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ORIGINAL PAPER

Fatty Acid Desaturation and Elongation Reactions of *Trichoderma* sp. 1-OH-2-3

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Abstract The fatty acid desaturation and elongation reactions catalyzed by *Trichoderma* sp. 1-OH-2-3 were investigated. This strain converted palmitic acid (16:0) mainly to stearic acid (18:0), and further to oleic acid (*c*9-18:1), linoleic acid (*c*9,*c*12-18:2), and α-linolenic acid (*c*9,*c*12,*c*15-18:3) through elongation, and Δ9, Δ12, and Δ15 desaturation reactions, respectively. Palmitoleic acid (*c*9-16:1) and *cis*-9,*cis*-12-hexadecadienoic acid were also produced from 16:0 by the strain. This strain converted *n*-tridecanoic acid (13:0) to *cis*-9-heptadecenoic acid and further to *cis*-9,*cis*-12-heptadecadienoic acid through elongation, and Δ9 and Δ12 desaturation reactions, respectively. *trans*-Vaccenic acid (*t*11-18:1) and *trans*-12-octadecenoic acid (*t*12-18:1) were desaturated by the strain through Δ9 desaturation. The products derived from *t*11-18:1 were identified as the conjugated linoleic acids (CLAs) of *cis*-9,*trans*-11-octadecadienoic acid and *trans*-9,*trans*-11-octadecadienoic acid. The product derived from *t*12-18:1 was identified as *cis*-9,*trans*-12-octadecadienoic acid. *cis*-6,*cis*-9-Octadecadienoic acid was desaturated to *cis*-6,*cis*-9,*cis*-12-octadecatrienoic acid by this strain through Δ12 desaturation. The broad substrate specificity of the elongation, and Δ9 and Δ12 desaturation reactions of the strain is useful for fatty acid biotransformation.

Keywords Desaturation · Elongation · Polyunsaturated fatty acid · Conjugated linoleic acid · *Trichoderma* sp.

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Introduction

Polyunsaturated fatty acids (PUFA) have various physiological effects. For example, those of the C-20 series such as dihomo-γ-linolenic acid (DGLA, *c*8,*c*11,*c*14-20:3), arachidonic acid (AA, *c*5,*c*8,*c*11,*c*14-20:4), and eicosapentaenoic acid (EPA, *c*5,*c*8,*c*11,*c*14,*c*17-20:5) are of interest as they are precursors for prostaglandins, thromboxanes, leukotrienes, and prostacyclins [1]. In addition, conjugated PUFAs (CPUFAs) have also attracted much attention as a novel type of biologically beneficial functional lipid in the last two decades. In particular, conjugated linoleic acid (CLA, 18:2 with conjugated double bonds) has been the most intensively investigated and was revealed to have beneficial effects on human and animal health [2].

Since natural sources rich in these PUFAs and CPUFAs are limited, recent investigations have been focused on microorganisms as alternative sources of them. Several groups have started to screen for microorganisms capable of accumulating lipids containing PUFAs and CPUFAs in

order to obtain more suitable sources for large-scale preparation of important nutritional components [3–5].

Previously, we found that a filamentous fungus, *Mortierella alpina* 1S-4 isolated from soil, is a potent producer of triacylglycerols containing AA, DGLA and EPA [6–8]. Mutants that are considered to be defective in (or to have low activity of) Δ5, Δ6, Δ12, Δ9 or ω3 desaturase have been derived from *M. alpina* 1S-4 [9–13], and many kinds of PUFA have been produced by means of these mutants. In fact, we have succeeded in their application to the industrial production of a triacylglycerol with a high content of AA [14]. Other than *Mortierella* sp., some groups investigated fatty acid compositions of *Trichoderma* sp. and reported that they accumulate unusual fatty acids such as hydroxylated fatty acids and conjugated fatty acids although the chemical structures of these fatty acids are still ambiguous[5, 15, 16]. We also isolated *Trichoderma* sp. AM076 from fresh water and revealed that the strain accumulated rarely occurring *cis*-9,*cis*-12-hexadecadienoic acid (*c*9,*c*12-16:2), when grown with palmitoleic acid (16:1) [17]. Moreover, the Δ9, Δ12, and Δ15 desaturases of the strain seemed to act on a variety of fatty acids [18].

In this study, we investigated the specificity of the desaturation and elongation reactions catalyzed by a mutant *Trichoderma* strain for its application for the production of PUFAs and CPUFAs. In particular, we focused on mutant strain 1-OH-2-3, which was derived from *Trichoderma* sp. AM076 exhibiting a high level of lipid accumulation, and evaluated its potential as a catalyst for fatty acid biotransformation.

Experimental Procedures

Chemicals

n-Tridecanoic acid (13:0), *n*-pentadecanoic acid (15:0), *n*-heptadecanoic acid (17:0), and a mixture of *trans*-vaccenic acid (*t*11-18:1) and *trans*-12-octadecenoic acid (*t*12-18:1) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Mead acid (MA, *c*7,*c*10,*c*13-20:3), AA, *cis*-6,*cis*-9,*cis*-12-octadecatrienoic acid ethyl ester (*c*6,*c*9,*c*12-18:3EE), and vaccenic acid (*c*11-18:1) were obtained from Funakoshi (Tokyo, Japan). *cis*-6,*cis*-9-Octadecadienoic acid ethyl ester (*c*6,*c*9-18:2EE) was obtained from Suntory Ltd. (Osaka, Japan). All other reagents were of analytical grade.

Microorganisms and Cultivation

The *Trichoderma* species (FA607, FA608, FA610, FA611, AM076, TU42 and 1-OH-2-3) were obtained from the AKU culture collection (Div. Appli. Life Sci., Kyoto University). Strains TU42 and 1-OH-2-3 were PUFA-accumulating mutants derived from *Trichoderma* sp. AM076 by nitrosoguanidine mutagenesis [17, 18]. All strains were inoculated into 4 ml of GY medium (1% glucose and 0.5% yeast extract; Oriental, Osaka, Japan), pH 6.0, with or without each fatty acid substrate, 0.80% (w/v), in a 20-ml Erlenmeyer flask, and then incubated with reciprocal shaking (120 rpm) at 28 °C for 7 days. All experiments were carried out in triplicate, and the averages of three separate experiments, which were reproducible within ±10%, are presented in the figures and tables.

Extraction, Esterification and Purification

The mycelia were harvested by filtration washed with 0.85% NaCl, and then dried at 100 °C overnight. The dried cells were directly transmethylated with 10% methanolic HCl, and the resultant fatty acid methyl esters were extracted with *n*-hexane and then analyzed by gas–liquid chromatography (GLC) [19]. The esters were quantitated using a Shimadzu GC-17A equipped with a flame ionization detector and a split injection system (split ratio, 1/50), and fitted with a capillary column (HR-SS-10, 50 m × 0.25 mm I.D.; Shinwa Kako, Kyoto, Japan). The column temperature was initially 180 °C, and then was raised to 220 °C at the rate of 2 °C/min and maintained at that temperature for 5 min. The injector and detector were operated at 250 °C. Helium was used as the carrier gas at 225 kPa/cm^2.

After conversion of the reaction products to their methyl esters, they were separated by reverse-phase high-performance liquid chromatography (HPLC, LC-10A; Shimadzu Co., Kyoto, Japan) on a Cosmosil 5C_{18} AR column (20 × 250 mm; Nacalai Tesque, Kyoto, Japan). The mobile phase was acetonitrile-H_2O (8:2 v/v) at the flow rate of 3.0 ml/min, and the effluent was monitored by means of ultraviolet detection (205 nm). The chemical structures of the fatty acid derivatives were determined by mass spectroscopy (MS), proton nuclear magnetic resonance (^{1}H–NMR) spectroscopy, ^{1}H–^{1}H chemical shift correlation spectroscopy (^{1}H–^{1}H COSY), ^{1}H clean-total correlation spectroscopy (TOCSY), and two-dimensional nuclear Overhauser effect spectroscopy (NOESY).

Preparation of Free Fatty Acids

Free fatty acids were prepared by heating the fatty acid methyl esters (50 mg) in a mixture of 5.0 ml of 7.0 N sodium hydroxide and 5.0 ml of methanol in a capped tube. After being heated in a boiling water bath for 1 h, the solution was acidified to pH 2.0 with 10% (w/v) sulfuric acid in water. The free fatty acids were extracted with diethylether. The organic extract was washed with water

and dried over anhydrous Na_2SO_4, and then the solvent was removed under vacuum with a rotary evaporator.

Preparation of Pyrrolidide Fatty Acids

Pyrrolidide derivatives were prepared by direct treatment of the isolated methyl esters with pyrrolidine-acetic acid (10:1 v/v) in a capped tube for 1 h at 100 °C, followed by extraction from the acidified solution according to the method of Andersson and Holman [20]. The dichloromethane extract was washed with water and dried over anhydrous Na_2SO_4, and then the solvent was removed under vacuum with a rotary evaporator.

GC–MS Analysis

GC–MS QP5050 with GC-17A (Shimadzu Co.) was used for mass spectral analyses. The GLC separation of fatty acid methyl esters was performed on a HR-SS-10 column as described above at the same temperature. MS was performed in the electron impact mode at 70 eV with a source temperature of 250 °C. Split injection (split ratio, 1/50) was performed with the injector port at 250 °C. The GLC separation of fatty acid pyrrolidide derivatives was performed on a HR-1 column (25 m × 0.5 mm I.D., Shinwa Kako) at 300 °C.

MS–MS Analysis

MS–MS analyses were performed on the free acids of the fatty acids with a Tandem Mass Spectrometer, JEOL HX110A/HX110A (Jeol Ltd, Tokyo, Japan). The ionization method comprised fast atom bombardment (FAB) and the accelerating voltage was 3 kV. Glycerol was used as the matrix.

^{1}H-NMR, ^{1}H-^{1}H COSY, NOESY and TOCSY Analyses

All NMR experiments were performed on a JEOL EX-400 (400 MHz at ^{1}H; Jeol Ltd.), and chemical shifts were assigned relative to the solvent signal. The fatty acid methyl esters were dissolved in dichloromethane-d_2 and the diameter of the tube was 5 mm.

Results and Discussion

Profiles of Fatty Acids Synthesized de novo by *Trichoderma* sp.

Table 1 shows the amounts and compositions of fatty acids synthesized de novo by the *Trichoderma* species. In strain 1-OH-2-3, *c*9-18:1 was a major unsaturated fatty acid accumulated due to a leaky defect in Δ12 desaturation. The strain 1-OH-2-3, however, accumulated much larger amounts of fatty acids compared with the others. *Trichoderma* sp. 1-OH-2-3 was selected for the following experiments to investigate the substrate specificity of desaturation and elongation reactions.

Fatty Acid Transformation by *Trichoderma* sp. 1-OH-2-3

13:0 Transformation

As shown in (Fig. 1b), four unknown peaks were detected on GLC when 13:0 was added to the medium. Based on comparison of their retention times on GLC and HPLC with those for standards, and on the results of mass spectral analysis, these unknown fatty acids were identified as 15:0,

Table 1 Fatty acid production and composition of *Trichoderma* species and mutants

Strains	Total FA (mg g^{-1} dry cell)	FA composition (%)[a]					
		16:0	16:1	18:0	*c*9-18:1	*c*9,*c*12-18:2	*c*9,*c*12,*c*15-18:3
FA607	44.7	36.4	2.7	3.3	9.9	38.4	9.3
FA608	50.4	58.4	6.5	4.2	9.7	18.4	2.8
FA610	16.7	41.7	1.5	2.0	7.0	38.5	9.3
FA611	41.9	49.1	1.9	4.6	12.6	27.5	4.3
TU42	31.7	54.7	ND	3.4	12.2	23.7	6.0
AM076	18.4	72.3	ND	5.4	7.3	15.0	ND
1-OH-2-3	102.9	47.3	4.1	3.7	22.3	18.9	3.7

All strains were grown in GY medium without fatty acid substrate at 28 °C for 1 week as described under "Experimental Procedures"

ND not detected

[a] *FA* fatty acid, *16:0* palmitic acid, *16:1* palmitoleic acid, *18:0* stearic acid, c*9-18:1* oleic acid, c*9,c12-18:2* linoleic acid, c*9,c12,c15-18:3* α-linolenic acid

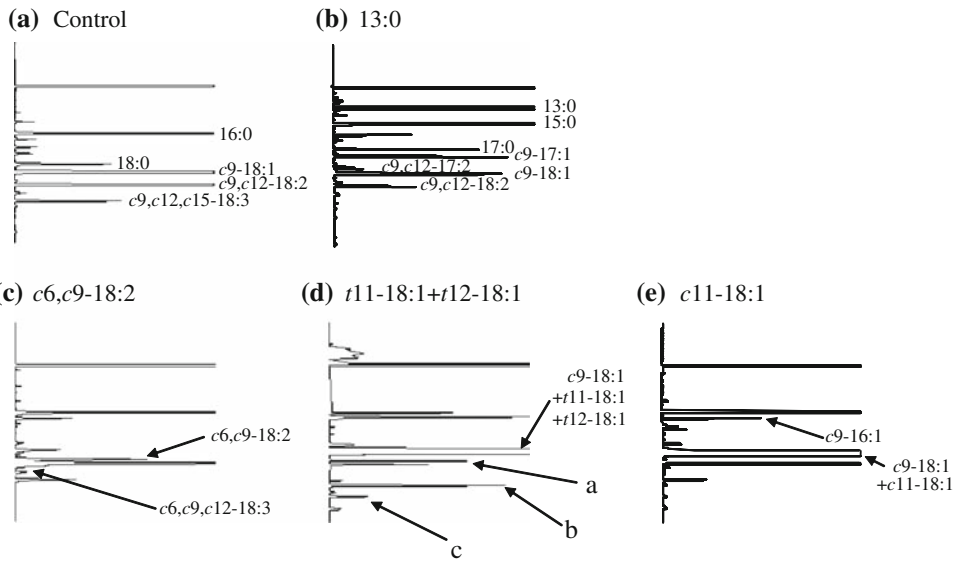

Fig. 1 GLC chromatogram of methyl esters of standard fatty acids (**a**) and those of fatty acids produced from 13:0 (**b**), *c*6,*c*9-18:2 (**c**), a mixture of *t*11-18:1 and *t*12-18:1 (**d**), and *c*11-18:1 (**e**) by *Trichoderma* sp. 1-OH-2-3

17:0, *c*9-17:1, and *c*9,*c*12-17:2. But the Δ15 desaturation product, *c*9,*c*12,*c*15-17:3, was not detected. These results suggest that 13:0 is elongated to 15:0, and further to 17:0. The resulting 17:0 is desaturated to *c*9-17:1 through Δ9 desaturation, and further to *c*9,*c*12-17:2 through Δ12 desaturation.

c6,c9-18:2 Transformation

An unknown peak was detected on GLC, when *c*6,*c*9-18:2 was added (Fig. 1c). Based on comparison of its retention times on GLC and HPLC to those for standards, and on the result of mass spectral analysis, this unknown fatty acid was identified as *c*6,*c*9,*c*12-18:3. This suggests that *c*6,*c*9-18:2 is desaturated to *c*6,*c*9,*c*12-18:3 through Δ12 desaturation. The Δ15 desaturation product, *c*6,*c*9,*c*12,*c*15-18:4, was not detected.

t11-18:1 and t12-18:1 Transformation

Unknown peaks, a, b, and c, were detected on GLC, when a mixture of *t*11-18:1 and *t*12-18:1 was added to the medium (Fig. 1d). These unknown fatty acid methyl esters were purified by HPLC and then subjected to structural determination by MS and NMR analysis. Pyrrolidide derivative of compound (a) showed a molecular weight of 333 (Fig. 2a). This result suggested that compound (a) is a C18 fatty acid containing two double bonds. Intervals of 12 atomic mass units (amu) were found between *m/z* 196 (C8) and 208 (C9), and *m/z* 236 (C11) and *m/z* 248 (C12), indicating double bonds at Δ9 and Δ12, respectively. Based on these results, and considering that fatty substrates comprise a mixture of *t*11-18:1 and *t*12-18:1, and that desaturases in microorganisms usually convert C–C single bonds to C=C double bonds in the *cis* conformation, compound (a) was deduced to be *c*9,*t*12-18:2.

The pyrrolidide derivative of compound (b) showed a molecular weight of 333 (Fig. 2b). This result suggested that compound (b) is a C18 fatty acid containing two double bonds. The FAB-MS data for the free fatty acids of compound (b) revealed a molecular weight of *m/z* 280 ($[M\text{-}H]^+$, 279). Typical fragments (*m/z*) for compound (b) were 127, 141, 167, 193, 207 and 208 (data not shown). The *m/z* 141, 167, and 193 fragments were derived through cleavage of single bonds between C8–C9, C10–C11, and C12–C13, numbered from the carboxyl group. The *m/z* 127 and 207 fragments, derived through cleavage of the single bond between the α and β positions from the double bond, were clearly detected. Hence, compound (b) was identified as a 9, 11 positional isomer of octadecadienoic acid. Furthermore, ^{1}H–NMR, ^{1}H–^{1}H COSY, NOESY, and TOCSY analyses were carried out to determine the geometric configuration of compound (b) (data not shown). Consequently, the data showed that compound (b) was *c*9,*t*11-18:2, a CLA isomer, which we reported previously to be produced by lactic acid bacteria from linoleic acid [21].

The pyrrolidide derivative of compound (c) likewise showed a molecular weight of 333 (Fig. 2c). This result again suggests that compound (c) is a C18 fatty acid containing two double bonds. Based on the composition determined from mass spectra, and the retention time on GLC relative to those of standard CLA (Sigma, USA) and CLA prepared by *Lactobacillus plantarum* AKU 1009a [21], compound (c) was strongly suggested to be *t*9,*t*11-18:2.

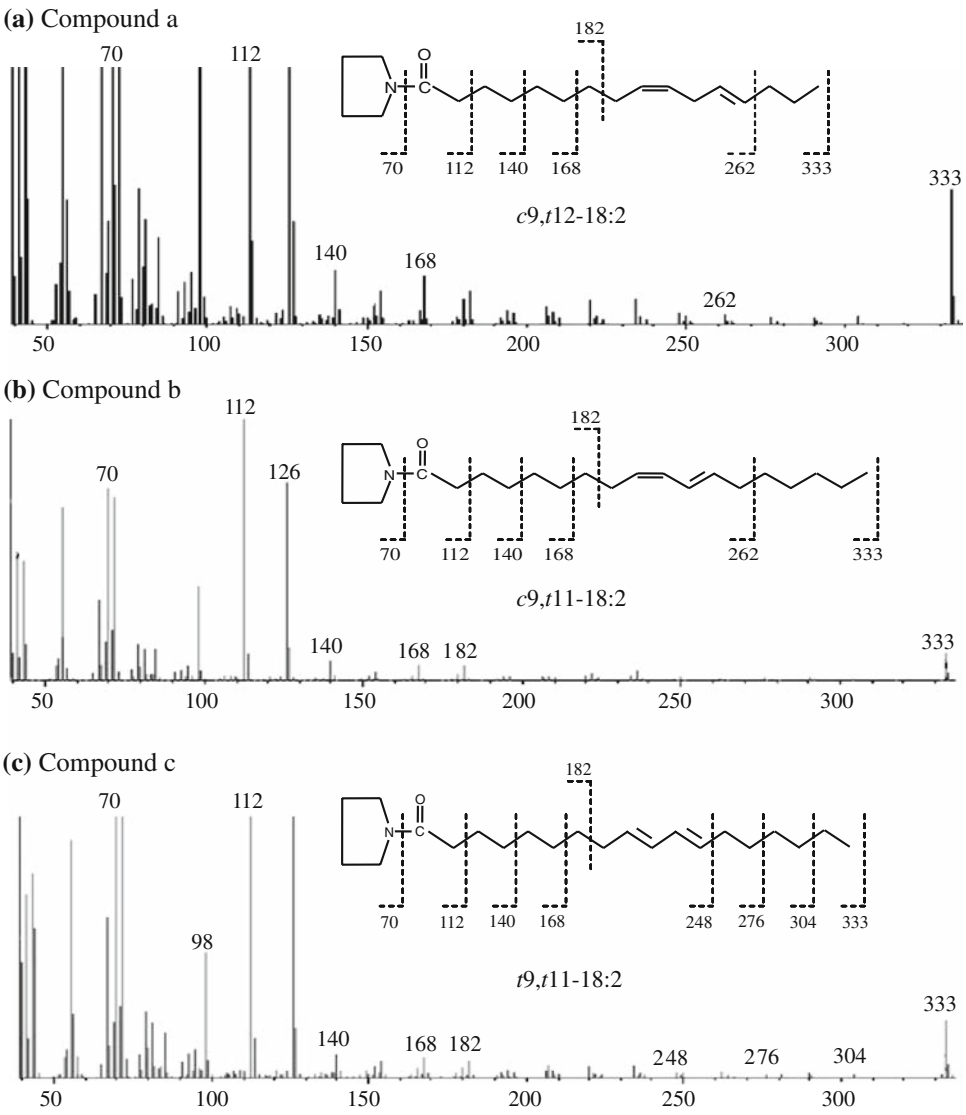

Fig. 2 GC–MS spectra of pyrrolidide derivatives of compounds (**a**), (**b**), and (**c**)

c11-18:1 Transformation

An unknown peak was detected on GLC when *c*11-18:1 was added (Fig. 1e). Based on the retention times on GLC and HPLC relative to those of standards, and the results of mass spectra analysis, this unusual fatty acid was identified as *c*9-16:1 (data not shown). The amount of *c*9-16:1 derived from *c*11-18:1 was much higher than that of de novo synthesized *c*9-16:1 (Fig. 1a, e). These results suggest that *c*11-18:1 is converted to *c*9-16:1 through *β*-oxidation. The Δ9 desaturation product, *c*9,*c*11-18:2, was not detected.

Production of Various Fatty Acids

Table 2 shows the amounts of typical fatty acids produced by strain 1-OH-2-3 for 7 days cultivation. When 0.80% of 17:0 was added to the medium, 40 μg/ml of *c*9-17:1 and 15 μg/ml of *c*6,*c*9-17:2 were produced, the proportions as to the total fatty acids being 0.67 and 0.26%, respectively. From 0.80% of *c*6,*c*9-18:2EE, 18 μg/ml *c*6,*c*9,*c*12-18:3 was produced, the proportion as to the total fatty acids being 2.49%. As to the production of CLA isomers, 45 μg/ml of *c*9,*t*11-18:2 and 19 μg/ml of *t*9,*t*11-18:2 were produced from 0.80% of *t*11-18:1 added to the medium, the proportions as to the total fatty acids being 0.70 and 0.30%, respectively. These results indicated that *Trichoderma* sp. 1-OH-2-3 exhibits desaturation activity with flexible substrate specificity, especially for Δ9 and Δ12 desaturation.

Previously, we reported that *Trichoderma* sp. AM076 produced *c*9,*c*12-16:2 and *c*9,*c*12,*c*15-16:3 from *c*9-16:1 and *c*9,*c*12-16:2 added to the culture medium, respectively [17, 18]. These results indicated that *Trichoderma* sp. have unique fatty acid transformation activities. In this report, we investigated the substrate specificity of the desaturation and elongation reactions catalyzed by a mutant strain

Table 2 Transformation of fatty acids by *Trichoderma* sp. 1-OH-2-3

Additional substrate	Newly generated fatty acids from added substrate (μg ml^{-1})					
	*c*9-17:1	*c*9,*c*12-17:2	*c*6,*c*9,*c*12-18:3	*c*9,*t*11-18:2	*t*9,*t*11-18:2	Others
*t*11-18:1	ND	ND	ND	45	19	35
*c*6,*c*9-18:2EE	ND	ND	18	ND	ND	ND
17:0	40	15	ND	ND	ND	ND

The fungus was grown in GY medium supplemented with 0.8% of each substrate for 7 days

ND not detected

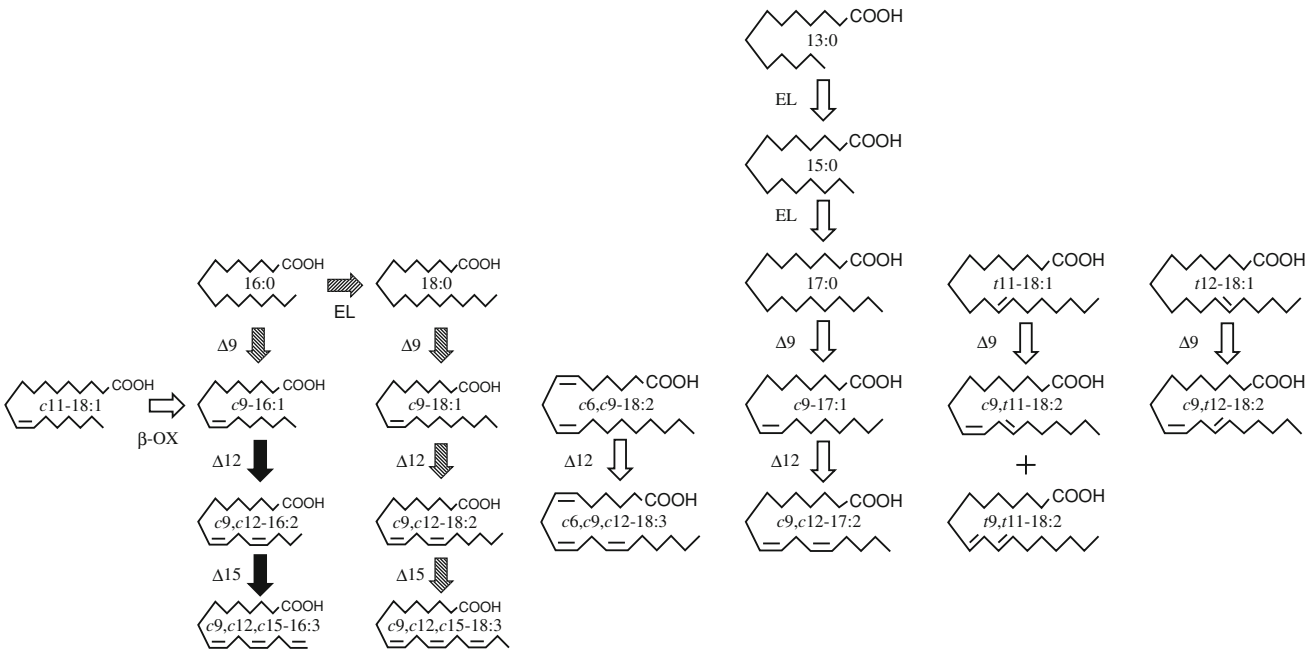

Fig. 3 Proposed pathway for FA transformation by *Trichoderma* sp. 1-OH-2-3. *EL* elongation, *Δ15* Δ15 desaturation, *Δ12* Δ12 desaturation, *Δ9* Δ9 desaturation, *β-OX* β-oxidation. The *gray arrows* indicate the de novo biosynthetic pathway. The *black arrows* indicate our previous observations [18]

1-OH-2-3 accumulating much higher amounts of fatty acid than parental strain.

The transformation of fatty acids by the mutant strain can be summarized as follows (Fig. 3): 13:0 is transformed to 15:0 and further to 17:0 through elongation. 17:0 is transformed to *c*9-17:1 and further to *c*9,*c*12-17:2 through Δ9 and Δ12 desaturation, respectively. *c*9,*c*12-17:2 is not further converted to *c*9,*c*12,*c*15-17:3 through Δ15 desaturation. *c*6,*c*9-18:2 is transformed to *c*6,*c*9,*c*12-18:3 through Δ12 desaturation. *c*6,*c*9,*c*12-18:3 was not converted to *c*6,*c*9,*c*12,*c*15-18:4 through Δ15 desaturation. *t*11-18:1 was transformed to *c*9,*t*11-18:2 and *t*9,*t*11-18:2 through Δ9 desaturation. *t*9,*t*11-18:2 might be produced from *c*9,*t*11-18:2 through spontaneous chemical isomerization or by the *cis-trans* isomerase of this strain. *t*12-18:2 was desaturated to *c*9,*t*12-18:2 through Δ9 desaturation. *t*11-18:1 was converted to CLA by strain 1-OH-2-3, while *c*11-18:1 was scarcely transformed.

In conclusion, we revealed that the substrate specificity of elongation, and Δ9 and Δ12 desaturation by mutant strain 1-OH-2-3 are broad and that the Δ9 desaturase of this strain showed an ability to produce specific CLA isomers (*c*9,*t*11-18:2 and *t*9,*t*11-18:2).

Recently, conjugated polyunsaturated fatty acids have attracted considerable attention because of their potentially beneficial effects. We reported that some lactic acid bacteria could convert linoleic acid to specific CLA isomers *c*9,*t*11-18:2 and *t*9,*t*11-18:2 [3]. Among these isomers, *c*9,*t*11-18:2 has been suggested to be one of the most important isomers in terms of biological activity because it is the major isomer in naturally occurring dairy products and it is incorporated into the phospholipid fraction of tissues of animals fed a mixture of CLA isomers [22]. Accordingly, methods for producing specific CLA isomers are strongly desired to evaluate the function of CLA isomers accurately. Though *t*11-18:1 (*trans*-vaccenic acid) is

converted to *c*9,*t*11-18:2 by mammalian cells through Δ9 desaturation, there have not been any reports that fungi can produce CLA isomers from *t*11-18:1 [23]. This is the important report of the production of specific CLA isomer, *c*9,*t*11-18:2, by fungi through Δ9 desaturation although further studies to increase the productivity and the selectivity are necessary.

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