Biohydrogenation of C_{20} polyunsaturated fatty acids by anaerobic bacteria

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Running foot line: Discovery of novel C_{20} polyunsaturated fatty acids

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Abbreviations

Arachidonic acid, AA; Docosahexaenoic acid, DHA; Eicosapentaenoic acid, EPA; Fast atom bombardment, FAB; ^{1}H clean-total correlation spectroscopy, TOCSY; ^{1}H-^{1}H-chemical shift correlation spectroscopy, COSY; High-performance liquid chromatography, HPLC; Gas-liquid chromatography, GC; Linoleic acid, LA; Mass spectroscopy, MS; Non-methylene-interrupted fatty acids, NMIFA; Polyunsaturated fatty acids, PUFAs; Proton nuclear magnetic resonance, ^{1}H-NMR; Vaccenic acid, VA; Two-dimensional nuclear Overhauser effect spectroscopy, NOESY

Abstract

The polyunsaturated fatty acids (PUFAs) include many bioactive lipids. The microbial metabolism of C_{18} PUFAs is known to produce their bioactive isomers, such as conjugated fatty acids and hydroxy fatty acids, but there is little information on that of C_{20} PUFAs. In this study, we aimed to obtain anaerobic bacteria for the ability to produce novel PUFA from C_{20} PUFAs. Through the screening of about 100 strains of anaerobic bacteria, *Clostridium bifermentans* JCM 1386 was selected as a strain with the ability to saturate PUFAs during anaerobic cultivation. This strain converted arachidonic acid (*cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid) and eicosapentaenoic acid (*cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17-eicosapentaenoic acid) into *cis*-5,*cis*-8,*trans*-13-eicosatrienoic acid and *cis*-5,*cis*-8,*trans*-13,*cis*-17-eicosatetraenoic acid, giving yields of 57% and 67% against the added PUFAs, respectively. This is the first report of the isolation of a bacterium transforming C_{20} PUFAs into corresponding non-methylene-interrupted fatty acids. We further investigated the substrate specificity of the biohydrogenation by this strain and revealed that it can convert two *cis* double bonds at the ω6 and ω9 positions in various C_{18} and C_{20} PUFAs into a *trans* double bond at the ω7 position. This study should serve to open up the development of novel potentially bioactive PUFAs.

Supplementary key words: arachidonic acid, fatty acid/metabolism, omega-3 fatty acids, lipids/chemistry, diet and dietary lipids, anaerobic bacteria, eicosapentaenoic acid, conjugated fatty acid, non-methylene-interrupted fatty acids (NMIFA)

INTRODUCTION

The polyunsaturated fatty acids (PUFAs) include many bioactive lipids that play an important role in the maintenance of biological functions in mammals (1, 2). The vast majority of PUFAs has 2 or more *cis* double bonds that are separated from each other by a single methylene group (known as methylene-interrupted fatty acids). They include two major subgroups (the ω3 and ω6 PUFAs) that have different functions (1-3). Arachidonic acid [*cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid (20:4, ω6), AA], which is the C_{20} PUFA of the ω6 class and is made from linoleic acid [*cis*-9,*cis*-12-octadecadienoic acid (18:2, ω6), LA], is involved in many cellular signaling mechanisms, and is also the precursor for the formation of 2-series of prostaglandins. On the other hand, eicosapentaenoic acid [*cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17-eicosapentaenoic acid (20:5, ω3), EPA], which is a C_{20} PUFAs of the ω3 class and is made from α-linolenic acid [*cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid (18:3, ω3)], is the precursor for the formation of 3-series of prostaglandins, and can competes with the effects of AA, such as the AA conversion to the prostaglandins. Unlike methylene-interrupted fatty acids, rare isomers of PUFAs, which have at least two double bonds that are separated by a single carbon-carbon bond (known as conjugated fatty acids) (4-7) or 2 or more methylene groups [known as non-methylene-interrupted fatty acids (NMIFA)] (8-10), have been found in several materials including plant oil. These rare PUFAs have been also reported to show interesting physiological effects (9, 11-15). Therefore, they have gained considerable attention, but natural sources rich in them are limited.

The partial hydrogenation of PUFAs is the process of converting PUFAs into the more saturated fatty acids and can produce NMIFAs from more readily available PUFAs. They can be mainly performed by chemical hydrogenation in industry and by microbial biohydrogenation in living organisms (16). Chemical partial hydrogenation is widely used to convert vegetable oils into foods such as margarine. The partial hydrogenation of vegetable oils produces various hydrogenated vegetable oils, including several isomers of octadecenoic acid (18:1), depending on the reaction conditions. In contrast, microbial biohydrogenation can selectively produce specific isomers (4-7). Thus, microbial biohydrogenation has several advantages over chemical hydrogenation.

Recently, some studies, including ours, have found that many anaerobic bacteria, such as *Lactobacillus* species, can produce conjugated linoleic acids from LA (4, 17-20). Further, we have revealed that lactic acid bacteria produce unique PUFAs from various C_{18} PUFAs through partial biohydrogenation (21-23). Thus, the biohydrogenation of C_{18} PUFAs has been widely studied. However, as far as we know, the biohydrogenation of other fatty acids, especially C_{20} PUFAs, has not been extensively studied so far.

In this paper, we report about the screening of anaerobic bacteria for the ability to transform C_{20} PUFAs through biohydrogenation. We found that *Clostridium bifermentans* JCM 1386 can specifically convert AA and EPA into their partially saturated fatty acids with a *trans* double bond at the ω7 position. We further found that other C_{18} and C_{20} PUFAs were also converted in a similar manner. Thus, we succeeded in the production of various C_{18} and C_{20} NMIFAs with a *trans* double bond at the ω7 position through the biohydrogenation by *C. bifermentans* JCM 1386, leading to the development of novel potentially bioactive PUFAs.

MATERIALS AND METHODS

Chemicals

LA and α-linolenic acid were purchased from Wako Pure Chemical (Osaka, Japan).

γ-Linolenic acid (*cis*-6,*cis*-9,*cis*-12-18:3), dihomo-γ-linolenic acid [*cis*-8,*cis*-11,*cis*-14-eicosatrienoic acid (20:3)], AA, and EPA were purchased from Sigma (St. Louis, USA). Docosahexaenoic acid [*cis*-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19-docosahexaenoic acid (22:6), DHA] was purchased from Cayman Chemical (MI, USA). All other chemicals used were of analytical grade and are commercially available.

Microorganism and cultivation

The identified anaerobic bacteria used for this study (Supplementary Table S1) were preserved in our laboratory (AKU Culture Collection, Division of Applied Life Science, Faculty of Agriculture, Kyoto University, Kyoto, Japan) and those obtained from other culture collections (JCM, Japan Collection of Microorganisms, Saitama, Japan; and ATCC, American Type Culture Collection, VA, USA). The unidentified anaerobic bacteria used for this study were isolated from pond, wastewater, fish viscera, and so on. The medium was GAM broth (pH 7.0) (Nissui Pharmaceutical co., Ltd., Tokyo, Japan) supplemented with 0.03% (w/v) LA, AA or 0.02% (w/v) EPA. Each strain was inoculated into 15 mL of the medium in screw-capped tubes (16.5 × 215 mm) and then incubated in an anaerobic chamber (98% nitrogen and 2% hydrogen) at 37°C for 2-3 days. After the cultivation, the culture medium was separated into supernatant and cells by centrifugation (8,000 *g*, 10 min), and the supernatant was used for lipid analysis.

Lipid analysis

Lipids were extracted from the supernatants with chloroform-methanol according to the procedure of Bligh-Dyer (24), and methylated with 4% methanolic-HCl at 50°C for 20 min. The resultant fatty acid methyl esters were extracted with *n*-hexane and analyzed by gas-liquid chromatography (GC) using a Shimadzu (Kyoto, Japan) GC-1700 gas chromatograph equipped with a flame ionization detector and a split injection system and fitted with a capillary column

(ULBON HR-SS-10, 50 m × 0.25 mm I.D., Shinwa Kako, Kyoto, Japan). The column temperature was initially 180°C and was raised to 220°C at a rate of 2°C/min and maintained at that temperature for 20 min. The injector and detector were operated at 250°C. Helium was used as a carrier gas at 0.97 ml/min.

Isolation, derivatization, and identification of products

For the isolation of the newly generated fatty acid in a culture of *C. bifermentans* JCM 1386 with 0.03% (w/v) AA (UK1), its methyl esters were purified by high-performance liquid chromatography (HPLC, monitored at 205 and 233 nm) using a Shimadzu LC-VP system fitted with a Cosmosil column 5C18-ARII (20 × 250 mm, Nacalai tesque, Kyoto, Japan). The mobile phase was acetonitrile-water (80:20, v/v) at a flow rate of 5.0 mL/min and the column temperature of 30°C. The fraction containing UK1 was further purified by HPLC on Inertsil ODS SQ5-1385 (4.6 × 250 mm, GL Science Inc., CA, USA) joined with Capcelpak C18 UG20 (4.6 × 250 mm, Shiseido, Tokyo, Japan). Acetonitrile-water (80:20, v/v) was used as the mobile phase at a flow rate of 1.2 mL/min. For the isolation of the newly generated fatty acid in a culture of *C. bifermentans* JCM 1386 with 0.02% (w/v) EPA (UK2), its methyl esters were purified using a same procedure as described for UK1 except that the mobile phase used for the latter step was acetonitrile-water (80:20, v/v) at a flow rate of 1.0 mL/min.

The chemical structures of purified fatty acids were determined by mass spectroscopy (MS), proton nuclear magnetic resonance (^{1}H-NMR), ^{1}H-^{1}H-chemical shift correlation spectroscopy (COSY), two-dimensional nuclear Overhauser effect spectroscopy (NOESY), and ^{1}H clean-total correlation spectroscopy (TOCSY).

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

All NMR experiments were performed on a BrukerBiospin DX-750 (750 MHz for ^{1}H) and chemical shifts were assigned relative to the solvent signal (dichloromethane-d_2).

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 screw-cap tubes for 1 h at 115°C followed by extraction according to the method of Andersson and Holman (25). The organic extract was washed with water and dried over anhydrous Na_2SO_4, and then the solvent was removed by a vacuum in a rotary evaporator.

GC-MS analysis

GC-MS QP5050 (Shimadzu) with a GC-17A gas chromatograph was used for mass spectral analysis. The GC separation of the methyl ester and the pyrrolidide derivatives was performed on an ULBON HR-1 column (25 m × 0.5 mm, Shinwa Kako) at 300°C. MS was used in the electron impact mode at 70 eV with a source temperature of 250°C. Split injection was employed with the injector port at 250°C.

MS-MS analysis

MS-MS analyses were performed on the free acids of the fatty acids with a JEOL-HX110A/HX110A tandem mass spectrometer. The ionization method was fast atom bombardment (FAB), and the acceleration voltage was 3 kV. Glycerol was used for the matrix.

RESULTS

Screening of anaerobic bacteria that have the ability to convert C_{20} PUFAs

The ability of anaerobic bacteria to convert the C_{20} PUFAs of EPA and AA during cultivation was investigated together with LA as a reference of C_{18} PUFA. We tested about 100 strains, including the identified bacteria, which belonged to genera such as *Megasphaera*, *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*, *Clostridium*, *Bacteroides*, *Eubacterium*, and so on (Supplementary Table S1), and the unidentified bacteria. The peaks of the PUFAs were identified by comparison with the retention time of the reference standards on GC analysis.

Of these bacteria, 2 strains of *Clostridium bifermentans* (JCM 1386 and JCM 7832) showed the activity to convert AA and EPA, while 5 strains (including the two C_{20} PUFAs converting strains mentioned above) belonging to the genera of *Clostridium* and *Propionibacterium* were found to have the ability to convert LA to vaccenic acid (*trans*-11-18:1, VA) (Table 1).

Figure 1 shows the GC chromatogram of methylated fatty acids produced by *C. bifermentans* JCM 1386 from AA, EPA, and LA as examples. When *C. bifermentans* JCM 1386 was cultured with AA or EPA, newly generated fatty acids [UK1 from AA (Fig. 1A) and UK2 from EPA (Fig. 1B)] were detected on the GC chromatogram of methylated fatty acids. The same reactions were observed when *C. bifermentans* JCM 7832 was cultured with AA or EPA (Table 1). However, *C. sporogenes* JCM 7849, *C. sporogenes* JCM 7850, and *P. acnes* JCM 6473 couldn’t convert AA and EPA.

As the concentration of the C_{20} PUFAs added grew, *C. bifermentans* JCM1386 showed higher activity than *C. bifermentans* JCM 7832 (data not shown). *C. bifermentans* JCM1386 was used for further analyses.

Identification of the newly generated fatty acid in a culture of *C. bifermentans* JCM 1386 with AA

When the lipids extracted from the medium after cultivation of *C. bifermentans* JCM 1386 with AA were analyzed by thin-layer chromatography, almost all lipids were present in the free form (data not shown). After complete esterification of the free form fatty acids products, the resulting methyl esters were isolated and used for structural analysis. The mass spectrum of the isolated methyl ester of UK1 exhibited a molecular weight of *m/z* 320, indicating that UK1 is C_{20} PUFA containing three double bonds. The molecular ion peak ($[M+Na]^+$, 343) obtained by FAB-MS analysis (FAB^+) of the methyl ester of UK1 was fragmented again by MS-MS [*m/z* (FAB^+, 8.00kV), 328(1), 314(2), 300(2), 299(3), 286(3), 285(4), 272(12), 258(1), 257(2), 232(3), 218(3), 217(28), 204(33), 190(1), 189(1), 164(35), 163(12), 150(3), 149(4), 124(5), 110(13), 109(68), 96(100), 82(6), and 81(40)]. The *m/z* 124, 150, 164, 190, 232, and 258 were derived from cleavage between single bonds 4-5, 6-7, 7-8, 9-10, 12-13, and 14-15, as numbered from the carboxyl group. The *m*/*z* 110, 150, 164, 204, 218, and 272 derived from the cleavage of single bonds between the α and β positions from the double bonds were detected. On the basis of the results of MS analyses, UK1 was identified as the geometrical isomers of 5,8,13-20:3.

^{1}H-NMR analysis also suggested that UK1 is an isomer of 20:3 (see Fig. 2). The signal intensity of L (5.36 ppm, *m*, 6H) indicates the existence of three double bonds in UK1. The sequence of the protons from the methyl end of the molecule was deduced A, B, E, L, L, J, L, L, G, C, F, L, L, H, D, and I or A, B, E, L, L, F, C, G, L, L, J, L, L, H, D, and I based on the pattern of crosspeaks in ^{1}H-^{1}H COSY analysis (see Fig. 2B). The sequence was confirmed as the latter one by the appearance of a crosspeak between J and H, but not J and E in TOCSY analysis (see Fig. 2C). Furthermore, NOESY analysis was carried out to identify the geometric configurations of double bonds. The positive crosspeaks appeared between G and J, and H and J, indicating that the two double bounds of Δ5 and Δ8 positions are in the *cis* configuration, whereas no positive crosspeak appeared between E and F, indicating that Δ13 position is in the *trans* configuration (see Fig. 3A). On the basis of the results of the above spectral analyses, UK1 was identified as *cis*5,*cis*-8,*trans*-13-20:3 (see Fig. 2A).

Identification of the newly generated fatty acid in a culture of *C. bifermentans* JCM 1386 with EPA

When the lipids extracted from the medium after cultivation of *C. bifermentans* JCM 1386 with EPA were analyzed by thin-layer chromatography, almost all lipids were present in the free form (data not shown). After complete esterification of the free form fatty acids products, the resulting methyl esters were isolated and used for structural analysis. The mass spectrum of the isolated methyl ester of UK2 exhibited a molecular weight of *m/z* 318. This result suggested that UK2 is C_{20} PUFAs containing four double bonds. The molecular ion peak ($[M+Na]^+$, 341) obtained by FAB-MS analysis (FAB^+) of the methyl ester of UK2 was fragmented again by MS-MS [*m/z* (FAB^+, 8.00kV), 326(4), 312(1), 311(1), 286(2), 272(6), 271(11), 258(1), 257(1), 232(2), 218(2), 217(22), 204(18), 190(1), 164(22), 150(2), 149(7), 124(5), 110(10), 109(57), 96(100), 82(4), 81(28)]. The *m/z* 124, 150, 164, 190, 232, 258, 286, and 312 were derived from the cleavage of single bonds 4-5, 6-7, 7-8, 9-10, 12-13, 14-15, 16-17, and 18-19 as numbered from carboxyl group. The *m/z* 110, 150, 164, 204, 218, 272, and 326 derived from the cleavage of single bonds between the α and β positions from the double bonds were detected. On the basis of the results of MS analyses, UK2 was identified as the geometrical isomers of 5,8,13,17-20:4. ^{1}H-NMR analysis also suggested that UK2 is an isomer of 20:4 (Fig. 4). The signal intensities of J (5.35 ppm, *m*, 6H) and K (5.42 ppm, *m*, 2H) indicate the existence of four double bonds in UK2. The sequence of the protons from the methyl end of the molecule was deduced A, E, J, J, F, E, K, K, D, B, E, J, J, H, J, J, F, C, and G based on the integration of COSY and TOCSY analyses (see Fig. 4B and C). NOESY spectrum revealed that the positive crosspeaks appeared between F and H, H and E, and F and E, and no positive crosspeak appeared between D and E, indicating that the three double bonds of Δ5, Δ8, and Δ17 position are all in *cis* configuration, and that the double bond of Δ13 position is in the *trans* configuration (see Fig. 3B). On the basis of the results of the above spectral analyses, UK2 was identified as *cis*5,*cis*-8,*trans*-13,*cis*-17-20:4 (see Fig. 4A).

Effects of AA and EPA concentration in the medium on their transformation by *C. bifermentans* JCM 1386

Effects of AA and EPA concentration on their transformation by *C. bifermentans* JCM1386 were investigated (see Fig. 5). When various concentrations of AA were added to the medium, the amount of UK1 production increased with increasing concentration of AA up to 0.42 mg/mL, giving a yield of 57% (0.24 mg/mL) against the added AA (0.42 mg/mL) (see Fig. 5A). When various concentrations of EPA were added to the medium, the amount of UK2 production increased with increasing concentration of EPA up to 0.18 mg/mL, giving a yield of 67% (0.12 mg/mL) against the added EPA (0.18 mg/mL) (see Fig. 5B). However, *C. bifermentans* JCM 1386 no longer produced UK2 when more than 0.24 mg/mL EPA was added.

Substrate specificity of polyunsaturated fatty acid transformation by *C. bifermentans* JCM 1386

To examine the substrate specificity of PUFAs transformation during the cultivation of *C. bifermentans* JCM 1386, free fatty acids of LA, α-linolenic acid, γ-linolenic acid, dihomo-γ-linolenic acid, AA, EPA, and DHA were added to the medium (see Fig. 6). *C. bifermentans* JCM 1386 could convert LA, AA, EPA, α-linolenic acid, γ-linolenic acid, and dihomo-γ-linolenic acid, but not DHA.

The GC-MS analysis of the products obtained from α-linolenic acid, γ-linolenic acid, and dihomo-γ-linolenic acid

The products from α-linolenic acid, γ-linolenic acid, and dihomo-γ-linolenic acid were analyzed by GC-MS (see Fig. 7). The spectrum of pyrrolidide derivative of the product from α-linolenic acid showed a molecular weight of *m/z* 333 and gaps of 26 amu between *m/z* 224 and 250, and between *m/z* 278 and 304, indicating that this is a C_{18} PUFA with double bonds at the ω3 and ω7 positions (11,15-18:2) (see Fig. 7A). The pyrrolidide derivative of the product from γ-linolenic acid showed a molecular weight of *m/z* 333 and gaps of 26 amu between *m/z* 154 and 180, and between *m/z* 222 and 248, indicating that the product is a C_{18} PUFA with double bonds at the ω7 and ω12 positions (6,11-18:2) (Fig. 7B). The pyrrolidide derivative of the product from dihomo-γ-linolenic acid showed a molecular weight of *m/z* 361 and gaps of 26 amu between *m/z* 182 and 208, and between *m/z* 250 and 276, indicating that the product is a C_{20} PUFA with double bonds at the ω7 and ω12 positions (8,13-20:2) (Fig. 7C). Thus, *C. bifermentans* JCM 1386 could convert C_{18} and C_{20} PUFAs with double bonds at the ω6 and ω9 positions into their corresponding NMIFAs by *C. bifermentans* JCM 1386 (see Fig. 8).

DISCUSSION

The studies on PUFAs conversion by anaerobic bacteria have been done with the primary aim to improve the quality of the ruminant products such as milk or meat. In the course of these studies, numerous PUFA-transforming bacteria, such as *Butyrivibrio fibrisolvens* (4), *Lactobacillus plantarum* (20-23), and *Bifidobacterium breve* (19), have been isolated, and their metabolic pathways of C_{18} PUFAs, such as LA and α-linolenic acid, have been revealed. However, the ability to transform C_{20} and C_{22} PUFAs has not been studied in detail, although there have been several reports that EPA and DHA are hydrogenated in the rumen *in vivo* (26) and disappear during incubations *in vitro* with mixed ruminal microorganisms (27, 28).

In this study, we found that *C. bifermentans* JCM 1386 could convert AA and EPA into *cis*-5,*cis*-8,*trans*-13-20:3 and *cis*-5,*cis*-8,*trans*-13,*cis*-17-20:4, respectively, which are NMIFAs with a *trans* double bond at the ω7 position (see Figs. 5 and 8). This is the first report of the isolation of the bacterium transforming C_{20} PUFAs into corresponding NMIFAs. Considering that similar reactions were observed with LA (see Fig. 1C), this strain can convert two *cis* double bonds at the ω6 and ω9 positions in PUFAs into a *trans* double bond at the ω7 position to generate the *trans* fatty acids regardless of the existence of double bonds at other positions. In addition, similar reactions were also observed of other C_{18} and C_{20} free PUFAs (α-linolenic acid, γ-linolenic acid, and dihomo-γ-linolenic acid) (see Fig. 6). They might be converted into the corresponding NMIFAs with a *trans* double bond at the ω7 position. However, *C. bifermentans* JCM 1386 could not convert DHA, indicating that C_{22} PUFAs might not be a substrate for this strain. Thus, we succeeded in the production of various C_{18} and C_{20} NMIFAs with a *trans* double bond at the ω7 position through the biohydrogenation by *C. bifermentans* JCM 1386.

NMIFAs are a class of PUFAs that has received attention because of their unique structure and physiological activity, and they have often been found in plant oils. Pinolenic acid (*cis*-5,*cis*-9,*cis*-12-18:3) and columbinic acid (*trans*-5,*cis*-9,*cis*-12-18:3) are C_{18} NMIFAs that were found in *Pinus koraiensis* and *Aquilegia hybrida*, respectively (8, 9). They are isomers of γ-linolenic acid and show various effects, such as the reduction of platelet aggregation by prostacyclin production, attenuation of the elevation of blood pressure, LDL-lowering and essential fatty acid activity (9, 11, 12). Podocarpic acid (*cis*-5,*cis*-11,*cis*-14-20:3) is a C_{20} NMIFA that was found in *Platycladus orientalis* oil (10). It has been reported to show a reduction in the AA concentration in the phosphatidylinositol fraction of rat liver (13), which functions in signal transduction, such as in the phospholipase C-signaling pathway (13, 29). Considering that PUFAs often show an isomer-specific function, novel NMIFAs are expected to show novel interesting physiological effects. Interestingly, several natural plant oils have a high content of PUFAs with a double bond at the ω7 position (30), and the biohydrogenation of PUFAs often produce PUFAs with a double bond at the ω7 position, such as VA (6, 7). These observations enable us to consider that a double bond at the ω7 position may become a key factor for a biological function. In this context, various C_{18} and C_{20} NMIFAs obtained in this study could be worthwhile. It is also noted that these NMIFAs were obtained in the high yield (approximately 60%). Therefore, this study could serve to open up the development of novel methods in the preparation of these rare possibly bioactive PUFAs.

Lipid metabolism by anaerobic bacteria is an attractive research area from the viewpoint of the role of the gut microbiota in relation to health of the host. Interestingly, obesity induced by a high-fat diet has been suggested as being associated with alterations of gut microbiota composition (31, 32). Dietary fats are metabolized by gut microbiota as well as by the host. It is noted that the biohydrogenation of fatty acids might function as a detoxification mechanism in bacteria, and PUFAs especially are more toxic than saturated fatty acids (18, 33). This suggests that the ability of the biohydrogenation of PUFAs might relate to the survival of gut bacteria when dietary intake of PUFAs is high. In addition, our recent research suggested the possibility that lipid metabolism by gut microbiota affects the health of the host by modifying fatty acid composition (23). Therefore, our evidence-based studies on lipid metabolism by gut bacteria, including *C. bifermentans* (in this study) and *Lactobacillus* (21-23, 34), should serve to maintain and improve the health of the host.

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Figure legends

Fig. 1. GC chromatograms of methyl esters of fatty acids produced by *C. bifermentans* JCM 1386 Cultivations were carried out in GAM broth with 0.03% (w/v) of arachidonic acid (AA) (A), eicosapentaenoic acid (EPA) (B), or linoleic acid (LA) (C) for 3 days. The lipid products were extracted from the supernatant and methylated as described in MATERIALS and METHODS. AA, EPA, and LA are converted to UK1, UK2, and vaccenic acid (VA), respectively.

Fig. 2. ^{1}H-NMR analysis of UK1 and structure of UK1 identified (A) Structure of methyl ester of UK1. (B) ^{1}H-^{1}H-chemical shift correlation spectroscopic (COSY) spectrum of the methyl ester of UK1. (C) ^{1}H clean-total correlation spectroscopic (TOCSY) spectrum of the methyl ester of UK1.

Fig. 3. Two-dimensional nuclear Overhauser effect spectroscopy (NOESY) spectra of the methyl esters of UK1 (A) and UK2 (B). The negative diagonal peaks are denoted in blue. The positive NOE crosspeaks are denoted in red.

Fig. 4. ^{1}H-NMR analysis of UK2 and structure of UK2 identified (A) Structure of methyl ester of UK2. (B) ^{1}H-^{1}H-chemical shift correlation spectroscopic (COSY) spectrum of the methyl ester of UK2. (C) ^{1}H clean-total correlation spectroscopic (TOCSY) spectrum of the methyl ester of UK2.

Fig. 5. Effects of fatty acid concentration for medium on fatty acids transformation by *C. bifermentans* JCM 1386 (A) **A**rachidonic acid (AA) and (B) eicosapentaenoic acid (EPA).

Cultivations were carried out with different concentrations of AA or EPA.

Fig. 6. Transformation of polyunsaturated fatty acids by *C. bifermentans* JCM 1386

Fig. 7. GC-MS spectra of pyrrolidide derivatives of the products from α-linolenic acid (A), γ-linolenic acid (B) and dihomo-γ-linolenic acid (C) The deduced structures are shown above the spectra.

Fig. 8. Pathway of polyunsaturated fatty acid transformation during cultivation of *C. bifermentans* JCM 1386 LA, linoleic acid. VA, vaccenic acid. AA, arachidonic acid. EPA, eicosapentaenoic acid.

Table 1: Screening results for the ability of transforming polyunsaturated fatty acids

Strain	No.	Produced fatty acid (mg/mL culture broth)		
		VA from LA	UK1 from AA	UK2 from EPA
Clostridium bifermentans	JCM 1386	0.06	0.12	0.11
Clostridium bifermentans	JCM 7832	0.05	0.13	0.10
Clostridium sporogenes	JCM 7849	0.11	-	-
Clostridium sporogenes	JCM 7850	0.16	-	-
Propionibacterium acnes	JCM 6473	0.12	-	-

Cultivations were carried out in GAM broth with 0.03% (w/v) of linoleic acid (LA), arachidonic acid (AA) or 0.02% (w/v) of eicosapentaenoic acid (EPA) for 3 days as described in MATERIALS AND METHODS. -, not detected. VA, vaccenic acid.

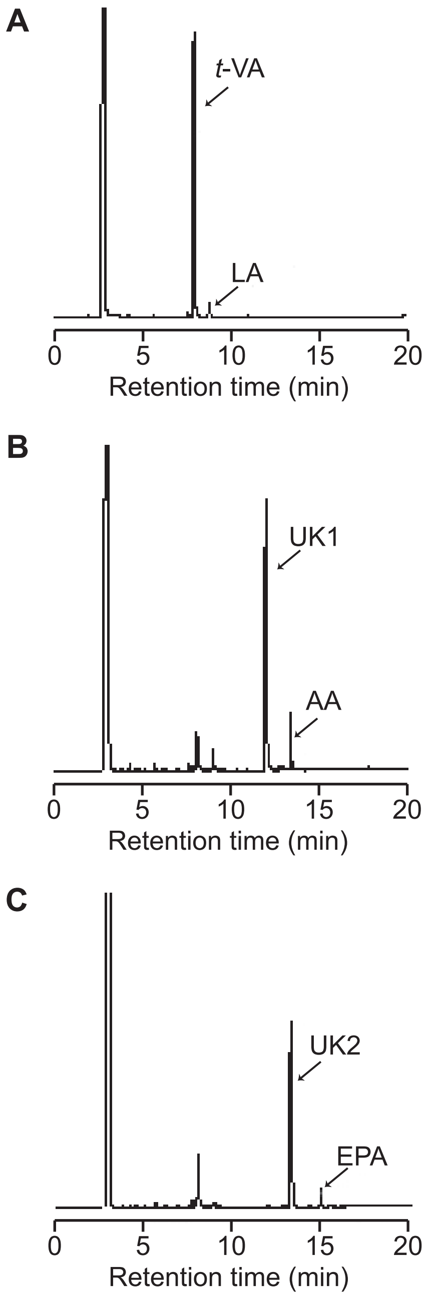


Fig. 1 Sakurama et al.

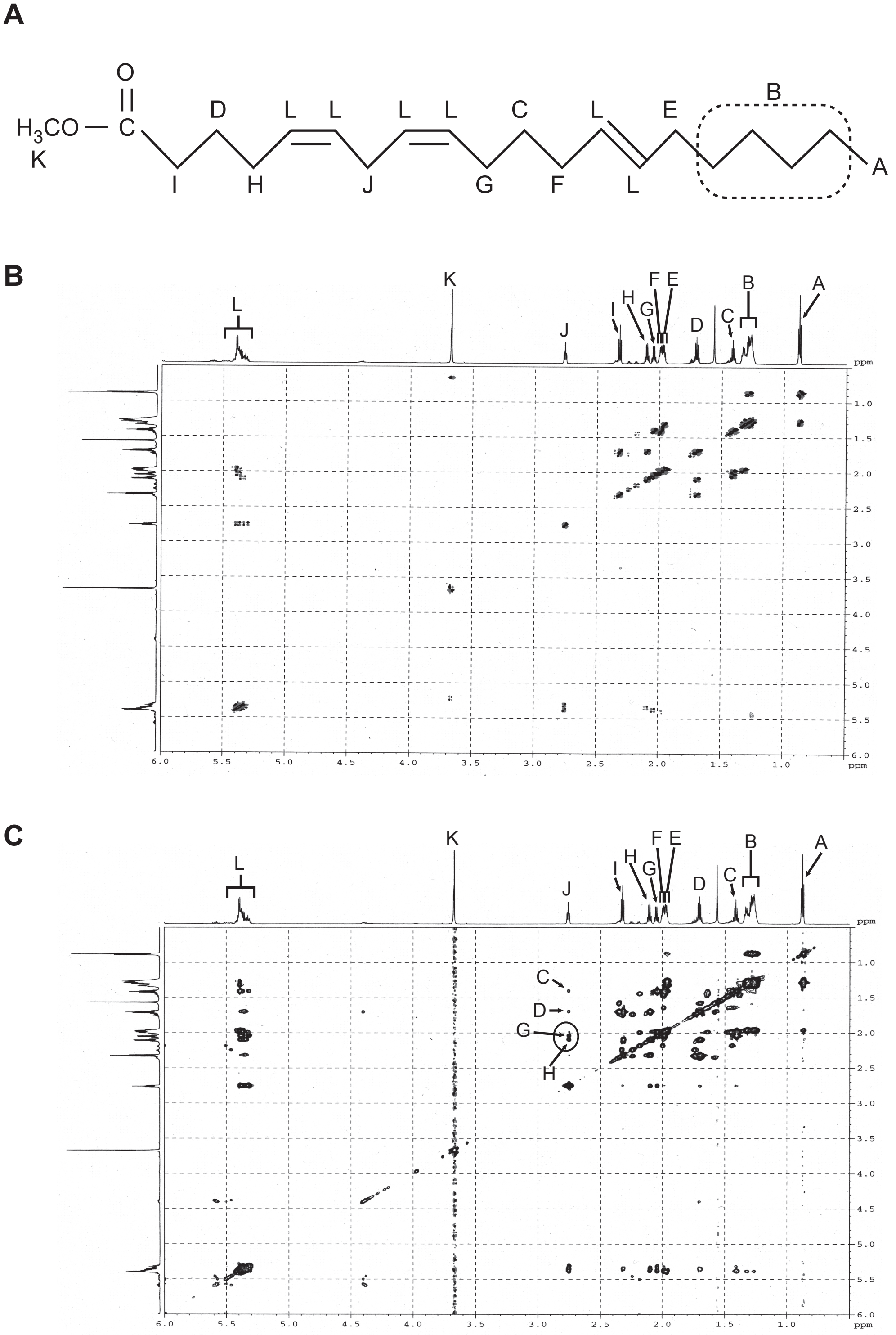


Fig. 2 Sakurama et al.

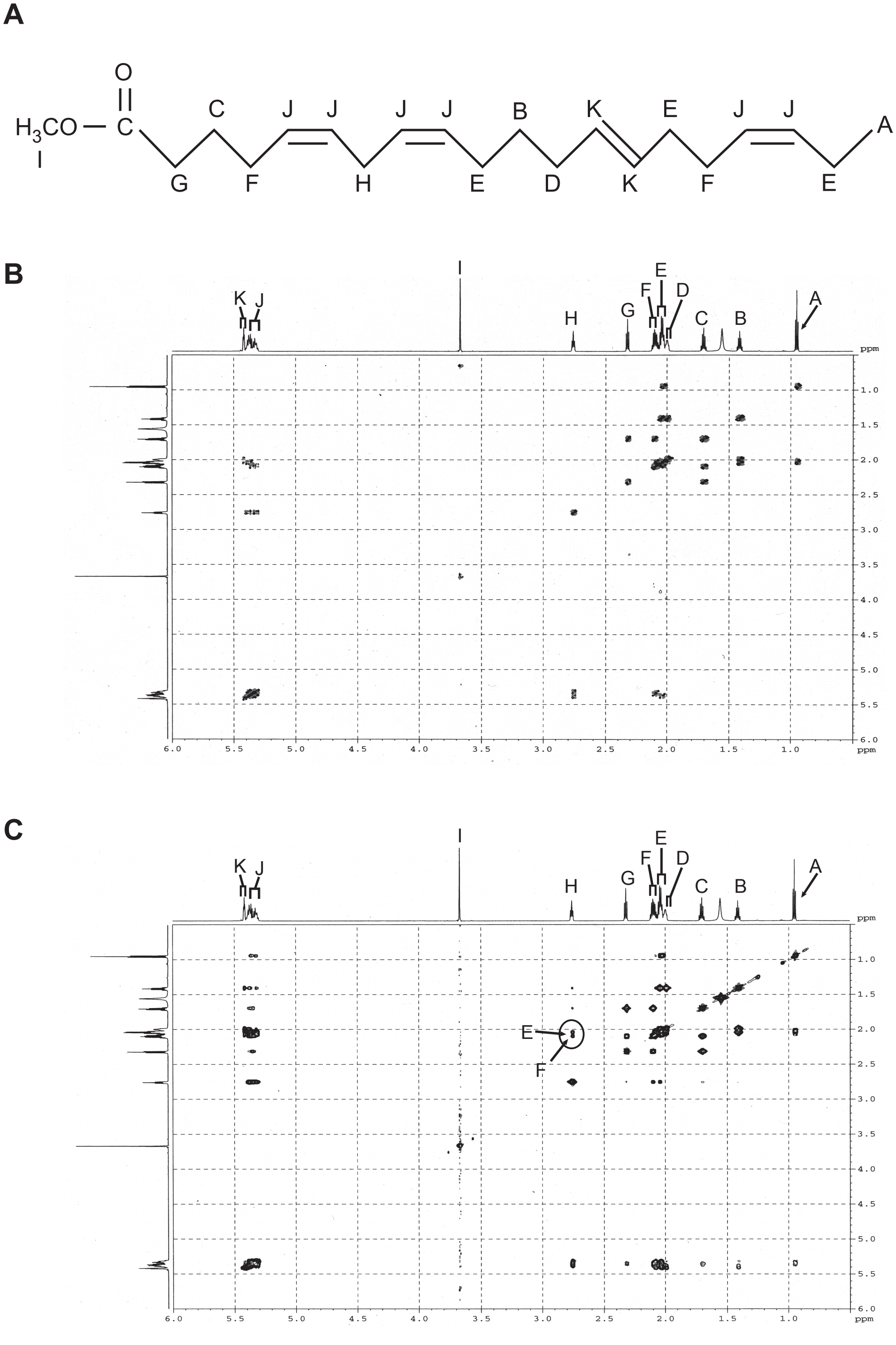


Fig. 3 Sakurama et al.

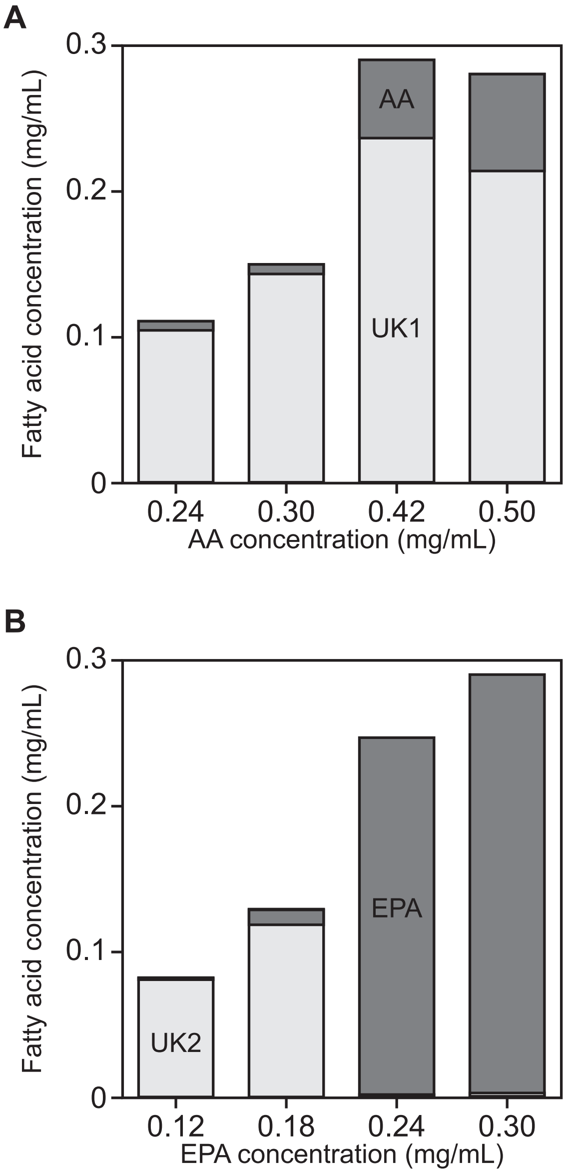


Fig. 4 Sakurama et al.

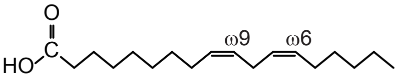
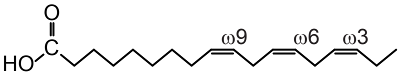
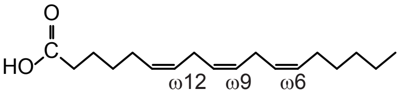
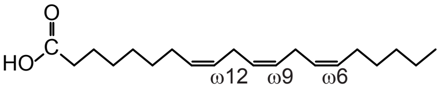
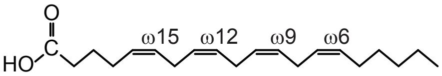
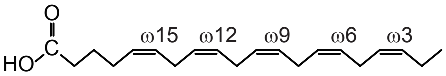
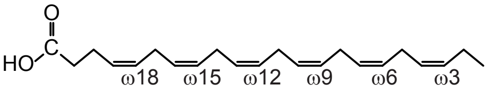
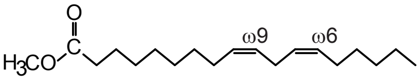
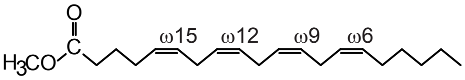
Form	Substrate	Structure	Transformation
Free	Linoleic acid (LA; 18:2 ω6)		+
	α-Linolenic acid (18:3 ω3)		+
	γ-Linolenic acid (18:3 ω6)		+
	Dihomo-γ-linolenic acid (20:3 ω6)		+
	Arachidonic acid (AA; 20:4 ω6)		+
	Eicosapentaenoic acid (EPA; 20:5 ω3)		+
	Docosahexaenoic acid (DHA; 22:6 ω3)		−
Methyl ester	Linoleic acid methyl ester		+
	Arachidonic acid methyl ester		+

Fig. 5 Sakurama et al.

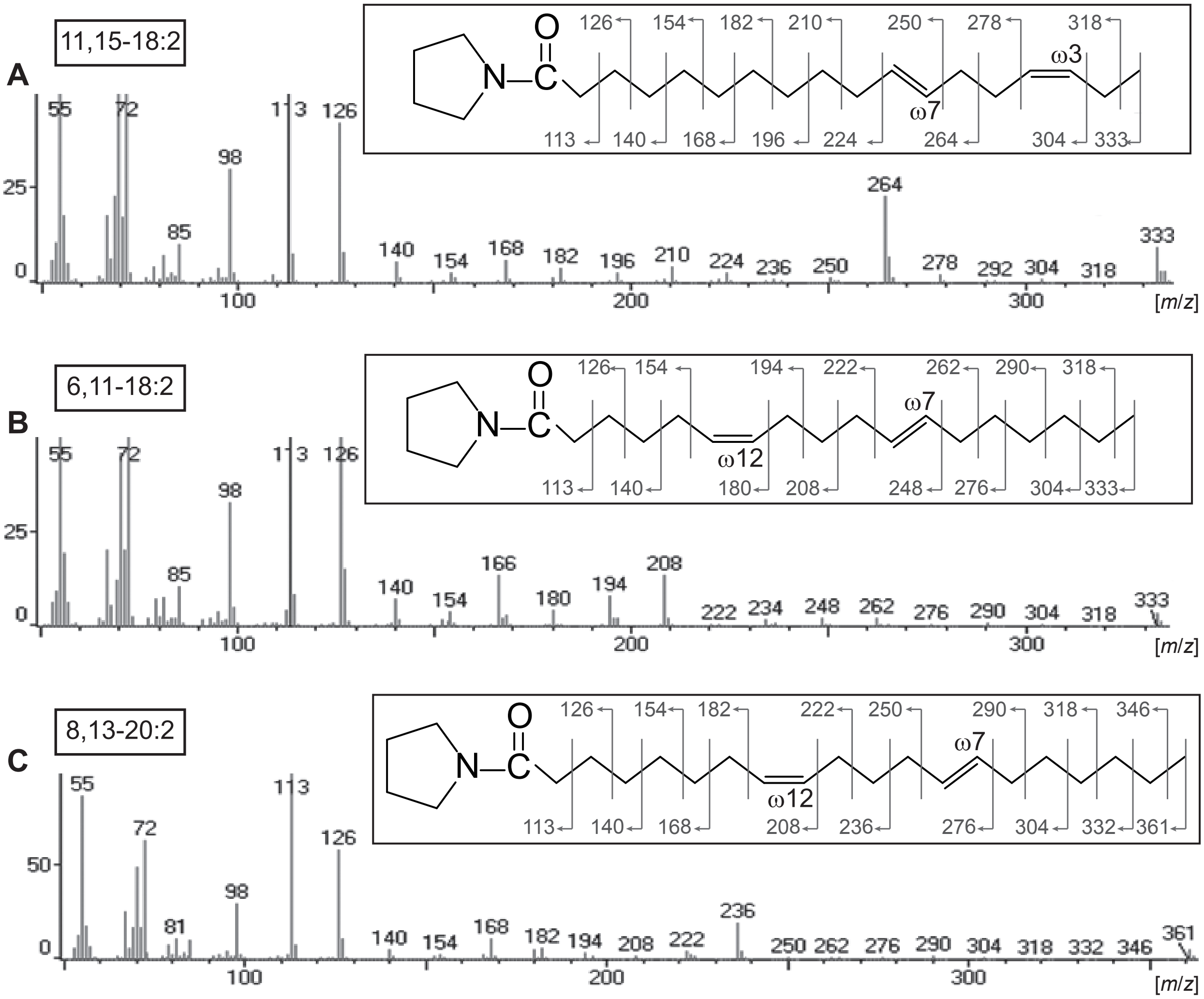


Fig. 6 Sakurama et al.

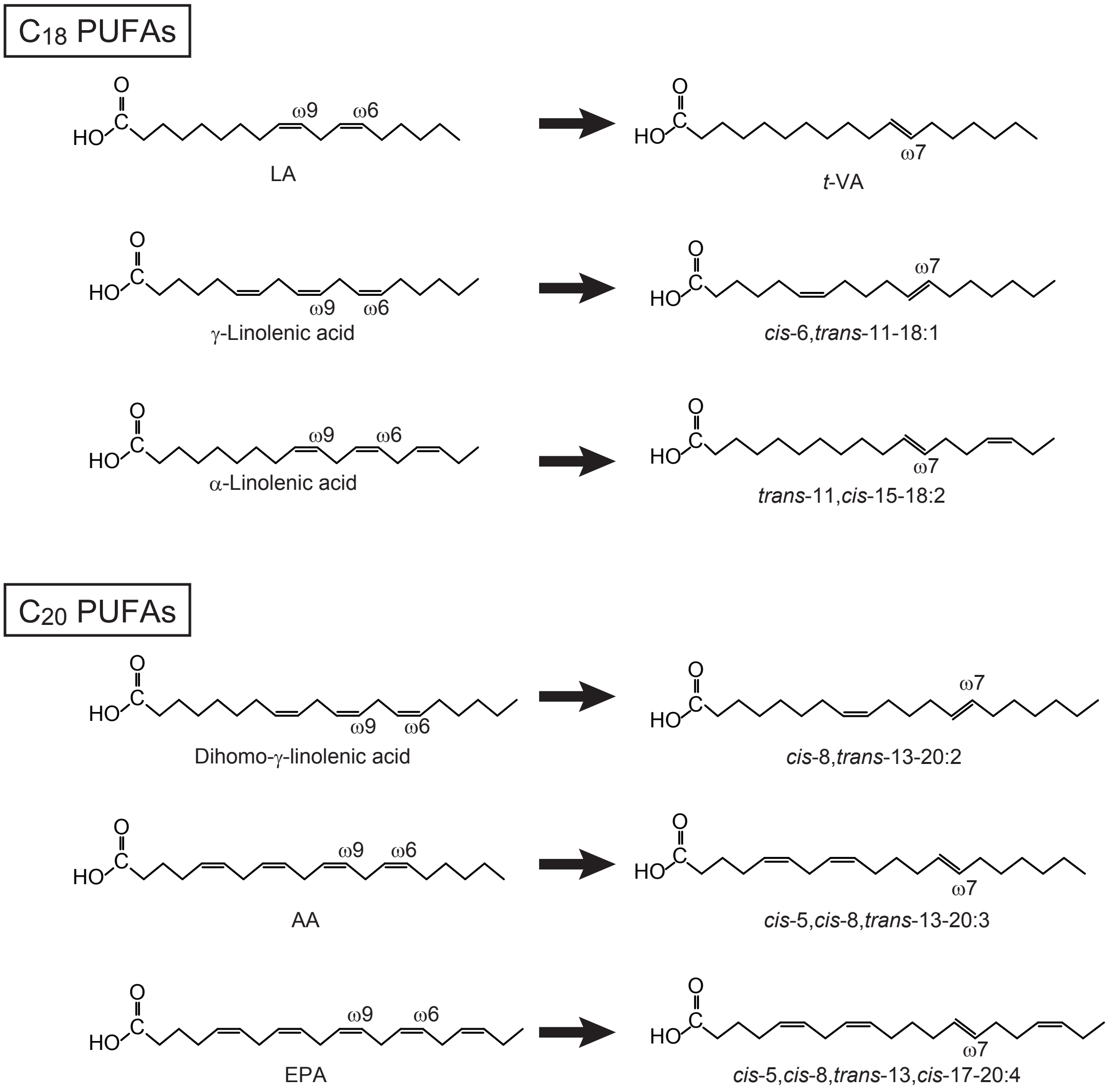


Fig. 7 Sakurama et al.

Supplementary Table S1: List of identified bacterial species used for screening

Acetobacterium	
Acetobacterium wieringae	
Anaerococcus	
Anaerococcus hydrogenalis	*Anaerococcus lactolyticus*
Anaerococcus prevotii	*Anaerococcus tetradius*
Anaerococcus vaginalis	
Atopobium	
Atopobium fossor	*Atopobium parvulum*
Bacteroides	
Bacteroides acidifaciens	*Bacteroides caccae*
Bacteroides distasonis	*Bacteroides fragilis*
Bacteroides merdae	*Bacteroides ovatus*
Bacteroides suis	*Bacteroides thetaiotaomicron*
Bifidobacterium	
Bifidobacterium adolescentis	*Bifidobacterium angulatum*
Bifidobacterium animalis	*Bifidobacterium asteroides*
Bifidobacterium bifidum	*Bifidobacterium boum*
Bifidobacterium breve	*Bifidobacterium catenulatum*
Bifidobacterium choerinum	*Bifidobacterium coryneforme*
Bifidobacterium cuniculi	*Bifidobacterium dentium*
Bifidobacterium longum	*Bifidobacterium merycicum*
Bifidobacterium minimum	*Bifidobacterium pseudocatenulatum*
Bifidobacterium pseudolongum	*Bifidobacterium pseudolongum* subsp. *globosum*
Bifidobacterium pseudolongum subsp. *pseudolongum*	*Bifidobacterium pullorum*
Bifidobacterium ruminantium	*Bifidobacterium subtile*
Bifidobacterium thermacidophilum	
Campylobacter	
Campylobacter rectus	
Clostridium	
Clostridium acetobutylicum	*Clostridium aminovalericum*
Clostridium baratii	*Clostridium beijerinckii*
Clostridium bifermentans	*Clostridium butyricum*
Clostridium cadaveris	*Clostridium clostridiiforme*
Clostridium cochlearium	*Clostridium cocleatum*
Clostridium difficile	*Clostridium ghonii*
Clostridium irregularis	*Clostridium oceanicum*
Clostridium perfringens	*Clostridium propionicum*
Clostridium ramosum	*Clostridium scindens*
Clostridium septicum	*Clostridium sporogenes*
Clostridium symbiosum	*Clostridium tyrobutyricum*
Collinsella	
Collinsella aerofaciens	*Collinsella intestinalis*
Collinsella stercoris	
Coprobacillus	
Coprobacillus cateniformis	
Eggerthella	
Eggerthella lenta	

Eubacterium	
Eubacterium barkeri	*Eubacterium budayi*
Eubacterium callanderi	*Eubacterium combesii*
Eubacterium cylindroides	*Eubacterium fissicatena*
Eubacterium hadrum	*Eubacterium limosum*
Eubacterium moniliforme	*Eubacterium multiforme*
Eubacterium nitritogenes	*Eubacterium nodatum*
Eubacterium saburreum	*Eubacterium tenue*
Fusobacterium	
Fusobacterium necrophorum subsp. *funduliforme*	*Fusobacterium varium*
Lactobacillus	
Lactobacillus catenaformins	*Lactobacillus crispatus*
Lactobacillus hamsteri	*Lactobacillus johnsonii*
Lactobacillus reuteri	*Lactobacillus ruminis*
Lactobacillus vitulinus	
Leuconostoc	
Leuconostoc mesenteroides subsp. *mesenteroides*	
Megasphaera	
Megasphaera cerevisiae	*Megasphaera elsdenii*
Mitsuokella	
Mitsuokella jalaludinii	*Mitsuokella multacida*
Peptoniphilus	
Peptoniphilus asaccharolyticus	*Peptoniphilus lacrimalis*
Propionibacterium	
Propionibacterium acidipropionici	*Propionibacterium acnes*
Propionibacterium arabinosum	*Propionibacterium intermedium*
Propionibacterium jensenii	*Propionibacterium pentosaceum*
Propionibacterium peterssonii	*Propionibacterium propionicum*
Propionibacterium thoenii	*Propionimicrobium lymphophilum*
Pseudoramibacter	
Pseudoramibacter alactolyticus	
Rarobacter	
Rarobacter faecitabidus	*Rarobacter incanus*
Rhodosporidium	
Rhodosporidium sphaerocarpum	
Rikenella	
Rikenella microfusus	
Ruminococcus	
Ruminococcus productus	
Selenomonas	
Selenomonas artemidis	*Selenomonas diae*
Selenomonas dianae	*Selenomonas flueggei*
Selenomonas infelix	*Selenomonas noxia*
Selenomonas ruminantium	*Selenomonas sputigena*