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Novel C₂₀ Conjugated Fatty Acids as Biohydrogenation Intermediates in *Paraclostridium bifermentans*

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ABSTRACT

In this paper, we report a detailed characterization of the eicosapentaenoic acid (EPA) hydrogenation pathway in *Paraclostridium bifermentans* JCM 1386^T, including the identification of novel intermediates of conjugated EPAs, *cis*-5,*cis*-8,*cis*-11,*trans*-13,*cis*-17-20:5 (CEPA1) and *cis*-5,*cis*-8,*trans*-11,*trans*-13,*cis*-17-20:5 (CEPA2) by nuclear magnetic resonance spectroscopy (NMR). EPA was converted to CEPA1 then subsequently to CEPA2. Moreover, the conversion from CEPA1 to CEPA2 was observed as an irreversible reaction. The saturation of CEPA1 and CEPA2 to *cis*-5,*cis*-8,*trans*-13,*cis*-17-20:4 appeared to occur sequentially, from CEPA1 to CEPA2 and then to *cis*-5,*cis*-8,*trans*-13,*cis*-17-20:4. The other C₂₀ fatty acids, arachidonic acid (AA), was hydrogenated in the same way by *P. bifermentans* JCM 1386^T, producing novel conjugated AAs of *cis*-5,*cis*-8,*cis*-11,*trans*-13-20:4 (CAA1) and *cis*-5,*cis*-8,*trans*-11,*trans*-13-20:4 (CAA2). C₁₈ and C₂₀ PUFAs with *cis*-ω6 and *cis*-ω9 double bonds were also converted to two corresponding conjugated fatty acid with *trans*-ω7 and *cis*-ω9 as well as *trans*-ω7 and *trans*-ω9 structures, which were further saturated (ω9 saturation) to *trans*-ω7 structures.

1 | Introduction

Conjugated fatty acids (CFAs) are positional and geometrical isomers of polyunsaturated fatty acids (PUFAs) that contain several conjugated double bonds. For the CFAs of linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid (18:2), LA), a conjugated linoleic acid (CLA) isomer, *cis*-9,*trans*-11-18:2 (CLA1), has been reported to exhibit numerous health benefits, including anti-cancer, anti-atherogenic, anti-obesity, and immune-modulating effects in animal models (Chen et al. 2025).

The bioconversion of LA to CLAs occurs naturally in the rumen during biohydrogenation. For example, the rumen anaerobic bacterium *Butyrivibrio fibrisolvens* is known for producing CLA1 as an intermediate in biohydrogenation (Kepler

et al. 1966). During biohydrogenation, LA is first converted to CLA1, followed by subsequent conversion to *trans*-vaccenic acid (*trans*-11-octadecenoic acid (18:1), *trans*-VA); this process yields stearic acid (octadecanoic acid) as an end product (Harfoot and Hazlewood 1997). Further studies involving pure cultures and defined substrates have found that some bacteria hydrogenate other C₁₈ PUFAs, including α-linolenic acid (*cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid (18:3), ALA) and γ-linolenic acid (*cis*-6,*cis*-9,*cis*-12-18:3, GLA) (Kishino et al. 2003, 2009, 2010).

Previous research from our lab has shown that *Lactobacillus plantarum* produces CLA1 and other isomers known as *trans*-9,*trans*-11-18:2 (CLA2) and *trans*-10,*cis*-12-18:2 (CLA3) from LA via a multicomponent enzyme system (Kishino et al. 2003, 2009, 2010, 2013; Kishino, Ogawa, et al. 2011;

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Kishino, Park, et al. 2011). However, despite this important characterization of the biohydrogenation pathways of C₁₈ PUFAs by bacteria, of the identity of bacteria that produce CFA from C₂₀ PUFAs such as conjugated eicosapentaenoic acids (CEPAs) remains incompletely elucidated.

In a previous study, we reported that an anaerobic bacteria, *Paraclostridium bifermentans* JCM 1386^T, hydrogenated C₂₀ PUFAs such as eicosapentaenoic acid (*cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17-eicosapentaenoic acid (20:5), EPA) (Sakurama et al. 2014). The strain can recognize *cis* double bonds at the ω6 and ω9 position of C₂₀ PUFAs and converted these double bonds to *trans* double bonds at the ω7 position. The aim of this study is to analyze the biohydrogenation pathway of this strain and to reveal the involvement of novel intermediates such as CEPAs. In doing so, we aim to propose new methods of producing CEPAs.

2 | Materials and Methods

2.1 | Chemicals

We purchased LA, ALA, GLA, stearidonic acid (*cis*-6,*cis*-9,*cis*-12,*cis*-15-18:4), EPA, arachidonic acid (*cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid (20:4), AA), ω3 docosapentaenoic acid (*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19-docosapentaenoic acid (22:5)), and docosahexaenoic acid (*cis*-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19-docosahexaenoic acid (22:6), DHA) from NU-CHEK-PREP Inc. (Elysian, USA). Dihomo-γ-linolenic acid (*cis*-8,*cis*-11,*cis*-14-eicosatrienoic acid (20:3)), CLA1, and CLA2 were purchased from Cayman Chemical Company (Michigan, USA). *cis*-11,*cis*-14,*cis*-17-20:3 was purchased from Biomol International, LP (Pennsylvania, USA). ω3 AA (*cis*-8,*cis*-11,*cis*-14,*cis*-17-20:4) was purchased from Larodan (Solna, Sweden). Columbinic acid (*trans*-5,*cis*-9,*cis*-12-18:3), and bovine serum albumin (BSA) were purchased from Larodan Fine Chemicals AB (Malmö, Sweden), Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), and Sigma-Aldrich (St. Louis, USA), respectively. All other chemicals used were of analytical grade and were commercially available.

2.2 | Preparation of Cell-Free Extracts of *P. bifermentans* JCM 1386^T

P. bifermentans JCM 1386^T was used for all cultivation experiments reported in this study. First, cultivation was carried out in anaerobic conditions at 37°C. To generate seed cultures or resting cell preparations, the focal strain was first inoculated into 15.0 or 30.0 mL samples of Gifu Anaerobic Medium (GAM) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). Here, 15.0 mL was used for seed cultures for time-course experiments and 30.0 mL for substrate specificity analyses. After inoculation, cultures were cultivated for 8 h (seed culture for substrate specificity analysis), overnight (seed culture for time-course experiment), or 24 h (for direct use as resting cell) as per experimental protocol. All cultures were performed under shaking at 120 strokes/min. Next, seed cultures were transferred into 1.6 L of GAM and cultured for 16 h (for substrate specificity analysis) with mixing by stir bar or 3 days without shaking (for time-course experiments). Cells were then

harvested by centrifugation (12,000g, 10 min), washed twice with physiological salt solution, and stored at −20°C until further use.

Cell-free extracts for substrate specificity analysis were prepared by sonicating cells resuspended in 20 mM potassium phosphate buffer (KPB, pH 6.5, two times the volume of the wet cell mass) for four cycles at 4°C for 5 min.

2.3 | Reaction Conditions

For resting cell experiments, a 1.0 mL reaction mixture was added to 15.0 mL test tubes. This mixture comprised 1.0 mg EPA (substrate) emulsified with 0.2 mg BSA in 0.1 M Tris-HCl (pH 8.0). Cells collected from 15.0 mL cultured medium were incubated for 24 h at 37°C with shaking (120 strokes/min) under anaerobic conditions controlled by O₂-absorbent AnaeroPacks (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan).

Substrate specificity experiments were carried out under the following conditions: 1.0 mL reaction mixture—comprising 1.0 mg substrate emulsified in 0.2 mg BSA in 20 mM KPB (pH 6.5) and 0.18 mL cell-free extract—was added to 15.0 mL test tubes and incubated at 37°C under shaking (120 strokes/min) in anaerobic conditions (AnaeroPacks) for 24 h.

Time-course experiments using resting cells were carried out as follows: 1.0 mL reaction mixtures were prepared containing:

- 0.27 mg EPA emulsified in 0.054 mg BSA,
- 0.078 mg purified CEPA1 (including 0.0055 mg CEPA2) emulsified with 0.017 mg BSA, or
- 0.002 mg purified CEPA2 emulsified with 0.4 μg BSA in 0.1 M KPB (pH 6.5).

Each mixture was incubated with 150 mg cell samples at 37°C without shaking under anaerobic conditions in an anaerobic chamber (atmosphere of 95% nitrogen and 5% hydrogen).

2.4 | Lipid Analyses

Lipids were extracted using chloroform-methanol following the Bligh and Dyer (1959) method. Extracted samples were then methylated with 10% methanolic-HCl at 50°C for 20 min. The resultant fatty acid methyl esters were first extracted with *n*-hexane, then analyzed by gas chromatography using a Shimadzu (Kyoto, Japan) GC-2025 gas chromatograph equipped with a flame ionization detector and split injection system and fitted with a capillary column (TC-70, 60 m × 0.25 mm I.D., GL Sciences, Kyoto, Japan). During analysis, the column temperature was initially held at 180°C for 10 min, then heated to 260°C at a rate of 5°C/min, after which it was maintained at 260°C for 4 min. The injector and detector were operated at 260°C. Helium was used as a carrier gas.

2.5 | Purification of Reaction Products

Fatty acids extracted from the reaction mixture were purified using high-performance liquid chromatography (HPLC, LC-VP

System, Shimadzu Co., Kyoto, Japan). Fatty acids were first fractionated on a Develosil C30-UG column (20.0×250 mm; C30-UG-5, Nomura Chemical, Aichi, Japan). Acetonitrile/water (8/2 by vol.) with 0.2% acetic acid was used as the mobile phase and was added at a flow rate of 15 mL/min. The effluent was monitored by ultraviolet detection (at 210 and 233 nm) using a photodiode array spectrophotometric detector (Shimadzu, M10A) equipped with a photodiode array.

After detection, the chemical structures of purified fatty acids were determined by mass spectroscopy (MS), proton nuclear magnetic resonance (^1H -NMR), ^1H - ^1H chemical shift correlation spectroscopy (^1H - ^1H COSY), carbon nuclear magnetic resonance (^{13}C -NMR), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC). In addition, fatty acids provided as substrates of the time-course experiment were also purified using HPLC under the same conditions.

2.6 | Pyrrolidine Derivatization

Methyl ester fatty acids were used for pyrrolidine derivatization. With methyl ester fatty acids, we added 200 μL of pyrrolidine and 50 μL of acetic acid to screw-capped test tubes (15.0 mL). The resulting mixture was incubated at 100°C for 1 h. After incubation, 2 mL methanol, 1 mL dichloromethane, and 1 mL H_2O were added, and the mixture was thoroughly vortexed. Next, fatty acids were extracted with *n*-hexane and washed with 1 mL H_2O . Finally, washed samples were evaporated and analyzed by GC-MS (see below).

2.7 | GC-MS Analysis

GCMS-QP2010 (Shimadzu, Kyoto, Japan) was used for mass spectral analysis. We performed GC separation of fatty acid methyl esters on a TC-70 column (for methyl ester derivatization) and a SPB-1 column (for pyrrolidine derivatization). The column temperature of the TC-70 column was initially held at 180°C for 10 min, then heated to 260°C at a rate of 5°C/min. It was maintained at 260°C for 4 min, during which the injector and detector were operated at 260°C. The column temperature of the SPB-1 column was held at 220°C and maintained there for 90 min. MS was conducted in electron impact mode with a source temperature of 200°C. Split injection was employed with the injector ports of the TC-70 and SPB-1 columns at 260°C and 300°C, respectively.

2.8 | Nuclear Magnetic Resonance Spectroscopy

We recorded ^1H and ^{13}C NMR spectra on a Bruker AVANCE III 500 spectrometer (500 MHz corresponds to ^1H , while 125 MHz corresponds to ^{13}C). Chemical shifts are reported in δ (ppm) with reference to residual solvent signals [^1H NMR: CDCl_3 (7.26); ^{13}C NMR: CDCl_3 (77.0)]. Signal patterns were indicated as *s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *quint*, quintet; *m*, multiplet; and *br*, broad peak.

3 | Results

3.1 | Transformation of EPA by *P. bifementans* JCM 1386^T

First, we conducted a reaction with EPA as the substrate using resting cells of *P. bifementans* JCM 1386^T. After a 24 h reaction period, two unknown fatty acids (named UK1 and UK2) were generated; GC analysis identified their respective peaks, with no others present (Figure 1). Next, UK1 and UK2 were purified by HPLC from the reaction mixture and subjected to structural analysis.

3.2 | Structural Analysis of UK1

^1H -NMR, H-H COSY, ^{13}C -NMR, HSQC, and HMBC spectra suggested that UK1 might be *cis*-5,*cis*-8,*cis*-11,*trans*-13,*cis*-17-20:5 (CEPA1) (Figure 2A). The geometrical configurations were determined as follows. The mass spectrum of UK1 methyl ester showed a molecular weight of m/z 316 and indicated that UK1 was a structural isomer of EPA that contained five double bonds (Figure 2B). ^1H -NMR analysis also suggested that UK1 is an isomer of 20:5 (Figure S1A). Signals H-5, H-6, H-8, H-9, H-11, H-12, H-13, H-14, H-17, and H-18 verified the existence of five double bonds in UK1. The sequence of the protons of the molecule was deduced based on the signal patterns of interaction between adjacent protons as observed by ^1H - ^1H COSY analysis (Figure 2C). Finally, ^{13}C -NMR analysis was carried out to identify the geometric configurations of double bonds (Figure S1B-D).

Except for the CO_2H and/or CH_3 end groups, the chemical shift did not appear to influence the allylic carbon atom means of 27.3 ppm for *cis* double bonds and 32.67 ppm for *trans*

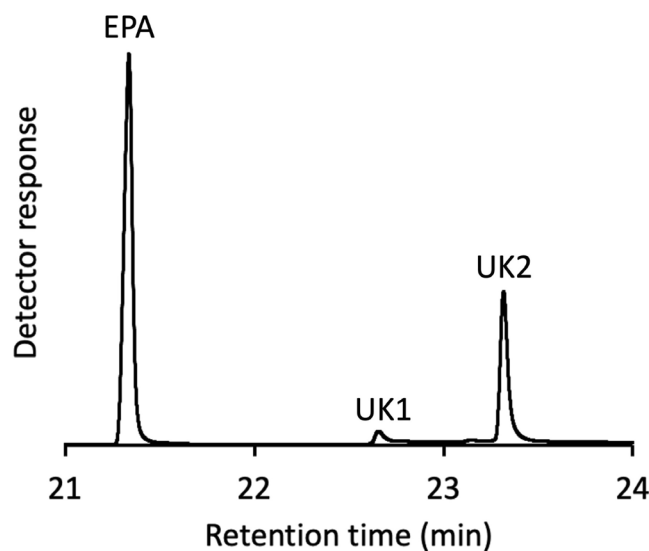


FIGURE 1 | GC chromatograms of methyl esters of fatty acids produced by *Paraclostridium bifementans* JCM 1386^T. Eicosapentaenoic acid (EPA) was used as the substrate, and UK1 and UK2 were the only products generated.

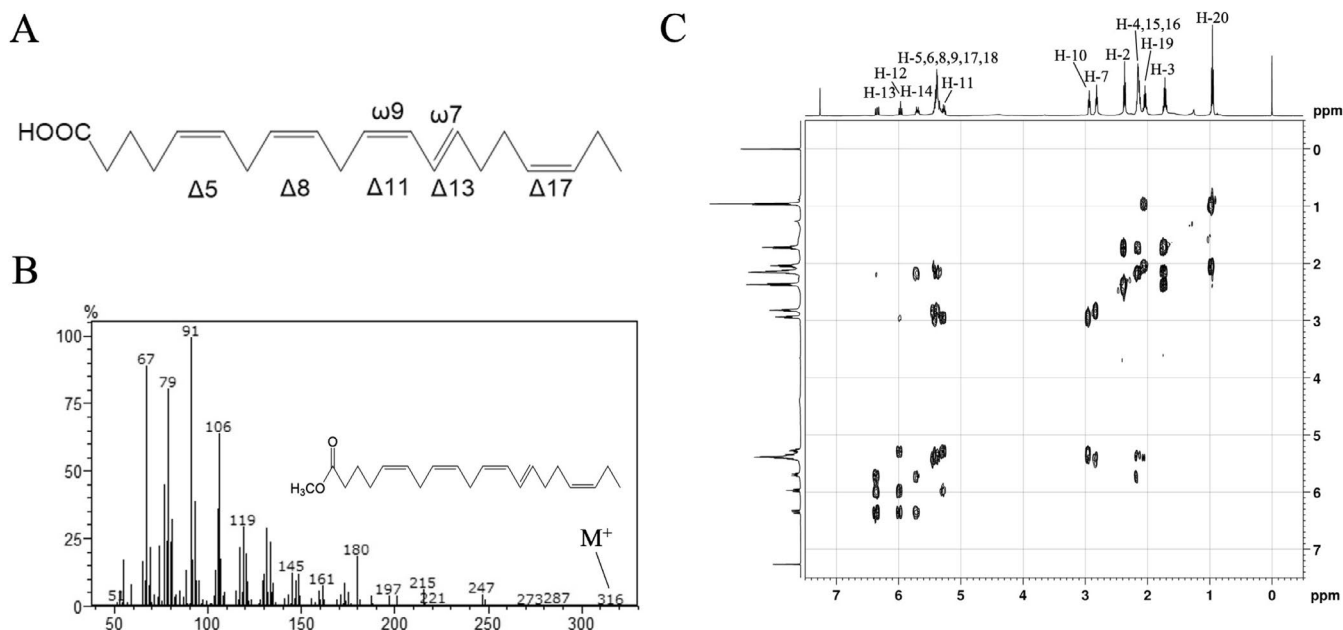


FIGURE 2 | Structural identification of UK1. (A) Structure of UK1. (B) Mass spectrum of UK1 methyl ester. (C) ^1H - ^1H COSY spectrum of UK1.

double bonds in ^{13}C -NMR analysis (Gunstone et al. 1977). According to our ^{13}C -NMR analysis, we observed a chemical shift of C-4 (26.5 ppm), C-7 (25.6 ppm), C-10 (26.0 ppm), and C-16 (27.0 ppm); moreover, $\Delta 5$, $\Delta 8$, $\Delta 11$, and $\Delta 17$ double bonds were all determined to be *cis* configurations. In contrast, the chemical shift of C-15 (33.0 ppm) showed that $\Delta 13$ double bonds were found in *trans* configurations; this may be due to the substituent effect on chemical shifts of allylic carbon (Figure S1B,C,G). Moreover, the mechanism responsible for steric compression—that is, chemical shifts between C-5 and C-6, C-8 and C-9, C-11 and C-12, C-17 and C-18—was all closely spaced, suggesting that the double bonds at the $\Delta 5$, $\Delta 8$, $\Delta 11$, and $\Delta 17$ positions were in the *cis* configuration. However, the chemical shifts between C-13 and C-14 (9.2 ppm) were widely separated, suggesting that the double bond of the $\Delta 13$ position was in the *trans* configuration (Figure S1D,G). Moreover, ^1H -NMR analysis suggested that the coupling constant between H-13 and H-14 was 14.8 Hz; this also indicated that $\Delta 13$ was in the *trans* configuration (Figure S1A,G). The carbon sequence of this molecule was deduced based on signal patterns observed during HSQC and HMBC analysis (Figure S1E,F). Moreover, pyrrolidine derivatization was also provided as the identification of the positions of C-C double bonds (Figure S2) (Andersson 1978).

3.3 | Structural Analysis of UK2

^1H -NMR, H-H COSY, ^{13}C -NMR, HSQC, and HMBC spectra suggested that UK2 was *cis*-5,*cis*-8,*trans*-11,*trans*-13,*cis*-17-20:5 (CEPA2) (Figure 3A). The geometrical configurations were determined as follows. The mass spectrum of UK2 methyl ester showed a molecular weight of m/z 316 and indicated that UK2 is a structural isomer of EPA that contains five double bonds (Figure 3B). ^1H -NMR analysis also suggested that UK2 is an isomer of 20:5 (Figure S3A). In addition, the H-5, H-6, H-8, H-9,

H-11, H-12, H-13, H-14, H-17, and H-18 signals confirmed the existence of five double bonds in UK2. The sequence of the protons in this molecule was deduced based on patterns of signal interaction between adjacent protons observed during ^1H - ^1H COSY analysis (Figure 3C). The *cis* and *trans* configurations were determined based on substituent effects in chemical shifts of allylic carbon relative to CEPA1 (Figure S3B-D). ^{13}C -NMR analysis indicated that the chemical shift of C-4 (26.4 ppm), C-7 (25.6 ppm), and C-16 (27.0 ppm), $\Delta 5$, $\Delta 8$, and $\Delta 17$ double bonds were all in the *cis* configuration. In contrast, the chemical shift of C-10 (30.3 ppm) and C-15 (32.7 ppm) indicated that the $\Delta 11$ and $\Delta 13$ double bonds were in the *trans* configuration (Figure S3B,C,G). The carbon sequence of this molecule was deduced based on signal patterns observed during HSQC and HMBC analysis (Figure S3E,F). Moreover, pyrrolidine derivatization was also provided as the identification of the positions of C-C double bonds (Figure S4).

3.4 | Time Course of EPA and CEPAs Transformation by Resting Cells of *P. bifermentans* JCM 1386^T

We carried out a time-course experiment to determine whether CEPA1 and CEPA2 are intermediates in the biohydrogenation of EPA (Figures 4 and 5). We first investigated a reaction profile using 0.27 mg/mL EPA as the substrate with the resting cells of *P. bifermentans* JCM 1386^T prepared under optimized culture conditions generating significant saturation activities (Figure 4). Over an initial 1 h reaction, we observed rapid generation of CEPA1, followed by relatively slower generation of CEPA2 as EPA decreased. After 14 h, we observed that both CEPA1 and CEPA2 started to decrease and *cis*-5,*cis*-8,*trans*-13,*cis*-17-20:4 began to increase (Figure 4). The *cis*-5,*cis*-8,*trans*-13,*cis*-17-20:4 had been identified in our previous research (Sakurama et al. 2014).

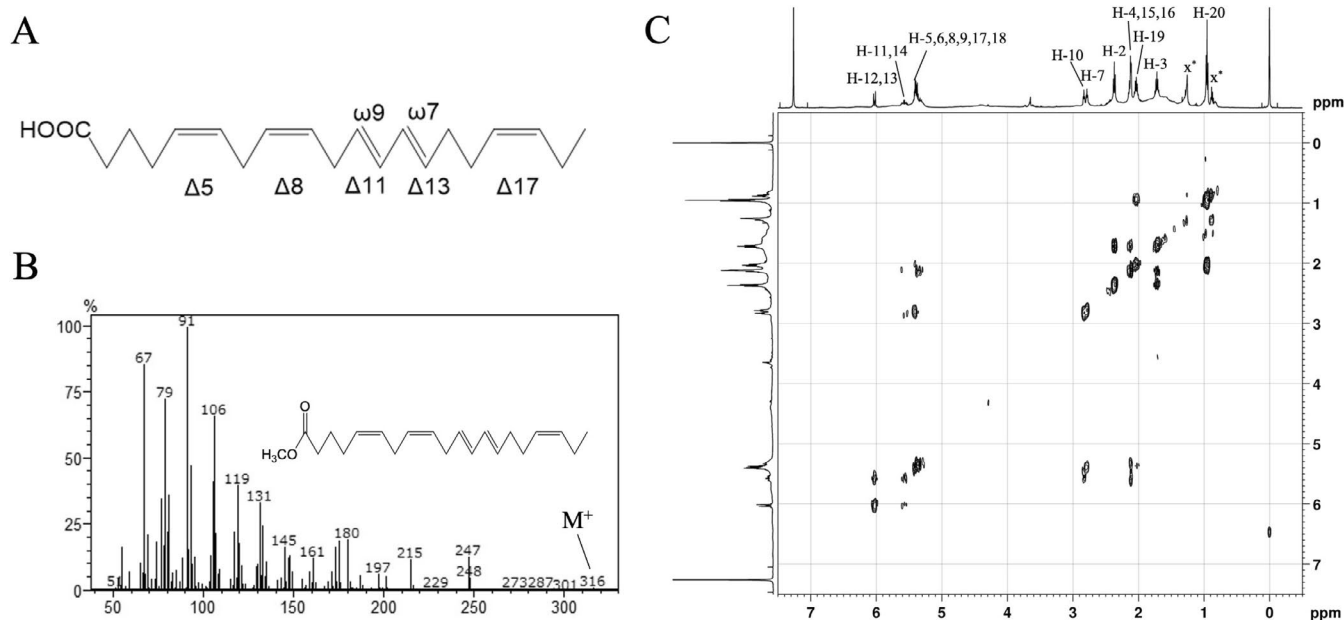


FIGURE 3 | Structural identification of UK2. (A) Structure of UK2. (B) Mass spectrum of UK2 methyl ester. (C) ^1H - ^1H COSY spectrum of UK2. *The noise originates from impurities.

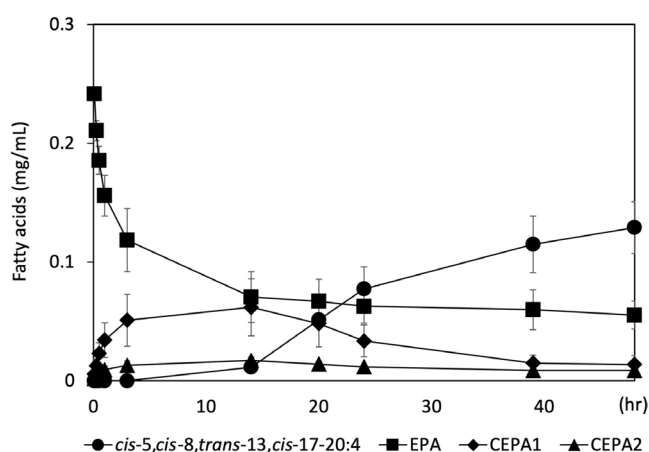


FIGURE 4 | Time course of EPA transformation by resting cells of *Paraclostridium bifermentans* JCM 1386^T over 48 h. CEPA1, *cis*-5,*cis*-8,*cis*-11,*trans*-13,*cis*-17-20:5; CEPA2, *cis*-5,*cis*-8,*trans*-11,*trans*-13,*cis*-17-20:5. Data represent three independent experiments ($n = 3$).

We then investigated the reaction profile using 0.078 mg/mL purified CEPA1, including 0.006 mg/mL CEPA2 (Figure 5A). After a 3 h reaction, CEPA1 decreased and CEPA2 generation was observed. After incubation for periods longer than 3 h, *cis*-5,*cis*-8,*trans*-13,*cis*-17-20:4 generation was observed as both CEPA1 and CEPA2 decreased (Figure 5A).

Next, we investigated the reaction profile using 0.002 mg/mL purified CEPA2 as a substrate (Figure 5B). After a 3 h reaction, the quantity of CEPA2 did not significantly change, and we did not observe CEPA1 generation. After incubation longer than 3 h, we observed the generation of *cis*-5,*cis*-8,*trans*-13,*cis*-17-20:4 as CEPA2 decreased. CEPA1 was not observed at any point during the time-course experiment (Figure 5B).

3.5 | Substrate Specificity in the Conversion by Cell-Free Extracts of *P. bifermentans* JCM 1386^T

To understand the substrate specificity and the products involved in PUFA conversion by cell-free extracts of *P. bifermentans* JCM 1386^T, we conducted the trials using various free form PUFAs as reaction substrates (Table 1).

Regarding AA transformation by cell-free extracts of *P. bifermentans*, we observed the generation of two novel CFAs (UK3 and UK4), then purified and analyzed them using GC-MS, ^1H -NMR, ^1H - ^1H COSY, ^{13}C -NMR, HSQC, and HMBC spectra (Figures S5 and S6). UK3 and UK4 were identified as *cis*-5,*cis*-8,*cis*-11,*trans*-13-20:4 (CAA1) and *cis*-5,*cis*-8,*trans*-11,*trans*-13-20:4 (CAA2), respectively.

In addition to EPA and AA, we also observed that C_{18} and C_{20} PUFAs with $\omega 6$ and $\omega 9$ double bonds were converted to two specific major products. The products of ALA and GLA were identified via comparison with previously identified standards (Kishino et al. 2009). Regarding other products, we identified the positions of C-C double bonds via GC-MS analysis with pyrrolidine derivatization (Figures S7–S12). Geometrical configurations were then inferred through deductive reasoning.

C_{22} PUFAs (e.g., $\omega 3$ -docosapentaenoic acid and DHA) were not transformed by cell-free extracts of *P. bifermentans* JCM 1386^T.

4 | Discussion

LA conversion by numerous ruminal bacteria, including *B. fibri-solvens* (Kepler et al. 1970) and *L. plantarum* (Kishino et al. 2013) is widely known and relatively well understood. Unlike these

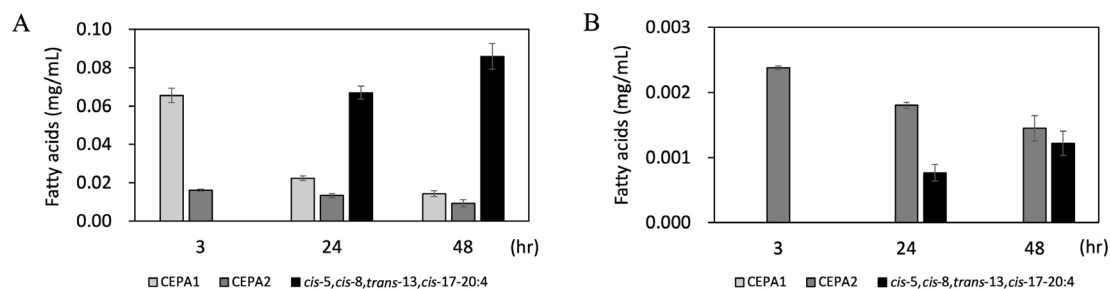


FIGURE 5 | Time course of the transformation of purified CEPAs by resting cells of *Paraclostridium bifermentans* JCM 1386^T. (A) Purified CEPA1 with a small amount of CEPA2 used as a substrate. (B) Purified CEPA2 used as a substrate. CEPA1, *cis*-5, *cis*-8, *cis*-11, *trans*-13, *cis*-17-20:5; CEPA2, *cis*-5, *cis*-8, *trans*-11, *trans*-13, *cis*-17-20:5. Data represent three independent experiments ($n = 3$).

TABLE 1 | Substrate specificity of PUFA conversion by cell-free extracts of *Paraclostridium bifermentans* JCM1386^T.

Substrates	Conversion	Products	
Linoleic acid (<i>cis</i> -9, <i>cis</i> -12-18:2)	+ ^a	<i>cis</i> -9, <i>trans</i> -11-18:2	<i>trans</i> -9, <i>trans</i> -11-18:2
α-Linolenic acid (<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-18:3)	+	<i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15-18:3	<i>trans</i> -9, <i>trans</i> -11, <i>cis</i> -15-18:3
γ-Linolenic acid (<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12-18:3)	+	<i>cis</i> -6, <i>cis</i> -9, <i>trans</i> -11-18:3	<i>cis</i> -6, <i>trans</i> -9, <i>trans</i> -11-18:3
Columbinic acid (<i>trans</i> -5, <i>cis</i> -9, <i>cis</i> -12-18:3)	+	<i>trans</i> -5, <i>cis</i> -9, <i>trans</i> -11-18:3 ^c	<i>trans</i> -5, <i>trans</i> -9, <i>trans</i> -11-18:3 ^h
Stearidonic acid (<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-18:4)	+	<i>cis</i> -6, <i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15-18:4 ^d	<i>cis</i> -6, <i>trans</i> -9, <i>trans</i> -11, <i>cis</i> -15-18:4 ⁱ
<i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17-Eicosatrienoic acid (<i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17-20:3)	+	<i>cis</i> -11, <i>trans</i> -13, <i>cis</i> -17-20:3 ^e	<i>trans</i> -11, <i>trans</i> -13, <i>cis</i> -17-20:3 ^j
Dihomo-γ-linolenic acid (<i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14-20:3)	+	<i>cis</i> -8, <i>cis</i> -11, <i>trans</i> -13-20:3 ^f	<i>cis</i> -8, <i>trans</i> -11, <i>trans</i> -13-20:3 ^k
ω3 Arachidonic acid (<i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17-20:4)	+	<i>cis</i> -8, <i>cis</i> -11, <i>trans</i> -13, <i>cis</i> -17-20:4 ^g	<i>cis</i> -8, <i>trans</i> -11, <i>trans</i> -13, <i>cis</i> -17-20:4 ^l
Arachidonic acid (<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14-20:4)	+	<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>trans</i> -13-20:4	<i>cis</i> -5, <i>cis</i> -8, <i>trans</i> -11, <i>trans</i> -13-20:4
Eicosapentaenoic acid (<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17-20:5)	+	<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>trans</i> -13, <i>cis</i> -17-20:5	<i>cis</i> -5, <i>cis</i> -8, <i>trans</i> -11, <i>trans</i> -13, <i>cis</i> -17-20:5
ω3 Docosapentaenoic acid (<i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19-22:5)	— ^b		
Docosahexaenoic acid (<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19-22:6)	—		

^aSubstrate was converted by the cell-free extracts.

^bSubstrate was not converted by the cell-free extracts.

^cStructure inferred via GC-MS analysis (Figure S8A,B) and deductive reasoning.

^dStructure inferred via GC-MS analysis (Figure S9A,B) and deductive reasoning.

^eStructure inferred via GC-MS analysis (Figure S10A,B) and deductive reasoning.

^fStructure inferred via GC-MS analysis (Figure S11A,B) and deductive reasoning.

^gStructure inferred via GC-MS analysis (Figure S12A,B) and deductive reasoning.

^hStructure inferred via GC-MS analysis (Figure S8C,D) and deductive reasoning.

ⁱStructure inferred via GC-MS analysis (Figure S9C,D) and deductive reasoning.

^jStructure inferred via GC-MS analysis (Figure S10C,D) and deductive reasoning.

^kStructure inferred via GC-MS analysis (Figure S11C,D) and deductive reasoning.

^lStructure inferred via GC-MS analysis (Figure S12C,D) and deductive reasoning.

bacteria, *P. bifermentans* JCM 1386^T can convert both C₁₈ and C₂₀ PUFAs.

In a previous study, we found that *P. bifermentans* JCM 1386^T hydrogenates C₂₀ PUFAs such as AA and EPA (Sakurama et al. 2014). In this study, we found that novel metabolites are produced in the biohydrogenation pathway of EPA and AA by

P. bifermentans JCM 1386^T. CEPA1 and CEPA2 were presumed to be EPA saturation intermediates, and CAA1 and CAA2 to be AA saturation intermediates. Moreover, we revealed pathways of EPA biohydrogenation using resting cells of *P. bifermentans* JCM 1386^T. EPA was found to be converted first to CEPA1 then subsequently to CEPA2. Furthermore, the conversion from CEPA1 to CEPA2 was observed to be irreversible.

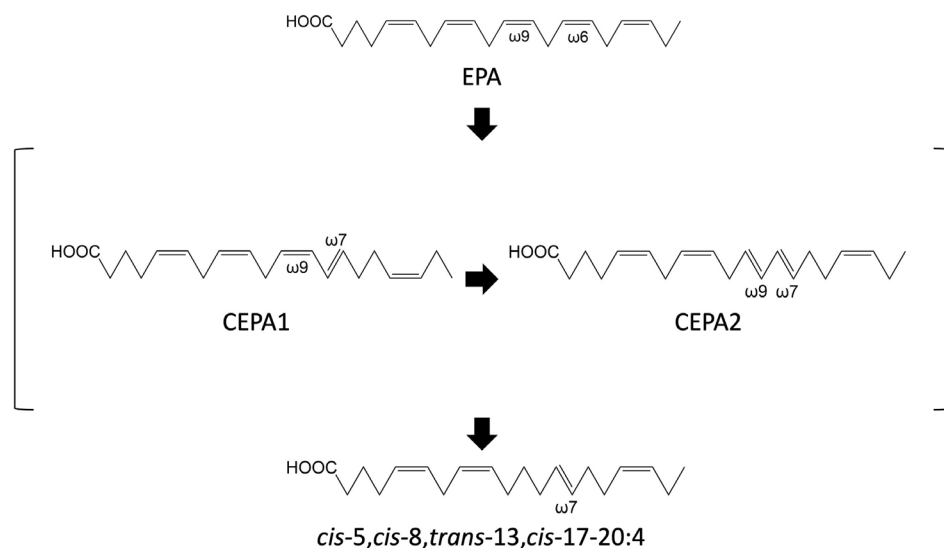


FIGURE 6 | Putative pathway of EPA transformation by *Paraclostridium bifermentans* JCM 1386^T.

The saturation of CEPA1 and CEPA2 to *cis*-5,*cis*-8,*trans*-13,*cis*-17-20:4 was observed apparently as the sequence reactions of CEPA1 to CEPA2 and further to *cis*-5,*cis*-8,*trans*-13,*cis*-17-20:4 (Figure 6).

As shown in Table 1, *P. bifermentans* JCM 1386^T converted the free form of both C₁₈ and C₂₀ PUFAs. Moreover, the results shown in Table 1 indicate that the PUFAs recognized as the substrates are C₁₈ and C₂₀ PUFAs with double bonds at the ω6 and ω9 positions, regardless of whether other double bonds were present (Table 1). Furthermore, *P. bifermentans* JCM 1386^T converted substrates to conjugated isomers with the following structure: one had a *trans* double bond at the ω7 position and a *cis* double bond at the ω9 position, while the other had *trans* double bonds at the ω7 and ω9 positions (Figure 6).

Previous reports detailed PUFA isomerases capable of converting C₂₀ PUFAs from *Clostridium sporogenes* (CSI), *Propionibacterium acnes* (PAI), and the red marine alga *Ptilota filicina* (PFI) (Peng et al. 2007; Hornung et al. 2005; Wise et al. 1994). For example, CSI cannot convert *cis*-8,*cis*-11,*cis*-14-20:3 (Peng et al. 2007). In contrast, our results suggest that *P. bifermentans* JCM 1386^T can indeed convert *cis*-8,*cis*-11,*cis*-14-20:3. PAI was found to convert C₂₀ PUFAs containing an ω6 double bond to CFAs with two or three conjugated double bonds (Hornung et al. 2005), while PFI could produce *cis*-5,*trans*-7,*trans*-9,*cis*-14,*cis*-17-20:5, with three conjugated double bonds, from EPA (Wise et al. 1994). Unlike these enzymes, *P. bifermentans* JCM 1386^T converts C₂₀ PUFAs to two CFAs, each of which has two conjugated double bonds. This indicates that *P. bifermentans* JCM 1386^T may have novel enzyme(s) that might be a potential tool for synthesizing novel bioactive CFAs such as CEPAs and CAAs. Further studies on what kind of enzyme(s) are involved in PUFA transformation by *P. bifermentans* JCM 1386^T may provide insight into the diversity of PUFA isomerases.

5 | Conclusion

EPA hydrogenation pathway to *cis*-5,*cis*-8,*trans*-13,*cis*-17-20:4 in *P. bifermentans* JCM 1386^T was found to include the novel

intermediates of conjugated EPAs, *cis*-5,*cis*-8,*cis*-11,*trans*-13,*cis*-17-20:5 and *cis*-5,*cis*-8,*trans*-11,*trans*-13,*cis*-17-20:5. C₁₈ and C₂₀ PUFAs with *cis*-ω6 and *cis*-ω9 double bonds were also converted to two corresponding conjugated fatty acids with *trans*-ω7 and *cis*-ω9 as well as *trans*-ω7 and *trans*-ω9 structures, which were further saturated to *trans*-ω7 structures.

Author Contributions

Shigenobu Kishino and Jun Ogawa conceived and designed the study. Juo-Ying Chen and Haruko Sakurama wrote the first draft of the manuscript. Juo-Ying Chen, Masaaki Kawai, Kousuke Mihara, and Natsumi Okada carried out the research. Si-Bum Park analyzed the data. All authors contributed to and approved the final draft of the manuscript.

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Ethics Statement

No humans or animals were used in this research.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** aocs70028-sup-0001-Supinfo.zip.