Conjugated Linoleic Acid Production from Linoleic Acid by Lactic Acid Bacteria

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ABSTRACT: After screening 14 genera of lactic acid bacteria, *Lactobacillus plantarum* AKU 1009a was selected as a potential strain for CLA production from linoleic acid. Washed cells of *L. plantarum* with high levels of CLA production were obtained by cultivation in a nutrient medium with 0.06% (wt/vol) linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid). Under the optimal reaction conditions with the free form of linoleic acid as the substrate, washed cells of *L. plantarum* produced 40 mg CLA/mL reaction mixture (33% molar yield) from 12% (wt/vol) linoleic acid in 108 h. The resulting CLA was a mixture of two CLA isomers, *cis*-9,*trans*-11 (or *trans*-9,*cis*-11)-octadecadienoic acid (CLA1, 38% of total CLA) and *trans*-9,*trans*-11-octadecadienoic acid (CLA2, 62% of total CLA), and accounted for 50% of the total FA obtained. A higher yield (80% molar yield to linoleic acid) was attained with 2.6% (wt/vol) linoleic acid as the substrate in 96 h, resulting in CLA production of 20 mg/mL reaction mixture [consisting of CLA1 (2%) and CLA2 (98%)] and accounting for 80% of total FA obtained. Most of the CLA produced was associated with the cells (*ca.* 380 mg CLA/g dry cells), mainly as FFA.

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KEY WORDS: Conjugated linoleic acid, CLA, lactic acid bacteria, *Lactobacillus, Lactobacillus plantarum*, linoleic acid.

Interest in conjugated linoleic acid (CLA), an octadecadienoic acid (18:2) with conjugated double bonds, has increased in the last two decades because of its unique physiological effects. It was reported that dietary CLA reduced carcinogenesis (1–4), atherosclerosis (5), and body fat (6), and had several other beneficial effects (7–9).

We investigated biological systems for CLA production and found that the washed cells of *Lactobacillus acidophilus* AKU 1137 produced CLA isomers from linoleic acid (10). They efficiently produced CLA from linoleic acid with 10-hydroxy-12-octadecenoic acid (HY), a hydroxylated octadecenoic acid (18:1) as a possible intermediate under microaerobic reaction conditions. Systems using lactic acid bacteria for CLA production were found to be advantageous for the following reasons: (i) specific isomers of CLA, i.e., *cis*-9,*trans*-11(or *trans*-9,*cis*-11)-18:2 (CLA1) and *trans*-9,*trans*-11-18:2 (CLA2), are obtained, whereas chemical synthesis produces a mixture of CLA isomers (11,12); (ii) CLA is accumulated in washed cells as the FFA, making it easy to recover, and the cells themselves can be used as the CLA source. These merits prompted us to search additional strains for practical production of CLA. We report here that *L. plantarum* AKU 1009a, which was selected through screening a wide range of lactic acid bacteria, produces large amounts of CLA even under aerobic conditions. Investigation of culture conditions to obtain active catalysts and optimization of reaction conditions for practical CLA production using *L. plantarum* AKU 1009a are also described.

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EXPERIMENTAL PROCEDURES

Chemicals. Standard samples of *cis*-9,*trans*-11(or *trans*-9,*cis*-11)-18:2 (CLA1), *trans*-9,*trans*-11-18:2 (CLA2), 10-hydroxy-*trans*-12-18:1 (HY1), and 10-hydroxy-*cis*-12-18:1 (HY2) were prepared as described previously (10). Linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid) and FA-free (<0.02%) BSA were purchased from Wako Pure Chemicals (Osaka, Japan) and Sigma Chemicals (St. Louis, MO), respectively. All other chemicals used were of analytical grade and were commercially available.

Microorganisms, cultivation, and preparation of washed cells. Lactic acid bacteria preserved in our laboratory (AKU Culture Collection, Faculty of Agriculture, Kyoto University, Kyoto, Japan) and those obtained from other culture collections (IAM, Institute of Molecular and Cellular Bioscience, The University of Tokyo, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; and JCM, Japan Collection of Microorganisms, Wako, Japan) were subjected to screening. For screening, strains were cultivated in MRS medium (10) supplemented with 0.06% linoleic acid. Each strain was inoculated into 15 mL of medium in screw-capped tubes (16.5 × 125 mm) and then incubated under O_2-limited conditions in sealed tubes for 24–72 h at 28°C with shaking (120 strokes/min). For optimization of culture conditions for *L. plantarum* AKU 1009a, cultivation was carried out essentially under the same conditions as described above. For optimization of reaction conditions and preparative CLA production, cultivation was carried out aerobically with 550 mL MRS medium containing 0.06% linoleic acid in 600-mL flasks for 24 h at 28°C with shaking (120 strokes/min). Cells were harvested by centrifugation (8,000 × *g*, 10 min), washed twice with 0.85% NaCl, and centrifuged again, then used as the washed cells for the reactions.

Reaction conditions. For screening and optimization of culture and reaction conditions, the reaction mixture, 1 mL, in test tubes (16.5 × 125 mm) was composed of 0.4% (wt/vol) linoleic acid complexed with BSA (0.08%, wt/vol), 0.1 M potassium phosphate buffer (KPB, pH 6.5), and 22.5% (wet cell, wt/vol) washed cells (corresponding to 3.2%; dry cell, wt/vol). The reactions were carried out microaerobically in an O_2-adsorbed atmosphere in a sealed chamber with O_2-absorbent (Aneropack "Kenki"; Mitsubishi Gas Chemical Co, Ltd., Tokyo, Japan), and gently shaken (120 strokes/min) at 37°C for 24 to 72 h. For investigation of the effects of linoleic acid concentration and cell concentration on the reaction, and for preparative CLA production, the reactions were carried out essentially under the same conditions as described above except that the volume of the reaction mixtures was 5 mL. All experiments were carried out in triplicate, and the averages of three separate experiments, which were reproducible within ±10%, are presented in figures and tables, except for Figure 3, where exact error limits are provided.

Lipid analyses. Lipids were extracted from the reaction mixture with chloroform/methanol (1:2, vol/vol) according to the procedure of Bligh and Dyer (13), and transmethylated with 10% methanolic HCl at 50°C for 20 min. The resultant FAME were extracted with *n*-hexane and analyzed by GLC as described previously (10). Extraction and fractionation into lipid classes were carried out essentially as described previously (14,15).

RESULTS AND DISCUSSION

Screening of lactic acid bacteria producing CLA from linoleic acid. The ability to produce CLA from linoleic acid was investigated using washed cells of lactic acid bacteria. The following observations obtained in our previous study using *L. acidophilus* AKU 1137 (10) were taken into consideration: (i) washed cells of *L. acidophilus* with high levels of CLA production were obtained by cultivation in a medium with linoleic acid, and (ii) production of CLA was only observed under microaerobic conditions (O_2 concentration was less than 1%). More than 250 strains were tested from the genera of *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Propionibacterium*, *Bifidobacterium*, *Weissella*, *Aquaspirillum*, *Enterococcus*, *Tetragenococcus*, *Aerococcus*, *Butyrivibrio*, and *Lactococcus*. Of these, strains belonging to the genera *Enterococcus*, *Pediococcus*, *Propionibacterium*, and *Lactobacillus* produced considerable amounts of two CLA isomers, i.e., *cis*-9,*trans*-11(or *trans*-9,*cis*-11)-18:2 (CLA1) and *trans*-9,*trans*-11-18:2 (CLA2). Table 1 summarizes the results with strains that produced more than 0.07 mg CLA/mL reaction mixture, most of which were lactobacilli. Either 10-hydroxy-*trans*-12-18:1 (HY1) or 10-hydroxy-*cis*-12-18:1 (HY2), possible intermediates of CLA biosynthesis from linoleic acid (10), were also found in all of these reaction mixtures. *Pediococcus acidilactici* AKU 1059 and *L. rhamnosus* AKU 1124 showed almost the same level of CLA production as *L. acidophilus* AKU 1137 (about 1.5 mg/mL reaction mixture). *Lactobacillus plantarum* AKU 1009a and *L. plantarum* JCM 1551 were found to produce CLA (sum of CLA1 and CLA2) at more than 1.5 mg/mL reaction mixture. Notably, *L. plantarum* AKU 1009a produced the highest amounts of CLA (3.41 mg/mL), and was used for further optimization of culture and reaction conditions.

Optimization of culture conditions for the preparation of washed cells of L. plantarum *with high CLA productivity.*

TABLE 1
Potential Strains for CLA Production from Linoleic Acid[a]

		FA (mg/mL reaction mixture)					
Strain	Origin	Other FA	LA	Total CLA	(CLA1:CLA2)	HY1	HY2
Enterococcus faecium	AKU 1021	0.09	0.72	0.10	(0.04:0.06)	0.02	0.06
Pediococcus acidilactici	AKU 1059	0.14	1.29	1.40	(1.00:0.40)	0.30	0.43
Propionibacterium shermanii	AKU 1254	0.11	1.42	0.11	(0.09:0.02)	—	0.07
Lactobacillus acidophilus	AKU 1137	0.14	0.24	1.50	(0.85:0.65)	0.11	0.07
L. acidophilus	IAM 10074	0.25	0.22	0.60	(0.18:0.42)	0.60	0.18
L. acidophilus	AKU 1122	0.09	0.91	0.12	(0.02:0.10)	—	0.02
L. brevis	IAM 1082	0.10	0.16	0.55	(0.23:0.32)	0.79	—
L. paracasei subsp. *paracasei*	IFO 12004	0.18	0.83	0.20	(0.05:0.15)	0.22	0.45
L. paracasei subsp. *paracasei*	JCM 1109	0.17	0.76	0.07	(0.02:0.05)	—	0.57
L. paracasei subsp. *paracasei*	AKU 1142	1.08	0.90	0.07	(0.04:0.03)	0.05	1.00
L. paracasei subsp. *paracasei*	IFO 3533	0.32	0.93	0.09	(0.05:0.04)	0.06	0.68
L. pentosus	AKU 1148	0.10	1.24	0.08	(0.05:0.03)	0.08	0.05
L. pentosus	IFO 12011	0.09	0.89	0.13	(0.10:0.03)	0.13	0.74
L. plantarum	AKU 1138	0.11	0.10	0.45	(0.10:0.35)	1.21	—
L. plantarum	AKU 1009a	0.07	0.06	3.41	(0.25:3.16)	0.11	0.16
L. plantarum	JCM 8341	0.18	0.43	0.19	(0.04:0.15)	0.27	0.40
L. plantarum	JCM 1551	0.36	0.02	2.02	(0.10:1.92)	0.02	0.46
L. rhamnosus	AKU 1124	0.10	0.22	1.41	(0.69:0.72)	0.13	0.15

[a]Reactions were carried out in 72 h as described in the Materials and Methods section. Other FA included myristic acid, palmitic acid, palmitoleic acid, oleic acid, vaccenic acid, and 2-hexy-1-cyclopropane-octanoic acid. LA, linoleic acid; CLA1, *cis*-9,*trans*-11- or *trans*-9,*cis*-11-18:2; CLA2, *trans*- 9,*trans*-11-18:2; HY1, 10-hydroxy-*trans*-12-18:1; HY2,10-hydroxy-*cis*-12 18:1; —, not detected.

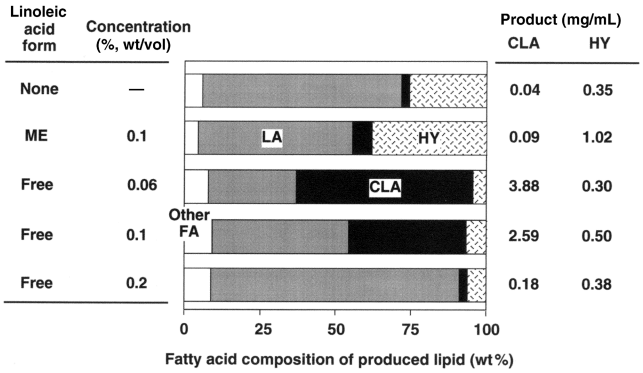

FIG. 1. Effects of linoleic acid on CLA production by *Lactobacillus plantarum* AKU 1009a. Cultivations were carried out in MRS medium with or without linoleic acid at the indicated concentrations. Reactions were carried out with linoleic acid as the substrate for 72 h as described in the Materials and Methods section. ME, linoleic acid methyl ester; Free, free linoleic acid; LA, linoleic acid; HY, hydroxylated octadecenoic acid, HY1 + HY2.

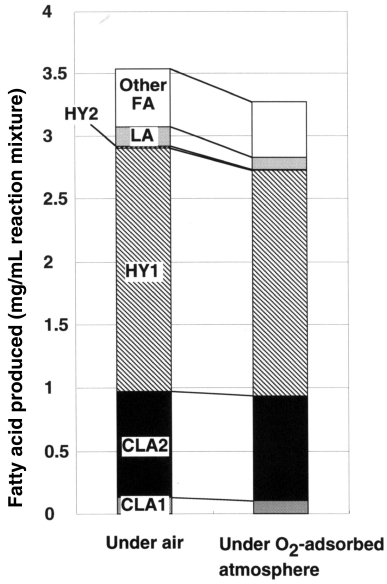

FIG. 2. Effects of oxygen on CLA production. Reactions were carried out in 24 h as described in the Materials and Methods section in an O_2-adsorbed atmosphere or under air. LA, linoleic acid; CLA1, *cis*-9,*trans*-11- or *trans*-9,*cis*-11-18:2; CLA2, *trans*-9,*trans*-11-18:2: HY1, 10-hydroxy-*cis*-12-18:1; HY2, 10-hydroxy-*trans*-12-18:1.

Lactobacillus plantarum AKU 1009a was easy to cultivate and showed a high growth rate even under aerobic conditions. To obtain washed cells with high CLA productivity, culture conditions were examined using MRS medium under aerobic conditions. When free linoleic acid was added to the MRS medium, CLA production increased markedly (Fig. 1). On the other hand, addition of linoleic acid methyl ester resulted in accumulation of the intermediate, HY. CLA production was the highest with addition of 0.06% linoleic acid to the medium (Fig. 1). Below 0.06%, linoleic acid did not affect cell growth, but higher concentrations of linoleic acid (0.2%) inhibited growth and decreased CLA productivity. The changes in CLA productivity during cultivation in MRS medium supplemented with 0.06% linoleic acid were monitored. The cells at late log phase showed significant productivity, but further cultivation resulted in a decrease in productivity. Washed cells obtained from late log-phase culture (24-h cultivation) were used for further optimization of reaction conditions.

Optimization of reaction conditions. (i) Effects of reaction pH: Reactions were carried out for 72 h in buffer systems of 0.1, 0.5, or 1.0 M acetate/sodium acetate buffer (pH 5.0, 6.0) or KPB (pH 6.5, 7.0, 7.5). CLA was most efficiently produced with 0.1 M KPB, pH 6.5. (ii) Effect of reaction temperature: Reactions were carried out for 72 h at different temperatures in the range of 20 to 52°C. CLA production increased with increasing temperature from 20 to 37°C, but decreased with higher temperature. At 52°C, neither CLA nor HY were produced. At 20°C, HY was produced in good yield, but CLA was not. (iii) Effects of substrate form: Free or methyl ester forms of linoleic acid were tested as substrates (0.5%, wt/vol) after treatment with BSA (0.5% wt/vol). BSA is a FFA carrier, increasing the solubility of FA in the reaction mixture. After 72-h reaction, the free form of linoleic acid was well converted to CLA (2.59 mg/ml), while the methyl ester was not (<0.05 mg/mL). The effect of the ratio of BSA to linoleic acid was also examined. The amount of CLA produced in 72-h reactions was not markedly changed with ratios of linoleic acid/BSA (weight ratio) between 5:2.5 and 5:1, but decreased with higher and lower ratios. (iv) Effects of oxygen: Reactions were carried out in an O_2-adsorbed atmosphere in test tubes in a sealed chamber with O_2-absorbent, or under air in open test tubes. The amounts of CLA produced and the FA compositions of the lipids produced were almost the same under both conditions. The results of 24-h reactions are presented in Figure 2. In our previous study using *L. acidophilus* AKU 1137, the presence of oxygen promoted oxidative metabolism, e.g., β-oxidation, and resulted in lower CLA production (10). Based on these findings, screening under microaerobic conditions was conducted in this study. However, the presence of oxygen did not affect CLA production from linoleic acid by *L. plantarum* AKU 1009a, resulting in easy control of the reaction conditions. *Lactobacillus plantarum* AKU 1009a may lack oxidative linoleic acid degradation activity. Strains with such high CLA-producing activity are promising for efficient production of CLA.

Effects of concentrations of linoleic acid and washed cells. Reactions were carried out for 48 h with 20% (wet cells, wt/vol) washed cells and different concentrations of linoleic acid in 5-mL reaction mixtures with a fixed ratio of linoleic acid/ BSA (weight ratio), 5:1. CLA production increased with increasing concentration of linoleic acid up to 2% (wt/vol) and reached a plateau (8.9 mg/mL) with higher concentrations, while HY production increased up to 5% (wt/vol) linoleic acid and reached a plateau (18.5 mg/mL) at higher concentrations.

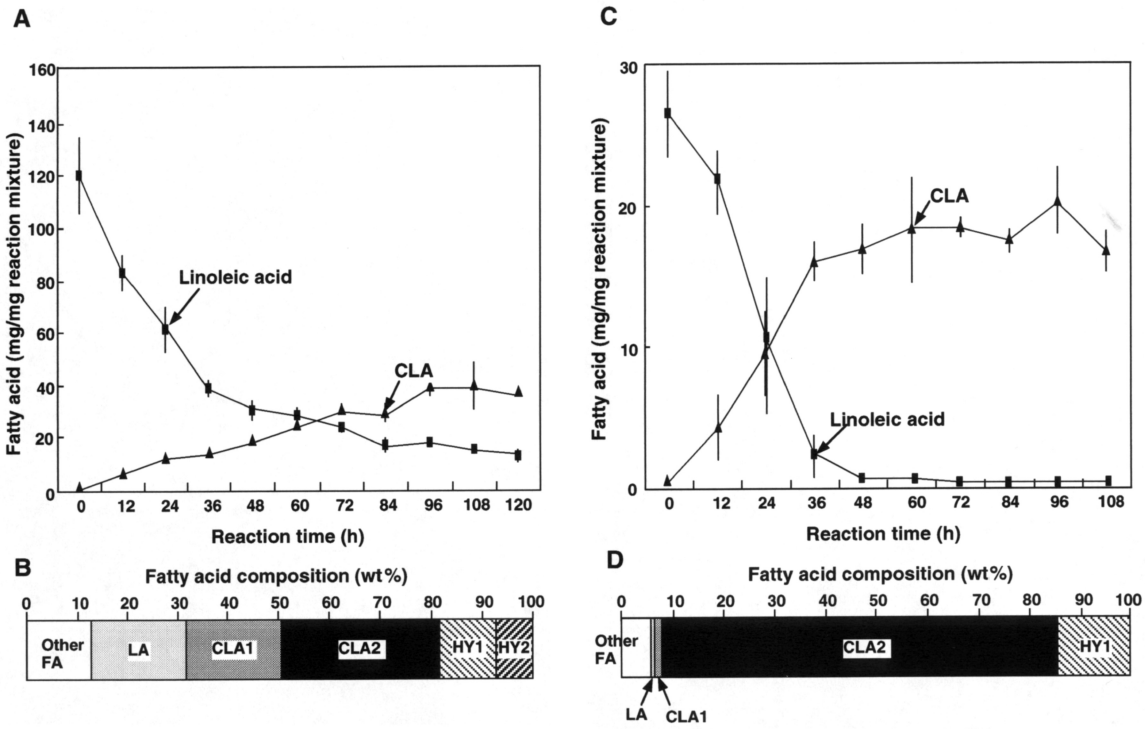

FIG. 3. Time course of preparative CLA production with 12 (A and B) or 2.6% (wt/vol) (C and D) linoleic acid as the substrate with 33 or 23% (wet wt/vol) washed cells, respectively, under the conditions described in the Materials and Methods section. (A) and (C): Time course of the reaction. (■), linoleic acid; (▲), CLA (*cis*-9,*trans*-11- or *trans*-9,*cis*-11- and *trans*-9,*trans*-11-18:2). (B) and (D): FA composition (wt%) of the lipid produced in 108- or 96-h reaction, respectively. For abbreviations, see Figure 2.

Reactions were carried out for 48 h with 6.9% (wt/vol) linoleic acid and different amounts of washed cells in 5-mL reaction mixtures. CLA production increased to 23.9 mg/mL with increasing amounts of washed cells up to 33% (wet wt/vol), which corresponded to 5% (dry wt/vol), but decreased slightly with greater amounts of washed cells.

Time course of preparative CLA production. The time course of CLA production from linoleic acid was monitored under two different conditions. With 12% (wt/vol) linoleic acid as the substrate and 33% (wet wt/vol) washed cells as the catalyst, the production of CLA reached a maximum (40 mg/mL) at 108 h and then gradually decreased (Fig. 3A). On the other hand, with 2.6% (wt/vol) linoleic acid as the substrate and 23% (wet wt/vol) washed cells as the catalyst, 80% (mol%) of the linoleic acid added was converted to CLA (20 mg/mL) in 96 h (Fig. 3C). FA compositions of the produced lipids are also presented in Figure 3. With a high substrate concentration (Fig. 3B), a large amount of CLA1 (15 mg/mL) was found, while with low substrate concentration (Fig. 3D) a significantly lesser amount of CLA1 (0.6 mg/mL) was observed and CLA2 became dominant (19.5 mg/mL). The proportions of CLA isomers changed depending on reaction conditions. Lower substrate concentration and longer reaction tended to increase CLA2 production. Investigation of the mechanism controlling the proportions of CLA isomers and the detailed analysis of the pathway of CLA production from linoleic acid are currently underway to produce one of the CLA isomers specifically.

Distribution and lipid classes of the FA produced by L. plantarum. The reaction mixture with 2.6% (wt/vol) linoleic acid as the substrate and 23% (wet wt/vol) washed cells as the catalyst was centrifuged after 108-h reaction and separated into supernatant and cells. The distribution and lipid classes of the FA produced in both supernatant and cells were analyzed (Table 2). Most of the FA (98.9%) were found in the cells (or associated with the cells), in which CLA was found as the most abundant FA. Of the CLA in the cells (or associated with cells), 53 and 41% were found in the FFA and nonpolar lipid fractions, respectively.

Biological systems are promising for the selective preparation of CLA isomers. Natural dietary sources of CLA are the meat and milk of ruminants and products made from them. In these materials, the predominant isomer is *cis*-9,*trans*-11-18:2, which accounts for over 75% of the total CLA (16,17). The *cis*-9,*trans*-11-18:2 is also produced from linoleic acid as an intermediate of biohydrogenation by rumen bacteria (18) and dairy starter cultures (19). However, the amounts of CLA in these materials are very low. The results presented here clearly show that *L. plantarum* AKU 1009a is a promising biocatalyst for CLA production. The produced CLA accumulated in the cells, reaching *ca.* 38% (w/w) of the dry cells obtained after the reaction.

TABLE 2
Distribution and Lipid Classes of FA Produced from Linoleic Acid by Washed Cells of *Lactobacillus plantarum*[a]

Fatty acid	FA concentration (mg/mL reaction mixture) after reaction	Distribution of FA in indicated lipid class (mol%) in: Supernatant FA[b]	Supernatant NL[c]	Supernatant PL[d]	Cells FA	Cells NL	Cells PL	Total
Linoleic acid	0.19	0.2	—[e]	—	0.1	0.1	0.4	0.8
CLA1	0.38	0.1	—	—	0.8	0.9	—	1.8
CLA2	16.33	0.5	—	—	39.7	30.4	4.4	75.0
HY1	1.32	Trace[f]	Trace	—	0.2	5.8	—	6.0
HY2	2.20	Trace	—	—	9.6	0.2	0.3	10.1
Other FA	1.33	0.1	0.1	0.1	5.2	0.6	0.2	6.3
Total	21.75	0.9	0.1	0.1	55.6	38.0	5.3	100

[a]*Lactobacillus plantarum* was cultivated in MRS medium with linoleic acid (0.06%, wt/vol) for 24 h. The reaction was carried out with 2.6% (wt/vol) linoleic acid as the substrate for 108 h under the conditions described in the Materials and Methods section. For abbreviations, see Table 1. Other fatty acids (mg/mL reaction mixture) were palmitic acid (0.02), oleic acid (0.75), vaccenic acid (0.43) and 2-hexy-1-cyclopropane-octanoic acid (0.13). NL, nonpolar lipids; PL, polar lipids; —, not detected; Trace, <0.05 mol%.

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