclassified as *Aurantiochytrium*), in which the disruption of one gene in the synthase pathway resulted in the loss of DHA and n-6 docosapentaenoic acid (C22:5 n-6, C22:$5^{\Delta 4, 7, 10, 13, 16}$), indicating that these PUFAs are produced solely by the PUFA synthase pathway (9, 10). Several genes encoding fatty acid desaturases and elongases, which may be involved in the standard pathway, have been isolated from thraustochytrids (11–13). However, the genes encoding the Δ12- and Δ15-fatty acid desaturases, which are key enzymes in the standard pathway, have not been identified in thraustochytrids. Thus, it is unclear whether the standard pathway is responsible for the production of PUFAs in thraustochytrids.

In this study, we demonstrate, for the first time, that two distinct pathways for the synthesis of PUFAs are active in *T. aureum*. This report is the first to describe the disruption of a fatty acid desaturase gene in thraustochytrids. Thus, this study opens the door for elucidating these entire biosynthetic pathways and the biological functions of PUFAs in thraustochytrids and facilitates the genetic modification of thraustochytrids for the production of beneficial PUFAs.

MATERIALS AND METHODS

Materials

Synthetic oligonucleotides were obtained from Hokkaido System Science (Hokkaido, Japan) and GeneNet (Fukuoka, Japan). D-(+)-Glucose and dry yeast extract were purchased from Nacalai Tesque (Kyoto, Japan). Restriction enzymes and a Ligation-Convenience Kit were purchased from Nippon Gene (Tokyo, Japan). The antibiotics neomycin (G418), hygromycin B, and blasticidin were purchased from Nacalai Tesque. SEALIFE was obtained from Marinetech (Tokyo, Japan). All other reagents were of the highest purity available.

Strains and culture

T. aureum ATCC 34304 was obtained from the American Type Culture Collection (USA). This strain was maintained on potato dextrose agar (PDA) plates (0.8% potato dextrose and 1.2% agar in 50% artificial sea water, ASW). The thraustochytrid was cultivated on GY medium consisting of 3% glucose, 1% yeast extract, and 1.75% SEALIFE.

Molecular cloning of TauΔ12des from *T. aureum* ATCC 34304

T. aureum was grown at 25°C in GY medium. Cells in the late logarithmic growth phase were harvested by centrifugation (3,500 × *g*, 4°C, 10 min), and the genomic DNA was extracted. The primers were designed based on our local genome database. The open reading frame (ORF) of the predicted Δ12-fatty acid desaturase was amplified with the forward primer Tw3-F1 and the reverse primer Tw3-R1. The primer sequences are listed in Supplementary Table I. PCR was then performed using these primers with *T. aureum* genomic DNA as a template in a master mix that included LA Taq DNA polymerase (Takara Bio Inc., Shiga, Japan). The amplified PCR products were purified and cloned into the pGEM-T Easy Vector (Promega, Tokyo, Japan) and sequenced. The full-length genomic DNA clone encoding a Δ12-fatty acid desaturase was named TauΔ12des.

Expression of the *tauΔ12des* in yeast

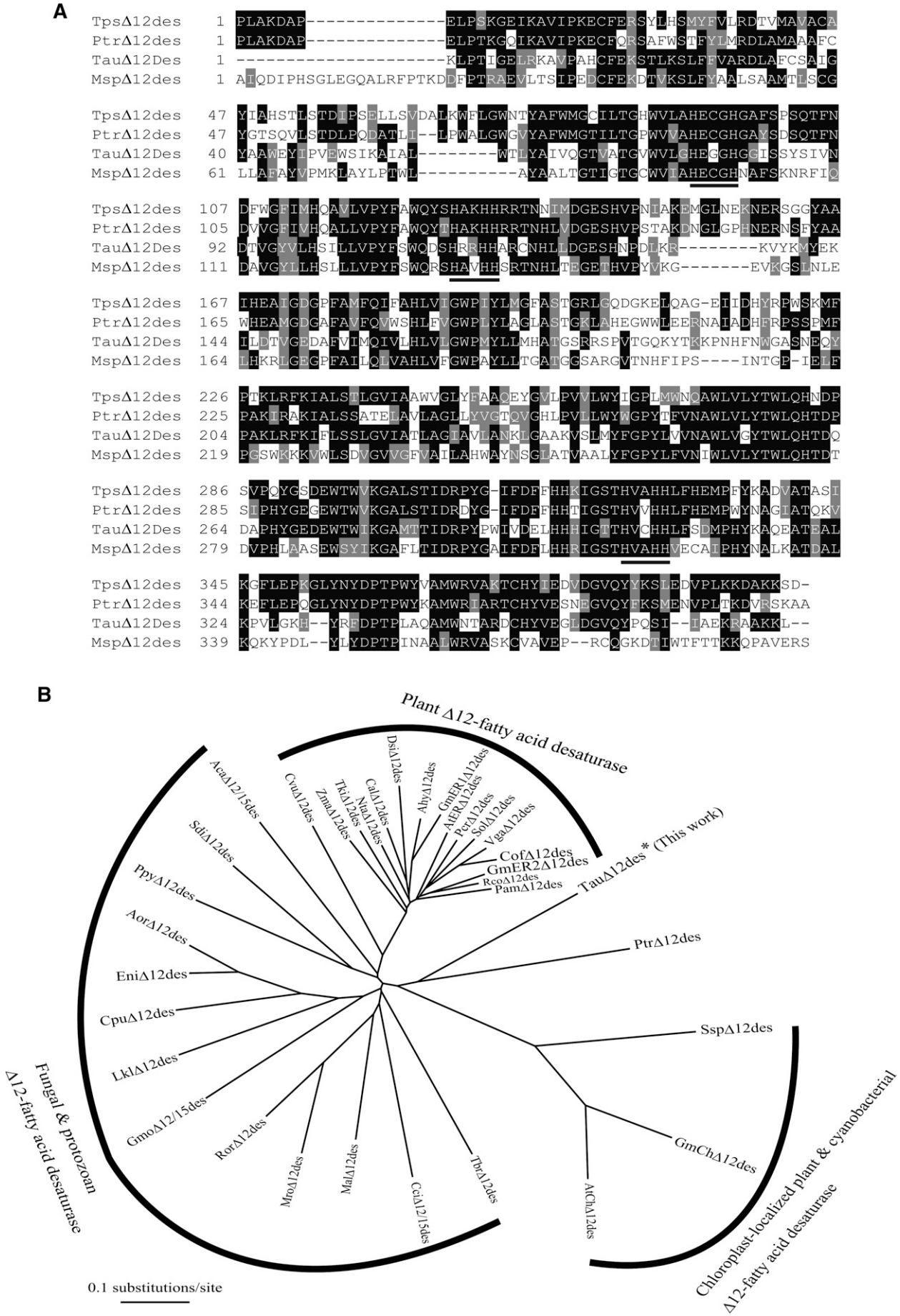
The ORF of the *tauΔ12des* was amplified by PCR using a 5′ primer containing a *Hin*dIII site (Tw3-*Hin*d3-F) and a 3′ primer containing an *Xba*I site (Tw3-*Xba*1-R) and genomic DNA as a template (98°C/20 s, 60°C/30 s, 72°C/1.5 min, 30 cycles). The PCR-amplified TauΔ12des ORF was digested with *Hin*dIII and *Xba*I and then purified and cloned into the same sites in pYES2/CT (Invitrogen, Carlsbad, CA). The resulting TauΔ12des expression vector, designated pYTauΔ12Des, was introduced into *S. cerevisiae* INVSc1 (Invitrogen) using the lithium acetate method (14). The transformants were selected by plating on synthetic agar plates lacking uracil (SC-ura). *S. cerevisiae* transformants harboring the *tauΔ12des* were cultured in SC-ura medium containing 2% glucose at 25°C for 3 days and then cultured for an additional 1 day in SC-ura medium containing 2% galactose. The cells were collected by centrifugation at 3,500 × *g* for 10 min.

Western blotting of FLAG-tagged TauΔ12des

The FLAG tag sequence was inserted immediately after the initiation codon of the TauΔ12des gene by PCR. The PCR was conducted using a forward primer containing the FLAG tag sequences (TD12d-FLAG-F, 5′- GGA AGC TTA TG*G ATT ACA AGG ATG ACG ATG ACA AGT GCA AG*G TCG ATG-3′) and the reverse primer Tw3-Xba1-R; the underlining and italics here indicate the *Hin*dIII site and the FLAG tag sequence, respectively. The PCR fragment was cloned directly into the yeast expression vector pYES2/CT and subsequently introduced into *S. cerevisiae* by the method described above. After the incubation of the transformants in SC-ura medium, the proteins were extracted, and a Western blotting assay was performed as described previously (15). Briefly, 10 μg of protein was loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane (0.45 μm) using a Bio-Rad Trans-Blot SD Cell. The membrane was incubated with 5% (w/v) skim milk in TBS buffer containing 0.1% Tween 20 (Tween-TBS) for 1 h at room temperature with constant agitation. After three washes with Tween-TBS, the membrane was incubated at room temperature for 3 h with an anti-DYKDDDDK tag monoclonal antibody (1:5,000; Wako, Osaka, Japan). The membrane was then washed with Tween-TBS three more times and incubated for 3 h at room temperature with an HRP-conjugated anti-mouse IgG [H+L] goat antibody (Nacalai Tesque; 1:10,000). The membrane was again washed thrice with Tween-TBS. Protein expression was visualized using a peroxidase staining kit (Nacalai Tesque; 1:20).

Targeted disruption of the *tauΔ12des* in *T. aureum*

The *tauΔ12des*-disruption mutants were generated by homologous recombination. Because *T. aureum* is apparently diploid, two different markers were used for the disruption of the gene in the two different alleles. The disruption constructs consisted of either the Hyg^r or Bla^r expression cassette sandwiched between the 1,001-bp 5′- and 3′-flanking sequences of the *tauΔ12des*. First, the 5′- and 3′-flanking sequences were amplified using the TD12d-up-F and TD12d-up-R primers and the TD12d-down-F and TD12d-down-R primers, respectively. Next, these amplified fragments were connected by fusion PCR and cloned into the pGEM-T Easy Vector. The Hyg^r and


A
TpsΔ12des 1 PLAKDAP----------ELPSKGEIKAVIPKECFERSYLHSMYFVLRDTVMAVACA
PtrΔ12des 1 PLAKDAP----------ELPTKGQIKAVIPKECFQRSAFWSTFYLMRDLAMAAAFC
TauΔ12Des 1 ----------KLPTIGELRKAVPAHCFEKSTLKSLFFVARDLAFCSAIG
MspΔ12des 1 AIQDIPHSGLEGQALRFPTKDDFPTRAEVLTSIPEDCFEKDTVKSLFYAALSAAMTLSCG
TpsΔ12des 47 YIAHSTLSTDIPSELLSVDALKWFLGWNTYAFWMGCILTGHWVLAHECGHGAFSPSQTFN
PtrΔ12des 47 YGTSQVLSTDLPQDATLI--LPWALGWGVYAFWMGTILTGPWVVAHECGHGAYSDSQTFN
TauΔ12Des 40 YAAWEYIPVEWSIKAIAL--------WTLYAIVQGTVATGVWVLGHEGGHGGISSYSIVN
MspΔ12des 61 LLAFAYVPMKLAYLPTWL----------AYAALTGTIGTGCWVIAHECGHNAFSKNRFIQ
TpsΔ12des 107 DFWGFIMHQAVLVPYFAWQYSHAKHHRRTNNIMDGESHVPNIAKEMGLNEKNERSGGYAA
PtrΔ12des 105 DVVGFIVHQALLVPYFAWQYTHAKHHRRTNHLVDGESHVPSTAKDNGLGPHNERNSFYAA
TauΔ12Des 92 DTVGYVLHSILLVPYFSWQDSHRRHHARCNHLLDGESHNPDLKR--------KVYKMYEK
MspΔ12des 111 DAVGYLLHSLLLVPYFSWQRSHAVHHSRTNHLTEGETHVPYVKG-------EVKGSLNLE
TpsΔ12des 167 IHEAIGDGPFAMFQIFAHLVIGWPIYLMGFASTGRLGQDGKELQAG-EIIDHYRPWSKMF
PtrΔ12des 165 WHEAMGDGAFAVFQVWSHLFVGWPLYLAGLASTGKLAHEGWWLEERNAIADHFRPSSPMF
TauΔ12Des 144 ILDTVGEDAFVIMQIVLHLVLGWPMYLLMHATGSRRSPVTGQKYTKKPNHFNWGASNEQY
MspΔ12des 164 LHKRLGEGPFAILQLVAHLVFGWPAYLLTGATGGSARGVTNHFIPS----INTGP-IELF
TpsΔ12des 226 PTKLRFKIALSTLGVIAAWVGLYFAAQEYGVLPVVLWYIGPLMWNQAWLVLYTWLQHNDP
PtrΔ12des 225 PAKIRAKIALSSATELAVLAGLLYVGTQVGHLPVLLWYWGPYTFVNAWLVLYTWLQHTDP
TauΔ12Des 204 PAKLRFKIFLSSLGVIATLAGIAVLANKLGAAKVSLMYFGPYLVVNAWLVGYTWLQHTDQ
MspΔ12des 219 PGSWKKKVWLSDVGVVGFVAILAHWAYNSGLATVAALYFGPYLFVNIWLVLYTWLQHTDT
TpsΔ12des 286 SVPQYGSDEWTWVKGALSTIDRPYG-IFDFFHHKIGSTHVAHHLFHEMPFYKADVATASI
PtrΔ12des 285 SIPHYGEGEWTWVKGALSTIDRDYG-IFDFFHHTIGSTHVVHHLFHEMPWYNAGIATQKV
TauΔ12Des 264 DAPHYGEDEWTWIKGAMTTIDRPYPWIVDELHHHIGTTHVCHHLFSDMPHYKAQEATEAL
MspΔ12des 279 DVPHLAASEWSYIKGAFLTIDRPYGAIFDFLHHRIGSTHVAHHVECAIPHYNALKATDAL
TpsΔ12des 345 KGFLEPKGLYNYDPTPWYVAMWRVAKTCHYIEDVDGVQYYKSLEDVPLKKDAKKSD-
PtrΔ12des 344 KEFLEPQGLYNYDPTPWYKAMWRIARTCHYVESNEGVQYFKSMENVPLTKDVRSKAA
TauΔ12Des 324 KPVLGKH--YRFDPTPLAQAMWNTARDCHYVEGLDGVQYPQSI--IAEKRAAKKL--
MspΔ12des 339 KQKYPDL--YLYDPTPINAALWRVASKCVAVEP--RCQGKDTIWTFTTKKQPAVERS
B
Plant Δ12-fatty acid desaturase
DsiΔ12des
AeaΔ12/15des
CvuΔ12des
ZmaΔ12des
TkiΔ12des
NtaΔ12des
CalΔ12des
AbyΔ12des
GmER1Δ12des
AtERΔ12des
PcrΔ12des
SolΔ12des
VgaΔ12des
CofΔ12des
GmER2Δ12des
RcoΔ12des
PamΔ12des
TauΔ12des * (This work)
SdiΔ12des
PpyΔ12des
AorΔ12des
EniΔ12des
CpuΔ12des
LklΔ12des
GmoΔ12/15des
RorΔ12des
MroΔ12des
MalΔ12des
CciΔ12/15des
TbrΔ12des
PtrΔ12des
SspΔ12des
GmChΔ12des
AtChΔ12des
Fungal & protozoan Δ12-fatty acid desaturase
Chloroplast-localized plant & cyanobacterial Δ12-fatty acid desaturase
0.1 substitutions/site

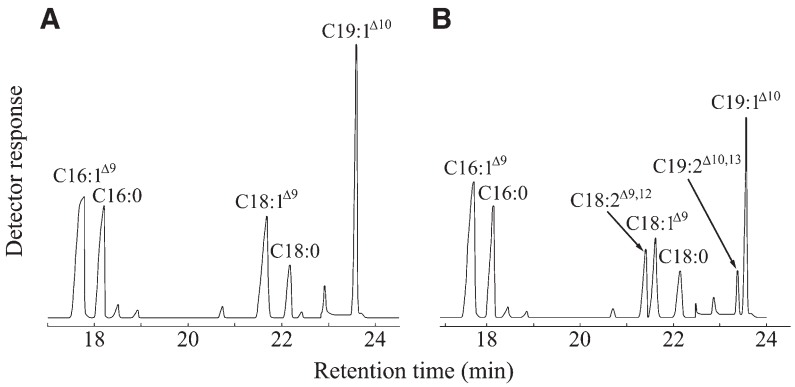


Fig. 2. GC analysis of FAMEs from the *S. cerevisiae* harboring *tauΔ12des*. Gas chromatograms showing the FAMEs extracted from *S. cerevisiae* transformed with (A) an empty vector, pYES2/CT (mock transformants) and (B) *tauΔ12des*-containing vector, pYTauΔ12Des (TauΔ12des transformants). The detector voltages were shifted from 1.20 to 1.50 kV at the retention time of 22.50 min. The arrows indicate the new peak observed in the TauΔ12des transformants (B). The cells were cultured in uracil-deficient SC medium containing 2% glucose at 25°C for 3 days and then cultured for an additional day in uracil-deficient SC medium containing 2% galactose with or without exogenous fatty acids. Fatty acids were added to the culture with 0.1% Tergitol. The fatty acids were extracted from freeze-dried cells and subjected to GC analysis as described in Materials and Methods.

Bla^r expression cassettes were cloned into the *Bgl*II site of the vector. The *ubiquitin* promoter and SV40 terminator were cloned from *T. aureum* ATCC 34304 and the pcDNA 3.1 Myc-His vector (Invitrogen), respectively. The *hyg*^{r} and *bla*^{r} were obtained from pcDNA 3.1/Hygro (Invitrogen) and pTracer-CV/Bsd/lacZ (Invitrogen), respectively. The primers used for the PCR amplification of these sequences are listed in Supplementary Table I. Homologous recombination was performed using the modified split marker system (16). The disruption construct was separated into 5′- and 3′-fragments by PCR and introduced into *T. aureum* cells by microprojectile bombardment. Cells in the logarithmic growth phase were harvested by centrifugation (3,500 × *g*, 4°C, 10 min) and spread on a PDA plate (15 × 60 mm) without antibiotics. The bombardment was performed in a PDS-1000/He Particle Delivery System (Bio-Rad) with DNA-coated gold microcarriers according to the manufacturer's instructions using the following bombardment conditions: pressure, 1,100 psi; target distance, 6 cm; vacuum, 26 inches Hg. Gold particles (0.6 μm in diameter) were coated with the disruption construct according to the manufacturer's instructions. The first allele of TauΔ12des was replaced with the disruption construct containing the Hyg^r expression cassette (first allele knock-out construct), and the second allele was replaced with the construct containing the Bla^r expression cassette (second allele knock-out construct). After the bombardment, the plate was incubated at 25°C for 3 h, after which the cells were collected and suspended in GY medium followed by respreading on a PDA plate containing antibiotics. The transformants were selected by their ability to grow on PDA plates containing hygromycin B or hygromycin B plus blasticidin. The concentrations of hygromycin B and blasticidin in the PDA plates were 2 mg/ml and 0.2 mg/ml, respectively.

Complementation of the *tauΔ12des*-disruption mutants with the *tauΔ12des*

To express the *tauΔ12des* in the *tauΔ12des*-disruption mutants, the Neo^r/TauΔ12des construct (see Fig. 5A) was prepared. For the control experiment, the *tauΔ12des* with the ubiquitin promoter/terminator was omitted from the expression construct (Neo^r construct; see Fig. 5B). The ubiquitin terminator was obtained from *T. aureum* ATCC 34304. The codons of Neo^r were adjusted to match the codon usage of *T. aureum* ATCC 34304. The primers used for the PCR amplification are listed in Supplementary Table I and in a previous report (15). The expression construct was introduced into *T. aureum* cells by the method described above. The cells were incubated on a PDA plate at 25°C for 3 h, after which the colonies were collected and spread on a PDA plate containing G418 at 2 mg/ml. After incubation at 25°C for 7 days, any colonies that appeared on the plates were regarded as putative transformants. The *T. aureum* transformants were cultured in GY medium containing G418 at 2 mg/ml at 25°C for 5 days. The cells were collected by centrifugation at 3,500 × *g* for 10 min.

Genomic PCR and Southern blot hybridization

Genomic PCR was performed using the Hyg-F and Hyg-R primers for the amplification of the *hyg*^{r}, the Bla-F and Bla-R primers for the *bla*^{r}, the forward primer ub pro-Tw3-F with the reverse primer ub term-Tw3-R for the *tauΔ12des*, and the forward primer 2F with the reverse primer pUC18-R for the Neo^r/TauΔ12des construct. For Southern blot hybridization, 1.5 μg of genomic DNA was digested with restriction enzymes at 37°C overnight. The digested DNA was separated on a 0.7% agarose gel and transferred onto a Hybond-N$^+$ membrane (GE Healthcare, Tokyo, Japan). The membrane was hybridized with a probe prepared using the DIG DNA Labeling Kit (Roche Diagnostics K.K., Mannheim, Germany). The probes were amplified with the KO up-probe-F1 and KO up-probe-R1 primers (for the 5′-flanking region), the KO down-probe-F3 and KO down-probe-R3 primers (for the 3′-flanking region), and the TD12d-probe-F1 and TD12d-probe-R1 primers (for the Neo^r/TauΔ12des construct). The genomic DNA hybridized with each probe was detected with the anti-Digoxigenin-AP Fab fragment and an NBT/BCIP stock solution (Roche Diagnostics K.K.).

Fig. 1. Alignment and phylogenetic tree of TauΔ12des. A: alignment of the deduced amino acid sequence of TauΔ12des with the sequences of diatom and picophytoplankton Δ12-fatty acid desaturases. TauΔ12des and Δ12-fatty acid desaturases from different origins were aligned using ClustalW 1.81 and the alignment was shaded in ESPript 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin /ESPript.cgi). Identical and similar amino acid residues are shown as white letters on a black background and in bold face with a black box, respectively. The histidine boxes that are commonly conserved in membrane fatty acid desaturases are underlined. TpsΔ12des, *Thalassiosira pseudonana* Δ12-fatty acid desaturase (XP_002292071); PtrΔ12des, *Phaeodactylum tricornutum* Δ12-fatty acid desaturase (3503348AJJ); TauΔ12des, *T. aureum* ATCC34304 Δ12-fatty acid desaturase (this study); and MspΔ12des, *Micromonas* sp. Δ12-fatty acid desaturase (XP_002507091). B: Phylogenetic analysis of Δ12- and bifunctional Δ12/Δ15-fatty acid desaturases. The scale bar represents a distance of 0.1 substitutions per site in the protein sequence. The abbreviations and origins of the desaturases used are summarized in Supplementary Table II.

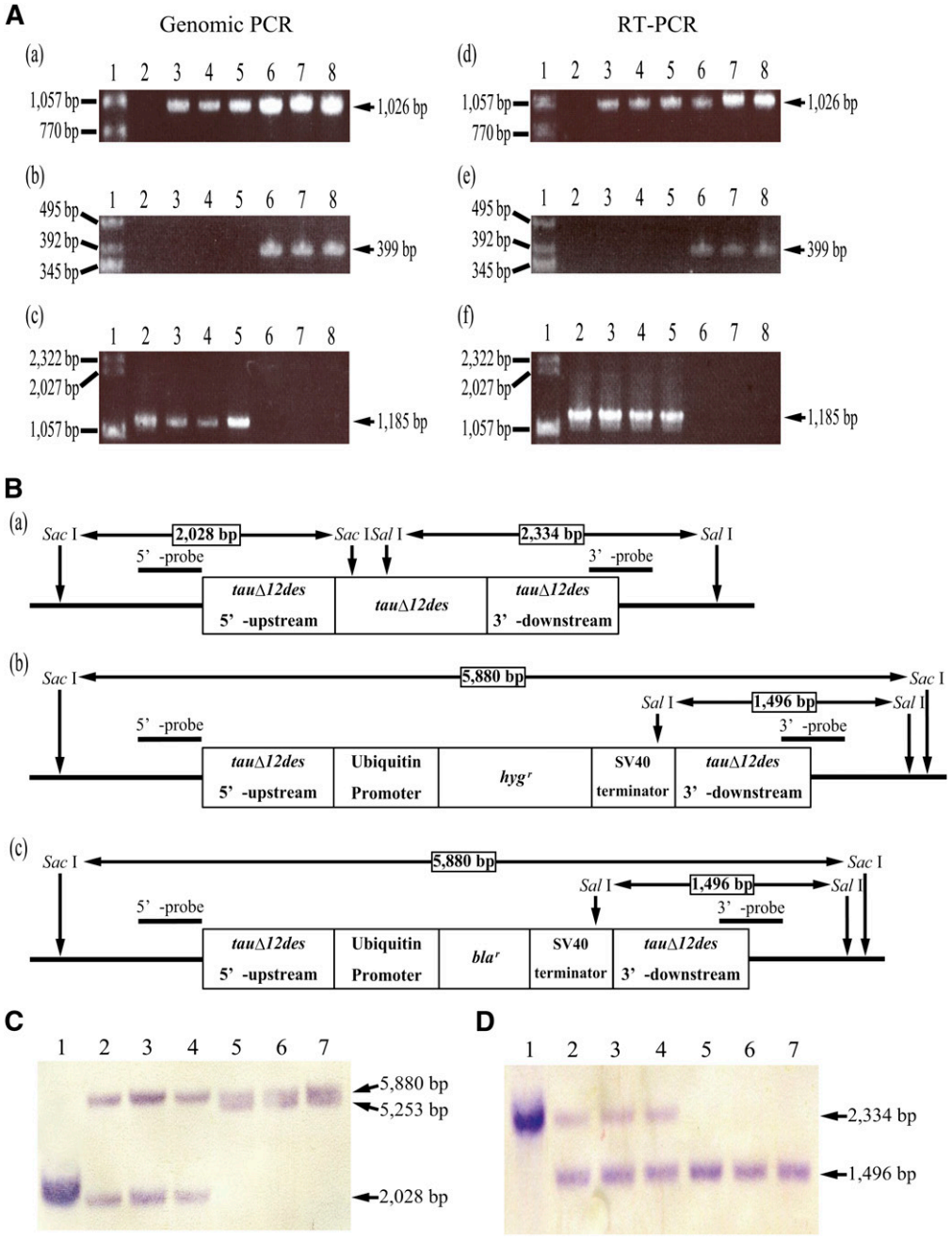


Fig. 3. Molecular characterization of *tauD12des*-disruption mutants. A: Genomic PCR (a–c) and RT-PCR (d–f) of wild-type and *tauΔ12des*-disruption mutants showing Hyg^r (a, d), Bla^r (b, e), and TauΔ12des (c, f) genes (a, b, c) or mRNAs (d, e, f). 1, λ*Hin*dIII digestion / ϕX174 *Hin*cII digestion marker; 2, wild-type *T. aureum*; 3–5, first-allele disruption mutants; 6–8, first- and second-allele disruption mutants. B: The restriction map for each disrupted allele. (a) Wild-type allele; (b) recombinant allele with Hyg^r expression cassette; (c) recombinant allele with Bla^r expression cassette. C, D: Southern hybridization showing the disruption of *tauΔ12des* using a 5′-upstream-specific probe (C) and a 3′-downstream-specific probe (D). 1, wild-type *T. aureum*; 2–4, first-allele disruption mutants; 5–7, first- and second-allele disruption mutants. Further details are provided in Materials and Methods.

Detection of Hyg^r, Bla^r, Neo^r, and TauΔ12des mRNA by RT-PCR

Total RNA was prepared from transformants grown in GY medium containing appropriate amounts of antibiotics with Sepasol RNA I Super (Nacalai Tesque), an RNeasy Mini Kit (QIAGEN, Tokyo, Japan), and DNaseI (Takara Bio Inc.) and used to produce first-strand cDNA with PrimeScript^{TM} Reverse Transcriptase (Takara Bio Inc.). PCR was performed using the Hyg-F and Hyg-R primers for the amplification of Hyg^r cDNA, the Bla-F and Bla-R primers for the Bla^r cDNA, the 3F and 4R primers for the Neo^r cDNA, and the ub pro-Tw3-F and ub term-Tw3-R primers for the TauΔ12des cDNA.

Growth curve and dry cell weight

Precultured cells (2.5 ml) were inoculated into 250 ml GY medium in a 500 ml flask. After incubating the culture at 25°C with

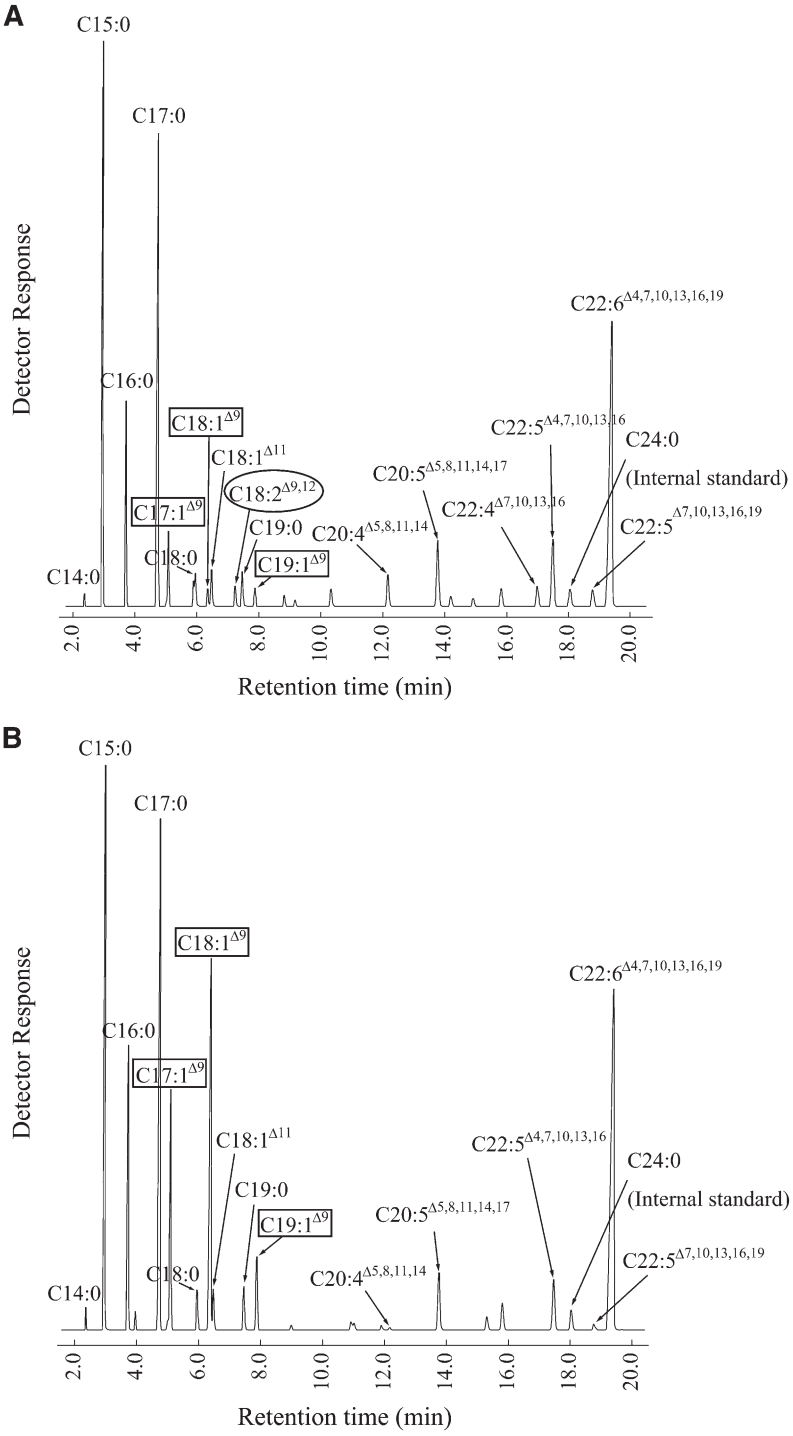


Fig. 4. GC of FAMEs from wild-type (A) and *tauΔ12des*-disruption mutants (B). The cells were cultured in a GY medium containing ampicillin at a concentration of 0.1 mg/ml at 25°C for 5 days. The fatty acids were extracted from freeze-dried cells and subjected to GC as described in Materials and Methods. Endogenous substrates for TauΔ12des, C17:1$^{\Delta 9}$, C18:1$^{\Delta 9}$, and C19:1$^{\Delta 9}$ are shown in the square, and C18:2$^{\Delta 9,\ 12}$ shown in the circle.

shaking at 150 rpm, the absorbance measurements were performed at a wavelength of 600 nm with an Ultrospec 3000 spectrophotometer. Optimal density at 600 nm is below 0.1 at the start for culture. Spectrophotometric readings of the optimal density were taken every hour. The dry cell weight was determined by transferring 10 ml of the culture to a preweighed centrifuge tube and then centrifuging at 3,500 × *g* for 10 min. The cell pellet was washed twice with 50% ASW and once with distilled water. The washed cell pellets were freeze-dried and weighed.

Fatty acid analysis

Precultured cells were incubated in a 50-ml flask containing 25 ml of GY medium at 25°C for 5 days with shaking at 150 rpm. The harvested cells were washed twice with 50% ASW and once with distilled water. The preparation and extraction of fatty acid methyl esters (FAMEs) were performed as described previously (15). The resulting FAMEs were analyzed by GC using the method described previously (15). The FAMEs were also subjected to GC-MS using a Shimadzu GC-MS QP-5000 (Shimadzu Co., Kyoto, Japan) equipped with a capillary column (DB-1, 0.25 mm i.d. × 30 m, film thickness 0.25 μm; Agilent). The column temperature was programmed to increase from 160°C to 260°C at 4°C/min. The injection-port temperature was 250°C. Using lignoceric acid (C24:0) as an internal standard, the FAME samples were analyzed and quantified based on their peak areas on the chromatogram relative to the peak area of the internal standard. Picolinyl esters

TABLE 1. The fatty acid contents (nmol/mg dry cell weight) of the wild-type strain and the *tauΔ12des*-disruption mutants. The means ± SD values based on three replicates are indicated (n = 3).

Fatty acids	Wild-type	First-allele KO	First- and second-allele KO
14:0	1.35 ± 0.179	1.44 ± 0.122	2.52 ± 0.308
15:0	63.0 ± 4.58	66.2 ± 5.25	78.9 ± 10.6
16:0	29.0 ± 4.16	32.1 ± 3.08	47.4 ± 10.5
17:0	81.8 ± 8.89	87.5 ± 9.39	87.0 ± 9.70
$17:1^{\Delta9}$	11.8 ± 0.573	17.9 ± 2.77	36.3 ± 4.46
18:0	11.5 ± 1.41	10.9 ± 0.756	10.3 ± 0.930
$18:1^{\Delta9}$	3.50 ± 0.272	7.61 ± 0.865	76.5 ± 2.14
$18:1^{\Delta11}$	6.09 ± 0.594	7.01 ± 1.16	6.01 ± 1.69
$18:2^{\Delta9, 12}$	4.50 ± 0.492	4.35 ± 0.260	ND
19:0	8.02 ± 0.775	8.11 ± 0.799	8.35 ± 0.961
$19:1^{\Delta9}$	4.20 ± 0.162	6.19 ± 0.665	13.8 ± 5.35
$20:4^{\Delta5, 8, 11, 14}$	8.37 ± 0.488	8.77 ± 0.491	0.226 ± 0.391
$20:5^{\Delta5, 8, 11, 14, 17}$	18.5 ± 1.14	20.6 ± 0.945	15.4 ± 0.713
$22:4^{\Delta7, 10, 13, 16}$	6.15 ± 0.793	5.90 ± 0.519	ND
$22:5^{\Delta4, 7, 10, 13, 16}$	20.3 ± 1.25	21.2 ± 1.26	15.3 ± 0.374
$22:5^{\Delta7, 10, 13, 16, 19}$	6.26 ± 0.801	6.54 ± 0.588	2.67 ± 0.516
$22:6^{\Delta4, 7, 10, 13, 16, 19}$	104 ± 6.79	114 ± 6.23	135 ± 30.4

ND, not detected (indicates values below the limit of detection).

prepared from the FAMEs as described previously (15) were subjected to GC-MS using the equipment described above. The column temperature was programmed to increase from 240°C to 260°C at 2.5°C/min, hold at 260°C for 15 min, and then increase to 280°C at 2.5°C/min.

Lipid extraction and the separation of lipid classes

Precultured cells were incubated in a 500-ml flask containing 200 ml of GY medium at 25°C for 5 days with shaking at 150 rpm. The cells were harvested by centrifugation at 3,000 × *g* for 10 min and washed twice with 50% ASW and once with distilled water. The total lipids were extracted using the Folch method (17) after freeze-drying the cells.

The separation of the total lipids into neutral lipid, glycolipid, and phospholipid fractions using a Sep-Pak Plus Silica cartridge (2 ml) and TLC analysis was performed as described previously (18). The FAMEs in each fraction were prepared and analyzed by GC as described above.

RESULTS

Molecular cloning of a Δ12-fatty acid desaturase from *T. aureum* ATCC 34304

Several fatty acid desaturase genes have been cloned from thraustochytrids (11–13); however, a Δ12-fatty acid desaturase gene has not been cloned from these organisms. In this study, we isolated a putative Δ12-fatty acid desaturase (TauΔ12des) gene from *T. aureum* ATCC 34304, as described in Materials and Methods. The gene, named *tauΔ12des*, contains a 1,185-bp ORF encoding a putative protein of 395 amino acids. The deduced amino acid sequence of the *tauΔ12des* exhibits a high degree of identity with Δ12-fatty acid desaturases found in diatoms and picophytoplankton, such as those from *Thalassiosira pseudonana* (41%) (XP_002292071), *Micromonas* sp. (44%) (XP_002507091), and *Phaeodactylum tricornutum* (41%) (3503348AJJ) (the number in parentheses indicates the sequence identity relative to TauΔ12des) (**Fig. 1A**). Three histidine boxes, which are conserved in almost all membrane-bound fatty acid desaturases, are found in the deduced amino acid sequence of TauΔ12des (Fig. 1A, underlined), whereas the cytochrome b_5 motif, a characteristic of front-end desaturases, is not present in the enzyme.

Phylogenetic analysis of TauΔ12des

The Δ12- and Δ12/Δ15-fatty acid desaturases have been classified into the following groups based on sequence similarity: a fungal and protozoan group, a plant group, a cyanobacterial group, and a chloroplast-localized plant group. The evolutionary relationships among TauΔ12des and other Δ12- and Δ12/Δ15-fatty acid desaturases were examined in a phylogenetic analysis. Although TauΔ12des was not clustered with any group, it was most closely related to the Δ12-fatty acid desaturase found in the diatom *P. tricornutum* (Fig. 1B).

TABLE 2. The fatty acid contents (nmol/mg lipid) in each lipid fraction of the wild-type strain and the *tauΔ12des*-disruption mutants. The means ± SD values based on three replicates are indicated (n = 3).

	Neutral lipid fraction		Glycolipid fraction		Phospholipid fraction	
Fatty acid	Wild-type	First- and second-allele KO	Wild-type	First- and second-allele KO	Wild-type	First- and second-allele KO
14:0	2.31 ± 2.04	6.48 ± 2.02	ND	0.540 ± 0.940	ND	ND
15:0	189 ± 89.0	235 ± 46.0	36.7 ± 21.2	22.5 ± 4.77	124 ± 15.0	116 ± 11.2
16:0	60.5 ± 27.6	74.9 ± 16.2	15.5 ± 8.72	10.0 ± 3.48	68.0 ± 5.23	53.0 ± 9.99
17:0	346 ± 114	251 ± 34.4	47.6 ± 21.6	18.7 ± 6.66	162 ± 10.2	91.5 ± 7.94
$17:1^{\Delta9}$	33.0 ± 18.2	137 ± 76.4	2.81 ± 0.700	15.0 ± 5.53	7.70 ± 1.64	82.7 ± 18.1
18:0	31.0 ± 20.1	30.0 ± 18.8	4.55 ± 4.40	1.78 ± 1.64	5.88 ± 1.22	ND
$18:1^{\Delta9}$	5.93 ± 3.12	322 ± 82.1	ND	26.5 ± 15.0	ND	96.6 ± 65.4
$18:1^{\Delta11}$	4.00 ± 4.66	3.93 ± 3.41	6.39 ± 3.66	6.66 ± 2.69	10.1 ± 8.92	10.3 ± 7.03
$18:2^{\Delta9, 12}$	8.47 ± 5.18	ND	ND	ND	1.34 ± 2.31	ND
19:0	29.5 ± 12.6	25.8 ± 8.95	2.47 ± 2.57	ND	ND	ND
$19:1^{\Delta9}$	9.25 ± 4.72	58.9 ± 15.4	ND	3.13 ± 1.12	ND	26.3 ± 18.3
$20:4^{\Delta5, 8, 11, 14}$	7.44 ± 5.02	ND	6.24 ± 3.49	ND	26.9 ± 13.8	1.85 ± 1.62
$20:5^{\Delta5, 8, 11, 14, 17}$	24.9 ± 11.1	7.30 ± 0.795	38.2 ± 6.42	17.2 ± 2.89	163 ± 18.6	99.5 ± 16.7
$22:4^{\Delta7, 10, 13, 16}$	8.37 ± 4.24	ND	ND	ND	1.22 ± 2.11	ND
$22:5^{\Delta4, 7, 10, 13, 16}$	20.4 ± 7.57	10.5 ± 3.86	10.8 ± 3.52	8.46 ± 3.44	120 ± 19.1	72.6 ± 5.88
$22:5^{\Delta7, 10, 13, 16, 19}$	18.1 ± 5.15	2.72 ± 2.73	3.01 ± 0.490	0.71 ± 1.23	13.6 ± 0.830	5.19 ± 0.550
$22:6^{\Delta4, 7, 10, 13, 16, 19}$	132 ± 41.9	82.1 ± 23.0	34.4 ± 11.4	28.0 ± 12.5	705 ± 59.8	702 ± 62.0

ND, not detected;(indicates values below the limit of detection).

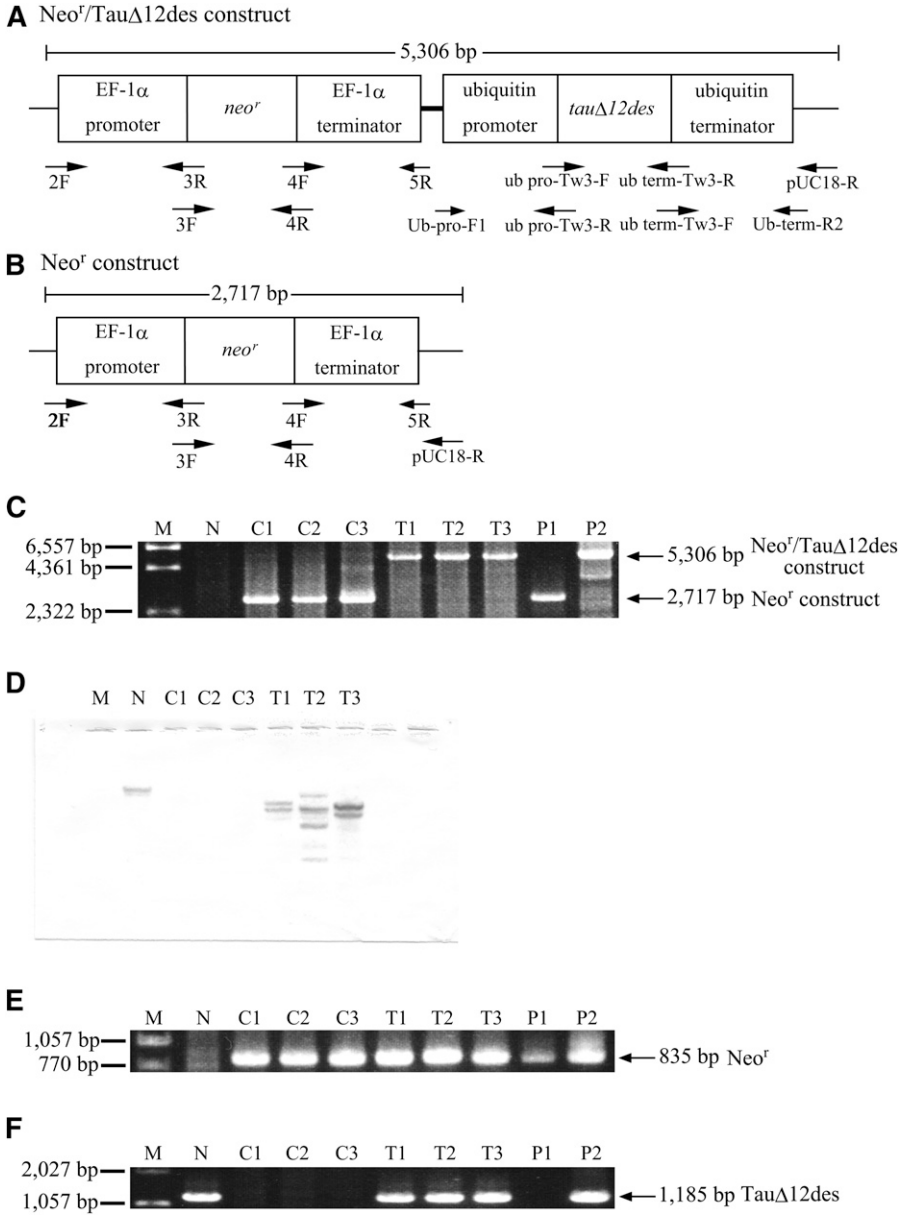


Fig. 5. Molecular characterization of TauΔ12des revertants. Neo^r/TauΔ12des construct (A) and Neo^r construct (B) were separately injected to the *tauΔ12des*-disruption mutants, and the transformants were obtained were designated TauΔ12des revertants and Neo^r transformants (control). The *tauΔ12des* and *neo^r* were driven with thraustochytrid-derived ubiquitin promoter/terminator and EF-1α promoter/terminator, respectively. The primers for fusion PCR of these constructs are shown below each construct. C: Genomic PCR showing the Neo^r and Neo^r/TauΔ12des constructs. D: Southern blot hybridization using a TauΔ12des-specific probe. E, F: RT-PCR amplifying Neo^r mRNA (E) and TauΔ12des mRNA (F). M, λ*Hin*dIII digestion / ϕX174 *Hin*cII digestion marker; N, negative control (wild-type *T. aureum*); C1–C3, Neo^r transformants (mock transformants); T1–T3, Neo^r/TauΔ12des transformants; P1, positive control (Neo^r construct); P2, positive control (Neo^r/TauΔ12des construct). These procedures are described in detail in Materials and Methods.

Exploring the specificity of TauΔ12des expressed in the budding yeast *S. cerevisiae*

To elucidate the specificity of TauΔ12des activity, a TauΔ12des expression construct (pYTauΔ12Des) and an empty-control construct (pYES2/CT) were separately introduced into the *S. cerevisiae* strain INVSc1, and the fatty acid compositions of these transformants were analyzed by GC using their corresponding FAMEs. The peak corresponding to the LA ($18{:}2^{\Delta 9,12}$) methyl ester standard was found in the GC spectra of the pYTauΔ12Des transformants (**Fig. 2B**) but not in those of the mock transformants (Fig. 2A). GC-MS analysis of the newly generated peak in the pYTauΔ12Des transformants revealed the presence of a molecular ion (*m/z* 294) and fragment ions identical to those of the LA methyl ester standard (supplementary Fig. IA, B). These results indicated that endogenous OA

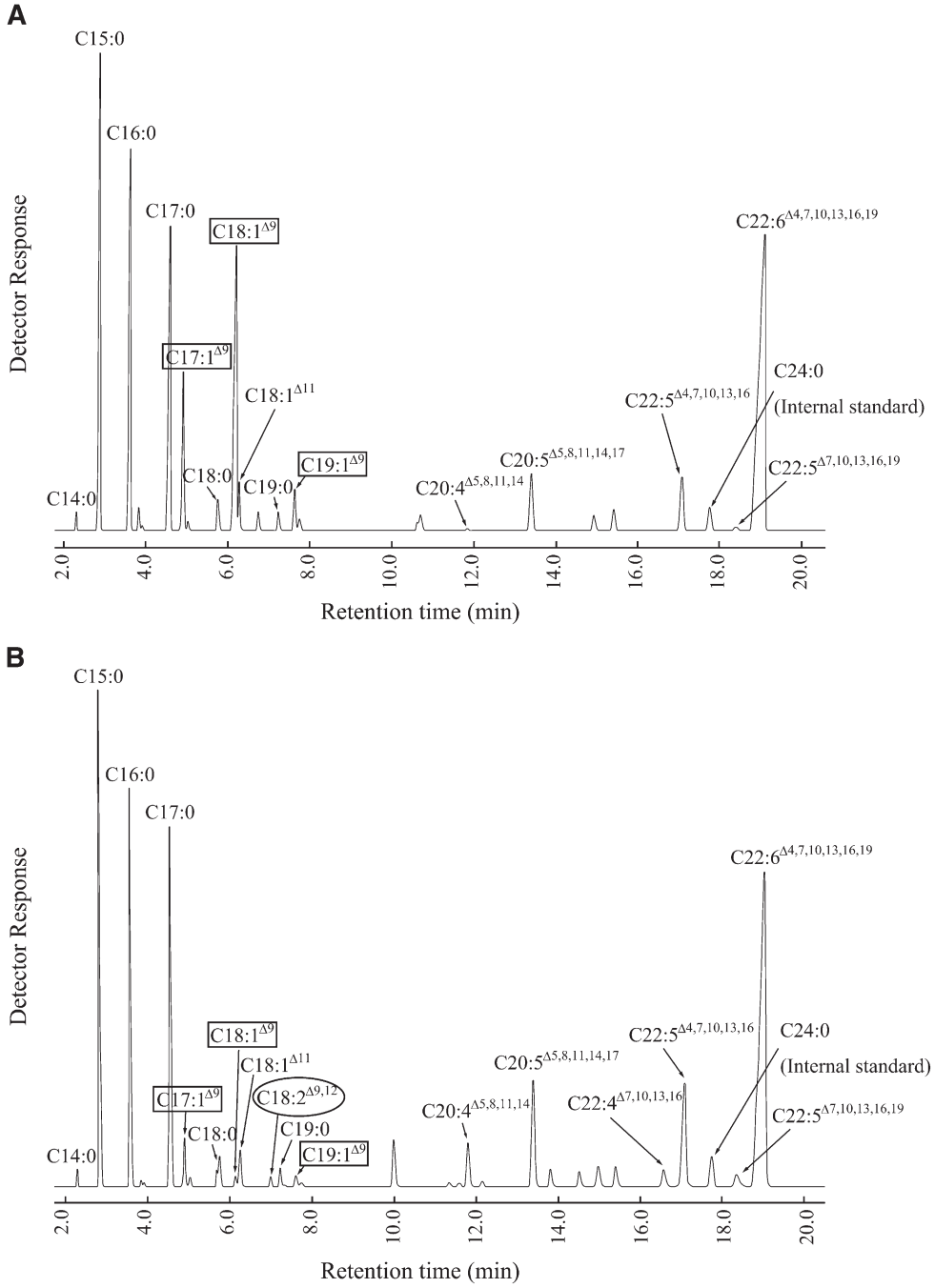


Fig. 6. GC analysis of FAMEs from Neo^r transformants (A) and TauΔ12des revertants (B). Neo^r construct and Neo^r/TauΔ12des construct were separately injected to the *tauΔ12des*-disruption mutants, and the transformants obtained were designated Neo^r transformants (A) and TauΔ12des revertants (B). Gas chromatograms showing the FAMEs extracted from (A) and (B). Endogenous substrates for TauΔ12des, C17:1$^{\Delta9}$, C18:1$^{\Delta9}$, and C19:1$^{\Delta9}$ are shown in the square, and C18:2$^{\Delta9,12}$ is shown in the circle. Details are described in Materials and Methods.

was converted into LA in the transformants harboring pYTauΔ12Des. Moreover, a new peak was generated in pYTauΔ12Des-harboring transformants, but not in mock transformants, when nonadecanoic acid (C19:1$^{\Delta10}$) was added to the culture (Fig. 2A, B). The new peak was determined to be nonadecadienoic acid (C19:2$^{\Delta10,13}$) by GC-MS (supplementary Fig. IIC, D). This result indicates that TauΔ12des is a Δ12-fatty acid desaturase with ν+3 regioselectivity. However, no double bonds were introduced into myristoleic acid (14:1$^{\Delta9}$), palmitoleic acid (16:1$^{\Delta9}$), heptadecenoic acid (17:1$^{\Delta10}$), elaidic acid (18:1$^{\Delta9\ trans}$), LA, γ-linolenic acid (C18:3$^{\Delta6,9,12}$), dihomo-γ-linolenic acid (C20:3$^{\Delta8,11,14}$), arachidonic acid (C20:4$^{\Delta5,8,11,14}$), or docosatetraenoic acid (C22:4$^{\Delta7,10,13,16}$) when they were added to cultures of pYTauΔ12Des-harboring transformants or mock transformants at 50 μM (data not shown). Taken

TABLE 3. The fatty acid contents (nmol/mg dry cell weight) of the KO/*neo*^{r} (the first- and second-allele KO + *neo*^{r}) and the revertant (the Neo^r/TauΔ12des transformants). The means ± SD values based on three replicates are indicated (n = 3).

Fatty acid	KO/*neo*^{r}	Revertant
14:0	1.71 ± 0.236	2.50 ± 1.68
15:0	40.2 ± 3.55	56.3 ± 22.4
16:0	49.1 ± 8.27	47.2 ± 8.38
17:0	32.1 ± 2.65	38.1 ± 9.73
17:1$^{\Delta 9}$	14.5 ± 0.873	12.1 ± 10.4
18:0	5.36 ± 1.11	5.29 ± 0.581
18:1$^{\Delta 9}$	50.2 ± 6.41	9.72 ± 7.84
18:1$^{\Delta 11}$	5.72 ± 0.616	4.31 ± 1.97
18:2$^{\Delta 9, 12}$	N.D.	1.96 ± 1.16
19:0	2.14 ± 0.302	2.30 ± 0.723
19:1$^{\Delta 9}$	4.49 ± 1.01	3.38 ± 2.92
20:4$^{\Delta 5, 8, 11, 14}$	0.384 ± 0.247	7.11 ± 0.509
20:5$^{\Delta 5, 8, 11, 14, 17}$	11.0 ± 1.50	21.7 ± 2.05
22:4$^{\Delta 7, 10, 13, 16}$	N.D.	3.02 ± 0.144
22:5$^{\Delta 4, 7, 10, 13, 16}$	13.7 ± 0.556	20.5 ± 2.15
22:5$^{\Delta 7, 10, 13, 16, 19}$	1.59 ± 0.274	3.08 ± 0.423
22:6$^{\Delta 4, 7, 10, 13, 16, 19}$	138 ± 3.87	114 ± 17.8

ND, not detected (indicates values below the limit of detection).

together, these data led to the conclusion that the *tauΔ12des* encodes a fatty acid desaturase with the dual specificities of a Δ12-fatty acid desaturase and a ν+3-fatty acid desaturase, which catalyze the conversions of C18:1$^{\Delta 9}$ to C18:2$^{\Delta 9, 12}$ and C19:1$^{\Delta 10}$ to C19:2$^{\Delta 10, 13}$, respectively.

Western blotting of FLAG-tagged TauΔ12des expressed in yeast

We examined the expression of TauΔ12des at the protein level when expressed in *S. cerevisiae*. Yeast cells expressing FLAG-tagged TauΔ12des were lysed and fractionated into microsomal and cytosolic fractions followed by analysis with Western blotting using an anti-DYKDDDDK tag antibody. A 45.3 kDa protein band was detected in the cell lysate and the microsomal fractions but not in the cytosolic fraction (supplementary Fig. II). This molecular weight was consistent with that estimated from the deduced amino acid sequence of TauΔ12des with a FLAG tag. These results indicate that TauΔ12des is classified as a microsomal fatty acid desaturase.

Generation of *tauΔ12des*-disruption mutants

To address the question of whether TauΔ12des is involved in the standard pathway in *T. aureum*, the *tauΔ12des* was disrupted in the thraustochytrid by homologous recombination using a disruption construct containing *hyg*^{r} or *bla*^{r} as a marker gene flanked with the 5′ and 3′ sequences of the TauΔ12des genomic locus (supplementary Fig. III). Because *T. aureum* ATCC 34304 appears to be diploid, two loci harboring the *tauΔ12des* should be disrupted by different marker genes to create a full deletion mutant. Transformants grown on GY medium containing hygromycin B (first-allele disrupted mutants) or hygromycin B plus blasticidin (first- and second-allele disrupted mutants) were subjected to genomic PCR and RT-PCR to confirm the disruption of the *tauΔ12des*. The 1,026-bp and 399-bp PCR products (corresponding to the *hyg*^{r} and *bla*^{r}, respectively) were detected in the first- and second-allele disrupted mutants (*tauΔ12des*-disruption mutants) but not in the wild-type strain (**Fig. 3Aa, Ab**). In contrast, a 1,185-bp PCR product (corresponding to *tauΔ12des*) was amplified in the wild-type strains and the first-allele disrupted mutants but not in the first- and second-allele disrupted mutants (Fig. 3Ac). Furthermore, RT-PCR revealed that transcripts of the *hyg*^{r} (1,026-bp) and the *bla*^{r} (399-bp), but not the *tauΔ12des*, were present in first- and second-allele disrupted mutants, whereas the transcript of *tauΔ12des* (1,185-bp) was detected in both the wild-type strains and in the first-allele disrupted mutants (Fig. 3Ad, Ae, Af). Transcripts of the *hyg*^{r}, but not the *bla*^{r}, were detected in the first-allele disrupted mutants, and no transcripts of the *hyg*^{r} or *bla*^{r} were detected in the wild-type strain.

Southern blot hybridization using the DIG-labeled 5′-upstream and 3′-downstream regions of the *tauΔ12des* as the probes was conducted to further characterize the *tauΔ12des*-disruption mutants (Fig. 3B). When hybridized with the 5′-upstream-specific probe, a single 2,028-bp band was detected in the wild-type strain, whereas 5,880- and 5,253-bp bands, corresponding to the two disruption constructs containing each marker gene, were detected in the first- and second-allele disrupted mutants, respectively (Fig. 3C). Hybridization with a 3′-downstream-specific probe resulted in the generation of single a 2,334-bp band in the wild-type strain, whereas a single 1,496 bp band was detected in the first- and second-allele disrupted mutants (Fig. 3D). These results clearly indicate that the *tauΔ12des* was disrupted by homologous recombination with the two marker genes and that *T. aureum* is diploid under the growth conditions used.

Characterization of the *tauΔ12des*-disruption mutants from the perspective of fatty acid biosynthesis

The compositions of the fatty acids in the wild-type strain and the *tauΔ12des*-disruption mutants were analyzed by GC using their methyl ester derivatives. The picolinyl esters, prepared from the FAMEs, were also analyzed by GC-MS to identify each fatty acid (data not shown). In contrast to the wild-type, the mutants had no LA (C18:2$^{\Delta 9, 12}$), the major product of TauΔ12des, whereas they accumulated a significant amount of OA (C18:1$^{\Delta 9}$), the major substrate for TauΔ12des (**Fig. 4A**, B; **Table 1**). Importantly, the downstream derivatives of LA in the standard pathway also decreased drastically in *tauΔ12des*-disruption mutants, except for DHA, which was instead slightly increased. Furthermore, the C17:1$^{\Delta 9}$ and C19:1$^{\Delta 9}$ contents significantly increased in the *tauΔ12des*-disruption mutants, indicating that these odd-chain fatty acids are substrates for TauΔ12des. The loss of Δ12-fatty acid desaturase activity was also confirmed by the metabolic labeling of mutants using ^{14}C-oleoyl-CoA (supplementary Fig. IVA); no ^{14}C-LA was found in the mutants, in contrast to the wild-type. The accumulation of OA and the decrease of LA and its downstream PUFAs in the standard pathway were observed not only in the total fatty acid fraction but also in each lipid class (i.e., neutral lipids, phospholipids, and glycolipids) of the *tauΔ12des*-disruption mutants (**Table 2**).

Fig. 7. Putative PUFA synthetic pathway in *T. aureum* ATCC34304. TauΔ12des functions in the standard pathway to convert OA into LA. On the other hand, DHA is primarily produced from the PUFA synthase pathway.

Despite the significant changes in the fatty acid profiles in the total fatty acid and complex lipid fractions, no difference was observed in cell growth between the wild-type strain and the *tauΔ12des*-disruption mutants under our cultivation conditions (Supplementary Fig. V).

Restoration of the fatty acid profile in revertants of the *tauΔ12des*-disruption mutants

To complement the *tauΔ12des* in the *tauΔ12des*-disruption mutants, a Neo^r/TauΔ12des-expression construct (**Fig. 5A**) was injected into the disruption mutants by microprojectile bombardment. As a control, a Neo^r-expression construct was injected into other *tauΔ12des*-disruption mutants (Fig. 5B). Transformants grown on GY medium containing G418 were selected as transformants and subjected to genomic PCR to determine whether a full-length Neo^r/TauΔ12des DNA was integrated into the genome of the transformants. As shown in Fig. 5C, a 5,306-bp PCR product (corresponding to the size of the Neo^r/TauΔ12des construct [Fig. 5A]) was detected in the transformants harboring Neo^r/TauΔ12des DNA (tentatively designated revertants), whereas a 2,717-bp PCR product (corresponding to the size of the Neo^r construct [Fig. 5B]) was detected in the transformants harboring the Neo^r control

construct (KO/*neo*^{r}). Southern blot hybridization using a TauΔ12des DNA probe confirmed that the *tauΔ12des* was integrated into the genomes of the revertants (Fig. 5D). Furthermore, RT-PCR revealed that transcripts of the *neo*^{r} (835 bp) and the *tauΔ12des* (1,185 bp) were present in the revertants, whereas the transcript of the *neo*^{r}, but not the *tauΔ12des*, was detected in the control (Fig. 5E, F). These results clearly indicate that *tauΔ12des* was integrated into the genome of the revertants and then transcribed into TauΔ12des mRNA.

The fatty acid compositions of the revertant and KO/*neo*^{r} were analyzed by GC using their respective FAMEs. In contrast to the KO/*neo*^{r}, the fatty acid profile of the revertants was restored to that of the wild-type strain (i.e., the levels of OA, LA, and its downstream PUFAs in the revertants were similar to those of the wild-type strains) (**Fig. 6A**, B; **Table 3**). Additionally, in vivo labeling with ^{14}C-oleoyl-CoA demonstrated the restoration of the Δ12-fatty acid desaturase activity in the revertants (supplementary Fig. IVB). These results clearly indicate that the change in the fatty acid profile in the *tauΔ12des*-disruption mutants was due to the loss of function of *tauΔ12des*.

It is shown in this study that TauΔ12des is the Δ12-fatty acid desaturase involved in the standard pathway and that this enzyme is primarily responsible for the conversion of OA into LA in *T. aureum*. Furthermore, DHA was found to be produced in *T. aureum* primarily independently of the standard pathway, possibly via the PUFA synthase pathway. In conclusion, two working pathways for the production of PUFAs in *T. aureum* were revealed through the analysis of a native Δ12-fatty acid desaturase.

DISCUSSION

Thraustochytrids, belonging to the protist kingdom Stramenopila, are microorganisms that constitute a promising alternative to fish oils as an industrial source of PUFAs. The fatty acid profiles differ among the different thraustochytrid genera (19). These different PUFA profiles may indicate the presence of different PUFA biosynthetic pathways in the various thraustochytrids. Several lines of evidence suggest the occurrence of two different pathways involved in the biosynthesis of PUFAs in thraustochytrids. The first, which is found in several marine bacteria, is the polyketide synthase-like pathway (the PUFA synthase pathway), comprising reiterative cycles including condensation, reduction, dehydration, and isomerization steps, with each step catalyzed by different enzymes. Three functional ORFs of the PUFA synthase pathway have been identified in *Schizochytrium* (now reassigned to *Aurantiochytrium*) (9, 20, 21). Lippmeier et al. (10) suggested that the PUFA synthase pathway is the sole system responsible for PUFA production in *Schizochytrium* because the disruption of an ORF of a PUFA synthase led to the loss of PUFAs in the thraustochytrids, which became PUFA-dependent auxotrophs. The other pathway, which is found in many organisms, including mammals, is the desaturase/elongase pathway (the standard pathway), comprising a series of alternating desaturation and elongation steps starting with saturated fatty acids that are produced in an FAS pathway. Although several desaturase and elongase genes have been cloned and characterized in thraustochytrids (11–13, 22), the direct evidence that such enzymes are operative in the standard pathway has not been obtained. In this study, we demonstrated that the standard pathway is functional in *T. aureum* ATCC 34304 by disrupting the gene encoding a Δ12-fatty acid desaturase, which is a key enzyme in the standard pathway for the production of n-3 and n-6 PUFAs.

In this study, we generated disruption mutants of *tauΔ12des* by replacing two *tauΔ12des* alleles with two different marker genes. The disruption construct was composed of the 5′ and 3′ regions of the *tauΔ12des* as homologous recombination sites and an antibiotic-resistance gene (*hyg*^{r} or *bla*^{r}) as a marker gene (Supplementary Fig. III). Molecular analysis of the *tauΔ12des*-disruption mutants showed that the *tauΔ12des* ORFs of two alleles were replaced by *hyg*^{r} or *bla*^{r} (Fig. 3). This result indicates that *T. aureum* is diploid, at least under the conditions used in this study. In contrast, *Schizochytrium* sp. ATCC 20888 appeared to be haploid (10).

Unexpectedly, the *tauΔ12des*-disruption mutants of *T. aureum* were indistinguishable from the wild-type strain in morphology and cell growth under the conditions used in this study (Supplementary Fig. V). However, the disruption of the *tauΔ12des* led to a dramatic change in the fatty acid profile, in which an increase of OA (C18:1$^{\Delta 9}$) was observed in combination with the disappearance of LA (C18:2$^{\Delta 9, 12}$) (Fig. 4; Table 1). Furthermore, the *tauΔ12des*-disruption mutants showed decreased levels of the n-6 and n-3 PUFAs that are downstream of LA in the standard pathway. In contrast, DHA levels were slightly increased in the disruption mutants. These results demonstrate that TauΔ12des functions in the standard pathway for the production of PUFAs, whereas DHA is primarily produced by a nonstandard pathway in *T. aureum*, possibly by the PUFA synthase pathway. However, we observed that the disruption of the PUFA synthase gene in *T. aureum* resulted in a marked decrease in DHA but not in other PUFAs, such as LA, ARA (C20:4$^{\Delta 5, 8, 11, 14}$) and EPA (C20:5$^{\Delta 5, 8, 11, 14, 17}$). A small amount of DHA was still present in the PUFA synthase-disrupted mutants, suggesting that DHA is produced not only by the PUFA synthase pathway but also by the standard pathway (data not shown). Neither TauΔ12des nor PUFA-synthase disruption mutants of *T. aureum* were auxotrophs, in contrast to PUFA-synthase mutants of *Schizochytrium* sp (10).

We observed the accumulation of C17:1$^{\Delta 9}$ and C19:1$^{\Delta 9}$ in the *tauΔ12des*-disruption mutants, and this accumulation was eliminated by introducing *tauΔ12des* into the disruption mutants. This result indicates that TauΔ12des also accepts odd-chain fatty acids as substrates. Chang et al. (23) identified odd-chain PUFAs in thraustochytrids and suggested that these PUFAs are synthesized through the standard pathway. The accumulation of C17:1$^{\Delta 9}$ and C19:1$^{\Delta 9}$ in *tauΔ12des*-disruption mutants supports their hypothesis.

The enzymes involved in the PUFA synthase pathway are cytosolic proteins, and the products are released from

the synthetic machinery as free fatty acids (20). In contrast, the membrane desaturases accept a wide range of acyl substrates (24, 25). Therefore, we expected that the fatty acid profiles of complex lipids from the *tauΔ12des*-disruption mutants would be somewhat different from those of the wild-type strain, as shown in filamentous fungus (26). However, no difference was observed between the wild-type strain and the disruption mutants in their fatty acid profiles of neutral lipids, phospholipids, and glycolipids (Table 2). Thus, it is possible that the fatty acids produced via the standard pathway are acyl-CoA forms that are directly incorporated into different complex lipids by various acyltransferases. In other words, the desaturases and elongases that constitute the standard pathway of the thraustochytrid accept the CoA forms of fatty acids as substrates. Another possibility is that the fatty acids in each lipid class are remodeled in *T. aureum* after their incorporation into complex lipids. Further studies are necessary to elucidate the acceptor specificity of TauΔ12des in vitro.

In conclusion, we present direct evidence that TauΔ12des functions in the standard pathway and is responsible for generating certain PUFAs in *T. aureum*, although DHA is primarily produced through the PUFA synthase pathway (**Fig. 7**). The results of this study also indicate that LA and its downstream products from the standard pathway are not necessary for the normal growth and morphology of *T. aureum* under our conditions because sufficient DHA is generated through the PUFA synthase pathway.

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