Profile of Polyunsaturated Fatty Acids Produced by *Thraustochytrium* sp. KK17-3

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ABSTRACT: More than 300 strains of microorganisms producing polyunsaturated fatty acids (PUFA) were newly isolated from coastal seawater in the Seto Inland Sea and around Iriomote Island, Japan, by the baiting method. The profiles of PUFA from docosahexaenoic acid (DHA)-producing strains could be classified into four types. A strain, named KK17-3, was chosen for further study owing to its high DHA content (52.1% of total fatty acid) and wide range of PUFA (76.1%) including arachidonic, eicosapentaenoic, and docosapentaenoic acids as well as DHA. Glucose and tryptone were the optimal carbon and nitrogen sources, respectively, in a medium with salinity at 75% that of seawater. The PUFA contents in polar lipids (22.1% of total lipid), in which the DHA content was 39.3%, were higher than those in neutral lipids and glycolipids. Molecular phylogenetic analysis of 18S rRNA gene sequences showed KK17-3 to be a thraustochytrid. It also was observed to possess a life cycle composed of vegetative cells without successive bipartition, zoosporangium, and zoospore stage. Classification by the chemotaxonomic criterion based on PUFA compositions also supported this assignment.

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KEY WORDS: 18S rRNA gene, docosahexaenoic acid, molecular phylogenetic analysis, polyunsaturated fatty acid, *Thraustochytrium*.

Polyunsaturated fatty acids (PUFA) with unique structural characteristics are distinguished by their functions not only in regulating cell physiology such as phase transition, membrane permeability and the behavior of membrane-bound proteins (1) but also in modulating the expression of certain genes (2). Therefore, it is not surprising that PUFA deficiencies lead to abnormalities in the skin, kidney, nervous network, immune and inflammatory reactions, cardiovascular system, endocrine control, and respiratory and reproductive systems (3,4). In particular, docosahexaenoic acid (DHA, C22:6n-3) is absolutely essential to humans owing to its functions in the brain and retina (5).

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The traditional commercial sources of PUFAs are fish oils. The relatively low proportion of PUFA in fish oils makes it difficult to produce DHA and other PUFA on a large scale. On that account, exploration of other sources of DHA as well as of other PUFA has drawn much research attention. To date, marine microorganisms belonging to achromatic stramenopiles such as thraustochytrids and labyrinthulids have been reported to produce significant quantities of DHA (3,6–14). Their different profiles of PUFA reflect taxonomic relationships. Molecular phylogenetic analysis (15,16) suggests that several regulatory mechanisms for PUFA biosynthesis exist in distinctive DHA-producing microbes. DHA can be synthesized by the conversion of arachidonic acid (AA, C20:4n-6), eicosapentaenoic acid (EPA, C20:5n-3), or docosapentaenoic acid (DPA, C22:5n-6) through their desaturation and elongation, catalyzed by fatty acid desaturases and elongases (3). It is desirable to obtain PUFA producers that accumulate not only DHA but also the intermediate fatty acids in order to illustrate the biosynthetic system for PUFA by molecular genetic approaches. For that purpose, we isolated more than 300 strains producing PUFA from seawater samples, and a strain, named KK17-3, was selected for the following studies.

MATERIALS AND METHODS

Microorganisms and medium. More than 300 strains of marine microorganisms were isolated from seawater samples taken in the Seto Inland Sea and around Iriomote Island in Japan by using pine pollen as a bait. The collected zoosporangia were cultivated on GPY-agar medium (2% glucose, 1% polypeptone, 0.5% yeast extract, 1.5% agar, pH 6.0) with a salinity equivalent to 50% that of sea water for 7 d at 28°C. Seawater salinity was taken to be 40 g/L. Salinity of solutions was adjusted by the addition of sea salts (No. S9883, Sigma Chemical Co., St. Louis, MO). For rapid analysis of fatty acids, the colonies on the agar medium were scraped, extracted, and directly treated with methanolic hydrochloride for gas–liquid chromatography (GLC) analysis as described below. GPY liquid medium (3% glucose, 1% polypeptone, 0.5% yeast extract) at 50% of seawater salinity was used as a basal medium for the following cultivations unless otherwise

indicated. An inoculum was prepared in a 200-mL baffled flask containing 30 mL of the basal medium and grown at 28°C for 48 h with rotary shaking at 160 rpm. An aliquot of the culture was transferred to 50 (2.5 mL) mL of fresh basal medium in a baffled flask (250 mL) and incubated at 180 rpm at 28°C for 3 d.

Lipid extraction and fraction analysis. The wet cells harvested from culture suspension by centrifugation at 3000 × *g* were washed two times with deionized water and used for extraction of lipid with a mixture of chloroform/methanol (2:1, vol/vol) as described previously (17). The extraction process was repeated two or three times to collect the total lipids as completely as possible. The total lipid was fractionated into neutral lipids, glycolipids, and polar lipids on a silica gel column (Wakogel C-200; Wako Chemical, Osaka, Japan) with chloroform, acetone, and methanol, respectively. The amount of each lipid fraction was determined gravimetrically after evaporation of the solvents. Lipid components were determined by thin-layer chromatography on a silica gel plate (Kieselgel 60; Merck, Darmstadt, Germany).

Analysis of the composition of fatty acid in lipid. The washed wet cells were heated at 105°C for 12–16 h until dryness and then measured gravimetrically. The dried cells were directly methyl-esterified with 10% HCl in methanol with addition of eicosanoic acid (C20:0) as an internal standard. The esterified samples were applied to a gas–liquid chromatograph (GC-17A; Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a split injector, using a TC-70 capillary column (GL Science, Tokyo, Japan) with temperature programming (190 to 220°C at 1°C/min). The fatty acid ester peaks were identified and calibrated with standard fatty acids. Data given are the averages of at least three determinations.

Continuous morphology observation. A drop of fresh cell culture was transferred into a concave depression on a sterile glass slide and then covered with a thin glass. Continuous observations were performed by microscope (Eclipse E600; Nikon, Tokyo, Japan). The image was captured by a CCD camera (Nikon) and recorded by a rewritable videodisc recorder.

Isolation and sequencing of 18S rRNA gene. Harvested cells were ground into powder by treatment with liquid nitrogen and then lysed with TNE buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 8.0). Following lysis, the genomic DNA was extracted by a standard phenol/chloroform method (18). To obtain almost complete 18S rRNA genes, we used polymerase chain reaction (PCR) protocols with amplification primers 18S1, 5′-TACCTGGTTGATCCTGCCAG-3′, and 18S12, 5′-CCTTCCGCAGGTTCACCTAC-3′ (16). A 50-μL sample of the PCR reaction mixture contained 20 mM of each deoxynucleotide triphosphate, 0.5 μM of each primer, 5 μL of 10× PCR buffer, 2 units of Taq polymerase (Takara Shuzo, Kyoto, Japan), and 2 μg of the genomic DNA. After initial denaturation at 95°C for 5 min, PCR amplification was performed in a thermal cycler (GeneAmp PCR System 9600; PE Corporation, Norwalk, CT) using a program of 30 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, followed by an extension for 10 min at 72°C. The PCR product was gel-purified and directly used for sequence analysis with a DNA autosequencer (GeneRapid Sequencing System; Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instruction. Sequences were determined over both strands of DNA fragment using the primers 18S1 and 18S12, and primers for internal region as described (16).

Molecular phylogenetic analysis. Nucleotide sequences of 18S rRNA genes from related microorganisms were obtained from the DNA Data Bank of Japan (DDBJ http://www.ddbj.nig.ae.jp/welcome.html). Sequences were aligned through a profile alignment process by Clustal W for Power PC, version 1.74 (19). The positions with gaps or undetermined or ambiguous bases were removed before the subsequent analyses and 1,549 nucleotides sites in 18S rRNA gene from KK17-3 were employed for the following analysis. When the mol% G+C content of the 18S rRNA varies greatly among operational taxonomical units, the estimated phylogeny may sometimes be unreliable (20). Therefore, the comparatively compact content of G+C was selected at the range from 39.9% (*Thraustochytrium pachydermum*) to 50.8% (strain KK17-3) to avoid the negative influence of distribution of G+C on phylogeny estimations. A phylogenetic tree was generated using the neighbor-joining (NJ) method (21) after a taxon *Cafeteria roenbergensis* was selected as an outgroup. NJ analysis was performed by using PAUP* version 4.0b4a (22). Distances were estimated by the maximum-likelihood method with Felsenstein's (F84) model (23). The statistical significance of the tree branches was assessed by 1000 bootstrap resamplings (24).

RESULTS AND DISCUSSION

PUFA profiles in DHA-producing isolates. The colonies of more than 300 microbial isolates were analyzed for their fatty acid compositions. Thirteen representative strains producing a significant amount of DHA were divided into four types according to their PUFA profiles as given in Table 1. PUFA from the third group, as exemplified by strain KK17-3, consisted of DHA, DPA, EPA, and AA, a pattern similar to that of *T. aureum* ATCC28211 (25). The content of PUFA was as high as 76.1% of the total fatty acids (TFA), and the ratio of DHA to TFA was as high as 52.1%. Strains H1-13 and H6-10 also produced a considerable amount of DHA although their DHA contents were relatively low. As for strains KH105 and KK251, the contents of PUFA (DHA + DPA) and DHA were lower than those in strain KK17-3. Although DHA contents in strains KH154 and KH155 were higher than that in KK17-3, they lacked AA in their PUFA. In addition, the contents of PUFA and DHA in strains H1-3 and H1-14 were relatively lower even though they could produce docosatetraenoic acid (C22:4n-6) in addition to DHA, DPA, EPA, and AA. In view of both the elucidation on the biosynthetic pathway and production of PUFA, we selected strain KK17-3 as a model strain, owing to both its high levels of DHA and its wide range of PUFA.

Optimization of conditions for cell growth and PUFA production in strain KK17-3. The medium salinity was first ex-

TABLE 1
Composition of Fatty Acids in Newly Isolated DHA-Producing Microbes

Strains	Principal fatty acid composition (%)[a]											Type of PUFA
	C15:0	C16:0	C17:0	C18:0	C18:1 (n-9)	C18:2 (n-6)	AA	EPA (n-3)	C22:4 (n-6)	DPA (n-6)	DHA (n-3)	
KH105	17.0	10.3	6.9	0.8	—	—	—	—	—	12.4	46.9	DHA/DPA
KK251	—	5.2	—	1.4	—	20.5	—	—	—	12.0	40.2	
KH154	5.8	5.3	8.5	0.4	—	—	—	3.9	—	15.7	58.7	DHA/DPA/EPA
KH155	5.7	5.0	7.7	—	—	—	—	5.4	—	16.6	59.7	
KK17-3	—	4.5	—	—	—	—	6.3	2.7	—	15.0	52.1	DHA/DPA/EPA/AA
H1-13	—	4.3	—	0.6	0.6	—	5.1	23.4	—	1.0	39.0	
H2-4	—	4.3	—	0.5	—	—	4.9	14.9	—	1.8	37.9	
H6-10	—	15.3	—	0.5	—	—	2.5	14.9	—	1.8	35.3	
H6-15	—	15.5	—	0.5	—	—	6.8	18.1	—	1.9	40.1	
H6-16	—	12.2	—	0.7	—	—	7.5	13.9	—	2.9	35.9	
H6-17	—	4.5	—	0.5	—	—	4.9	14.0	—	2.7	38.1	
H1-3	—	15.8	—	4.8	4.1	2.1	8.7	9.0	1.1	3.7	23.6	DHA/DPA/EPA/AA
H1-14	—	25.6	—	9.0	7.5	—	4.5	3.9	2.5	18.2	11.6	+ C22:4n-6

[a]Dashes indicate less than 0.3%. DHA, docosahexaenoic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; PUFA, polyunsaturated fatty acid.

amined since the strain KK17-3 was isolated from seawater. Table 2 showed that a salinity of 75% that of seawater was optimal to obtain the highest cell growth (5.7 g/L), TFA yield (690 mg/L), and DHA yield (169 mg/L). KK17-3 showed a slight resistance to high salinity, up to 200% that of seawater, which was similar to the excellent DHA-producer, *Schizochytrium limacinum* SR21 (26), whereas *T. aureum* did not grow at that salinity (27). The PUFA profiles, however, did not obviously change over the range of 50–200% of seawater salenity. Such constant proportion of PUFA may help to maintain the physicochemical functions of lipid membranes across a wide range of osmotic pressures.

As shown in Table 3, of the carbohydrates tested, glucose was the preferred carbon source for cell growth (biomass yield 5.5 g/L), TFA yield (545 mg/L), and DHA yield (145 mg/L). Most strains producing large amounts of PUFA are heterotrophic and utilize glucose as an effective carbon source, except for *T. roseum* ATCC28210, which assimilates starch rather than glucose (8). Strain KK17-3 did not utilize linseed or sesame oil as a sole carbon source (data not shown). This is in contrast to *T. aureum* ATCC34304 (9) and *S. limacinum* SR21 (26) which could make good use of linseed oil or oleic acid as well as glucose. As for nitrogen sources, tryptone was preferable in view of biomass production (7.1 g/L), TFA yield (843 mg/L), and DHA yield (233 mg/L). Additionally, inorganic compounds such as NH_4NO_3 and $NaNO_3$ could be utilized efficiently by KK17-3. In general, polypeptone and yeast extract are suitable for most PUFA producers (9,12,14,26,27). On the other hand, Table 3 revealed that different nitrogen sources had little effect on the proportion of PUFA except for the relatively lower DHA contents in the presence of corn steep liquor and urea or the relatively high DPA contents in case of NH_4Cl.

Lipid compositions of strain KK17-3. Cells cultivated for 96 h (4% glucose, 1% tryptone, salinity at 75% that of seawa-

TABLE 2
Effect of Salinity in Mediium on Cell Growth and PUFA Production in Strain KK17-3

Salinity (%)	Biomass (g/L)	TFA[a] yield (mg/L)	DHA yield (mg/L)	PUFA in TFA (%, w/w)			
				AA	EPA	DPA	DHA
0	0.2	103	6.9	7.4	1.4	6.7	6.7
25	1.9	214	35.3	8.3	3.1	11.9	16.5
50	4.5	518	118.6	8.6	2.5	9.6	22.9
75	5.7	690	169.1	9.3	1.6	8.5	24.5
100	5.7	639	155.9	11.0	1.6	8.2	24.4
150	5.5	537	136.9	14.0	2.4	12.3	25.5
200	4.7	449	118.8	10.9	3.3	14.0	26.5
250	1.5	179	54.6	10.2	1.2	8.5	30.5
300	0.6	64	9.2	4.6	1.0	9.9	14.4

[a]TFA, total fatty acids; for other abbreviations see Table 1.

TABLE 3
Effect of Carbon and Nitrogen Scources on Growth and PUFA Production in Strain KK17-3

Medium	Biomass (g/L)	TFA yield (mg/L)	DHA yield (mg/L)	PUFA in TFA (%, w/w)			
				AA	EPA	DPA	DHA
Carbon source (4%)[a]							
Galactose	2.5	109	27.6	13.1	3.4	13.9	25.2
Glycerol	3.8	288	97.4	14.0	2.3	11.9	33.8
Saccharose	3.7	333	88.5	13.9	3.9	10.4	26.6
Maltose	2.0	194	50.2	16.1	4.5	12.4	25.9
Starch	2.4	198	48.9	11.0	4.2	15.7	24.7
Raffinose	1.2	88	16.2	12.7	3.7	17.1	18.4
Glucose	5.5	545	145.4	13.3	2.8	13.6	26.7
Nitrogen source (1%)[b]							
Yeast extract	4.5	451	129.4	10.1	3.9	9.6	28.7
Tryptone	7.1	843	232.8	9.1	2.7	9.9	27.6
Corn steep liquor	3.9	289	50.4	10.1	1.9	13.8	17.4
NH_4NO_3	5.8	515	134.8	9.4	3.5	7.2	26.2
$NaNO_3$	5.4	394	104.3	8.7	4.6	12.8	26.4
$(NH_4)_2SO_4$	1.6	186	47.1	10.4	2.9	9.3	25.2
NH_4Cl	1.1	124	30.0	11.3	2.1	17.7	24.1
Urea	2.3	183	26.2	16.4	2.8	8.6	14.3

[a]1% polypeptone and 0.5% yeast extract as nitrogen sources at 75% of seawater salinity.
[b]4% glucose as carbon source at 75% of seawater salinity. For abbreviations see Table 1.

ter) produced DHA with a yield of 321 mg/L, and the lipid content in cells was 19.9%. The total lipid was fractionated into neutral lipids (71.2%), glycolipids (6.7%), and polar lipids (22.1%) by silicic acid column chromatography. The profile of fatty acids in each fraction is shown in Table 4. It is obvious that the contents of PUFA in polar lipids were higher than those in neutral lipids or glycolipids. In fact, PUFA, which are highly specialized membrane components and mainly exist in the form of phospholipids, play a key role in regulating membrane fluidity in response to high salinity and temperature variation (3). In turn, this allows the range of complex membrane functions to continue. Additionally, a high degree of unsaturation of the fatty acids in phospholipids may be a structural necessity for their function as membrane components since enzymes involved in the PUFA biosynthesis, such as fatty acid desaturases, are membrane-bound proteins (3).

Phylogenetic position of strain KK17-3. Strain KK17-3 was observed to possess a life cycle with vegetative cells, zoosporangium, and zoospore stages (Fig. 1). Spherical vegetative cells were 8–13 μm in diameter. A zoosporangium with the size of 15–23 μm in diameter could produce 20–50 zoospores with diameters of 4.5–7.0 μm, but successive bipartition to give tetrads of vegetative cells was not observed. Therefore, the strain KK17-3 belonged to a genus other than *Schizochytrium*, which proliferates in the vegetative stage by successive bipartition (28,29).

For molecular phylogenetic analysis of KK17-3, its 18S rRNA gene was amplified by PCR using genomic DNA as a template. The resultant fragment was 1793 base pairs in length (DDBJ accession number AB052556). A direct comparison between 18S rRNA genes from strain KK17-3 and the reported strains in thraustochytrids and labyrinthulids revealed the presence of a signature insertion region shared by the thraustochytrid phylogenetic group (16) (data not shown). This specific insertion sequence was found at nucleotide positions 1499–1515 of the 18S rRNA gene from KK17-3 whereas strains in the labyrinthulid phylogenetic group and other stramenopiles lacked this domain.

A phylogenetic tree (Fig. 2), which was generated by the NJ method, indicated that strain KK17-3 was closest to *S. ag-*

TABLE 4
Fatty Acid Profiles in Lipid Fractions from the Strain KK17-3 and Their Comparisons with *Schizochytrium aggregatum*

Lipid fractions	Principal fatty acids composition (%)										Lipid content in biomass (%, w/w)
	C15:0	C16:0	C16:1n-7	C18:0	C18:1n-9	C18:2n-6	AA	EPA	DPA	DHA	
KK17-3											19.9
Neutral lipids	5.5	22.4	9.6	2.9	4.3	2.9	9.9	3.0	11.2	28.2	14.2
Glycolipids	6.5	28.5	10.8	4.2	6.9	3.2	8.5	3.0	9.0	22.9	1.3
Polar lipids	3.5	14.6	6.4	0.8	2.4	1.4	13.0	3.7	15.0	39.3	4.4
S. aggregatum[a]											
Neutral lipids	ND	16.5	5.7	5.8	41.2	15.0	Trace	1.3	Trace	4.2	0.8
Glycolipids	ND	17.0	12.3	6.3	24.6	12.4	3.0	6.7	1.5	4.1	0.5
Polar lipids	ND	10.1	3.6	8.0	19.8	11.9	1.5	15.9	7.7	12.4	0.4

[a]Data cited from Reference 10. ND, not determined; for other abbreviations see Table 1.

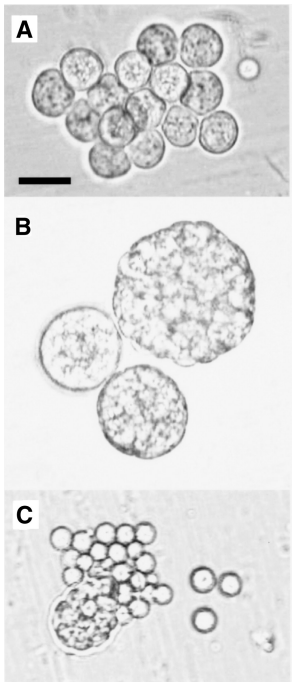

FIG. 1. Microphotographs of *Thraustochytrium,* sp. KK17-3. (A) Vegetative cells. (B) Zoosporangia. (C) Zoospores being released from zoosporangium. A bar in panel A equals 10 µm and applies to all panels.

gregatum. Indeed, their similar and relatively high G+C contents (50.8% for KK17-3 and 50.1% for *S. aggregatum*) and a high bootstrap value on the branch between them and *T. kinnei* (92%) might support their closest relationship. However,

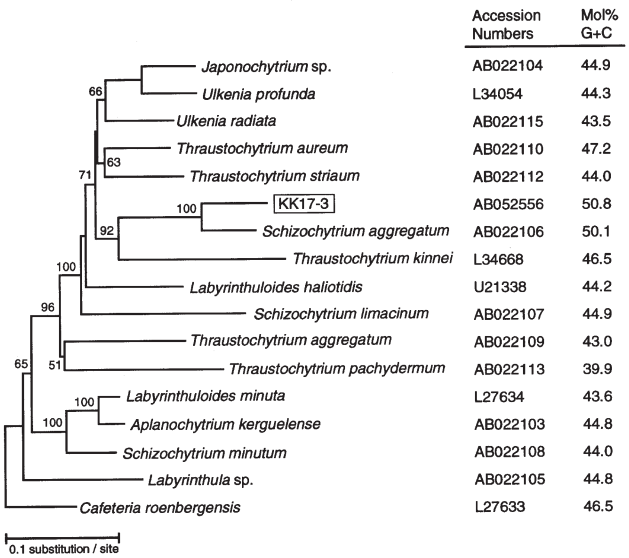

FIG. 2. Phylogenetic tree based on 18S rRNA sequences with *Cafeteria roenbergensis* as outgroup. The neighbor-joining (NJ) tree was constructed from the distances estimated by the maximum likelihood (ML) method with F84 models. The numbers at each internal branch show bootstrap values only for the nodes supported by more than 50% of 1000 replicates.

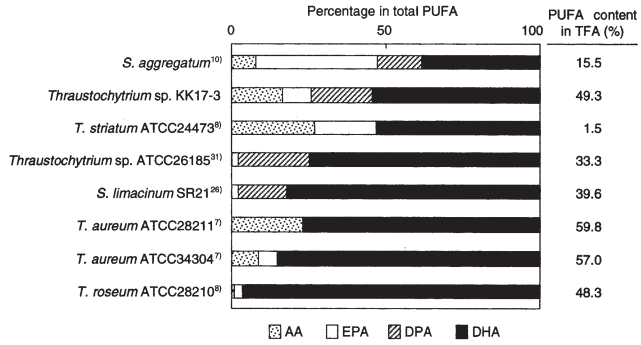

FIG. 3. Comparison of polyunsaturated fatty acid (PUFA) profiles between *Thraustochytrium* sp. KK17-3 and other reported docosahexaenoic acid (DHA) producers. Data for strain KK17-3 are from Table 2. TFA, total fatty acids; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid. The superscripted numbers stand for reference numbers.

a homology score from their sequences was less than 88%, which was not high enough to regard them as the same genus. Differences were also obvious in their modes of successive bipartitioning of vegetative cells, contents of cellular lipids, and PUFA profiles in their lipid fractions as described in Table 4. In particular, the neutral lipids from *S. aggregatum* contained only trace amounts of AA and DPA, which were major fatty acid components in the neutral lipids from KK17-3 (Table 4). In addition, strain KK17-3 exhibited several characteristics often recognized in the genus *Thraustochytrium* (16), which include the lack of motility in vegetative cells and the absence of amoeboid cells. We therefore concluded that the strain KK17-3 could be assigned to the genus *Thraustochytrium*. A further assignment of its species identity is ongoing.

The 18S rRNA gene is generally thought to accurately reflect phylogenetic differences among organisms at the level of genus and species. Furthermore, the profile of PUFA has been shown to be an effective chemotaxonomic criterion for classification and identification of the marine microorganisms producing PUFA (15,30), as demonstrated in this study. Figure 3 shows that the strain KK17-3 can be distinguished from the reported DHA-producers with regard to either the types or amounts of PUFA. Other strains in the genus *Thraustochytrium* either lacked DPA or produced lower content of total PUFA. As for *S. aggregatum* with a PUFA profile similar to the strain KK17-3, its total content of PUFA was much lower. Moreover, *S. limacinum* SR21 produced a trace of AA and EPA. DHA as a typical long-chain PUFA is synthesized by elongating, desaturating, and oxidizing the precursor fatty acids such as EPA, DPA, and tetracosahexaenoic acid (C24:6n-3) (32). However, the pathways and the regulation of PUFA biosynthesis are not well understood in marine microorganisms. Therefore, it is conceivable that our newly isolated strain *Thraustochytrium* sp. KK17-3 could be used to elucidate DHA biosynthesis by comparative analysis with a variety of DHA-producing microorganisms. The requisite experiments are being carried out.

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