Polyunsaturated fatty acid saturation by gut lactic acid bacteria affecting host lipid composition

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In the representative gut bacterium *Lactobacillus plantarum*, we identified genes encoding the enzymes involved in a saturation metabolism of polyunsaturated fatty acids and revealed in detail the metabolic pathway that generates hydroxy fatty acids, oxo fatty acids, conjugated fatty acids, and partially saturated *trans*-fatty acids as intermediates. Furthermore, we observed these intermediates, especially hydroxy fatty acids, in host organs. Levels of hydroxy fatty acids were much higher in specific pathogen-free mice than in germ-free mice, indicating that these fatty acids are generated through polyunsaturated fatty acids metabolism of gastrointestinal microorganisms. These findings suggested that lipid metabolism by gastrointestinal microbes affects the health of the host by modifying fatty acid composition.

biohydrogenation | hydratase | fatty acid isomerase | conjugated linoleic acid | lipid nutrition

Dietary fats are metabolized not only by humans but also by microbes in our gastrointestinal tracts. Microorganisms in the gastrointestinal tract interact with their host in many ways and contribute significantly to the maintenance of host health (1). Lipid metabolism by gastrointestinal microbes generates multiple fatty acid species, such as conjugated fatty acids and *trans*-fatty acids, that can affect host lipid metabolism (2). However, lipid metabolism by gastrointestinal microbes has not been explored in detail. Saturation metabolism of polyunsaturated fatty acids, a representative mode of lipid metabolism by gastrointestinal microbes, is a detoxifying metabolism of anaerobic bacteria, such as lactic acid bacteria, that reside in colon and intestine. This process transforms growth-inhibiting free polyunsaturated fatty acids into less toxic free saturated fatty acids (3). This saturation metabolism generates characteristic fatty acids (e.g., conjugated fatty acids and *trans*-fatty acids, which are well known to present in ruminant-derived foods and exert various physiological activities).

"Conjugated fatty acid" is a collective term for positional and geometric isomers of fatty acids with conjugated double bonds. In particular, conjugated linoleic acids (CLAs), such as *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA, reduce carcinogenesis (4), atherosclerosis (5), and body fat (6). With regard to lipid metabolism, CLA is a potent peroxisome proliferator-activated receptor (PPAR)α agonist (7), and treatment with CLA increases the catabolism of lipids in the liver of rodents (8). Based on these findings, CLA is now commercialized as a functional food for control of body weight, especially in the United States and European countries.

On the other hand, consumption of *trans*-fatty acids increases the risk of coronary heart disease by increasing LDL and reducing HDL cholesterol levels (9). Consequently, *trans*-fatty acids are considered to be harmful for health, and nutritional authorities have recommended that consumption of *trans*-fatty acids be reduced to trace amounts (10). Therefore, it is important to control fatty acid saturation processes that generate these fatty acids (11); however, the precise metabolic pathway and enzymes involved have not been clearly identified.

Our analyses on conjugated fatty acid synthesis in representative gut bacteria, the lactic acid bacteria (12–15), demonstrated that *Lactobacillus plantarum* AKU 1009a (AKU Culture Collection, Faculty of Agriculture, Kyoto University) can transform the *cis*-9,*cis*-12 diene structure of C18 fatty acids such as linoleic acid, α-linolenic acid, and γ-linolenic acid into the conjugated diene structures *cis*-9,*trans*-11 and *trans*-9,*trans*-11 (16–21). In addition, this strain can saturate these conjugated dienes into the *trans*-10 monoene. Our subsequent metabolic analysis indicated that 10-hydroxy-12-octadecenoic acid is an intermediate of CLA synthesis, and further investigations of hydroxy fatty acid metabolism by lactic acid bacteria revealed that CLA is produced from hydroxy fatty acids such as ricinoleic acid in castor oil (22–25). In cell-free extracts from this strain, we identified the enzymes involved in CLA synthesis (26). Three enzymes, CLA-HY, CLA-DH, and CLA-DC, are necessary for synthesis of conjugated fatty acids such as CLA. Only the combined action of these three enzymes can generate CLA from linoleic acid, with 10-hydroxy-*cis*-12-octadecenoic acid arising as an intermediate

Significance

Microorganisms in the gastrointestinal tract interact with their host in many ways. Lipid metabolism by gastrointestinal microbes generates multiple fatty acid species that can affect host health. In the representative gut bacterium *Lactobacillus plantarum*, we revealed a fatty acid metabolism, saturation metabolism of polyunsaturated fatty acid, that generates hydroxy fatty acids, oxo fatty acids, conjugated fatty acids, and partially saturated *trans*-fatty acids as intermediates. Furthermore, fatty acid analysis in mice suggests that the fatty acid metabolism by gastrointestinal microbes modifies fatty acid composition of the host. Therefore, functional investigations of lipid metabolisms of gastrointestinal microbes may provide new methods for improving our health by altering lipid metabolism related to the onset of metabolic syndrome.

Author contributions: S.K., S.S., and J.O. designed research; S.K., M.T., S.-B.P., A.H., N.K., J.K., R.I., Y.I., and M.A. performed research; S.K., S.-B.P., J.K., H.K., M.A., H.A., K.U., J.S., S.T., K.Y., and S.S. analyzed data; and S.K., M.T., and J.O. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AB812091).

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(Fig. 1*B*, *3*). The reactions catalyzed by each enzyme, however, were not revealed in those studies. Through genomic analysis in *L. plantarum* WCFS1, we found that *cla-dh* (GenBank accession no. NC_004567; region: 59613-60473) and *cla-dc* (GenBank accession no. NC_004567; region: 60505-61350) are located in a cluster with another gene, *cla-er* (GenBank accession no. NC_004567; region: 61378-62031) (Fig. 1*A*). In light of this, we tried to identify the function of the gene product (CLA-ER) together with those of CLA-HY, CLA-DH, and CLA-DC.

Results and Discussion

Based on the sequence of the *L. plantarum* WCFS1 genome, we designed primers to amplify the CLA-ER gene from *L. plantarum* AKU 1009a genomic DNA. The PCR-amplified product, *cla-er* (GenBank accession no. AB812091), consisted of 654 bp and encoded a protein whose amino acid sequence is 99.8% identical to the homologous sequence from *L. plantarum* WCFS1. We transformed the resulting plasmid containing *cla-er* from *L. plantarum* AKU 1009a, pCLA-ER, into *Escherichia coli* Rosetta2 (DE3) to generate *E. coli* Rosetta/pCLA-ER. The gene product, CLA-ER, had a molecular mass of ~25 kDa (including a His tag) and could be detected in soluble cell-free extracts of *E. coli* Rosetta/pCLA-ER. We examined the function of CLA-ER in fatty acid metabolism using purified CLA-ER in combination with other purified enzymes (CLA-HY, CLA-DH, and CLA-DC) from the corresponding transformants (*E. coli* Rosetta/pCLA-HY, *E. coli* Rosetta/pCLA-DH, and *E. coli* Rosetta/pCLA-DC).

In a reaction with these four enzymes (CLA-HY, CLA-DH, CLA-DC, and CLA-ER) as catalysts, *cis*-9,*trans*-11-CLA (CLA1), *trans*-9,*trans*-11-CLA (CLA2), 10-hydroxy-*cis*-12-octadecenoic acid,

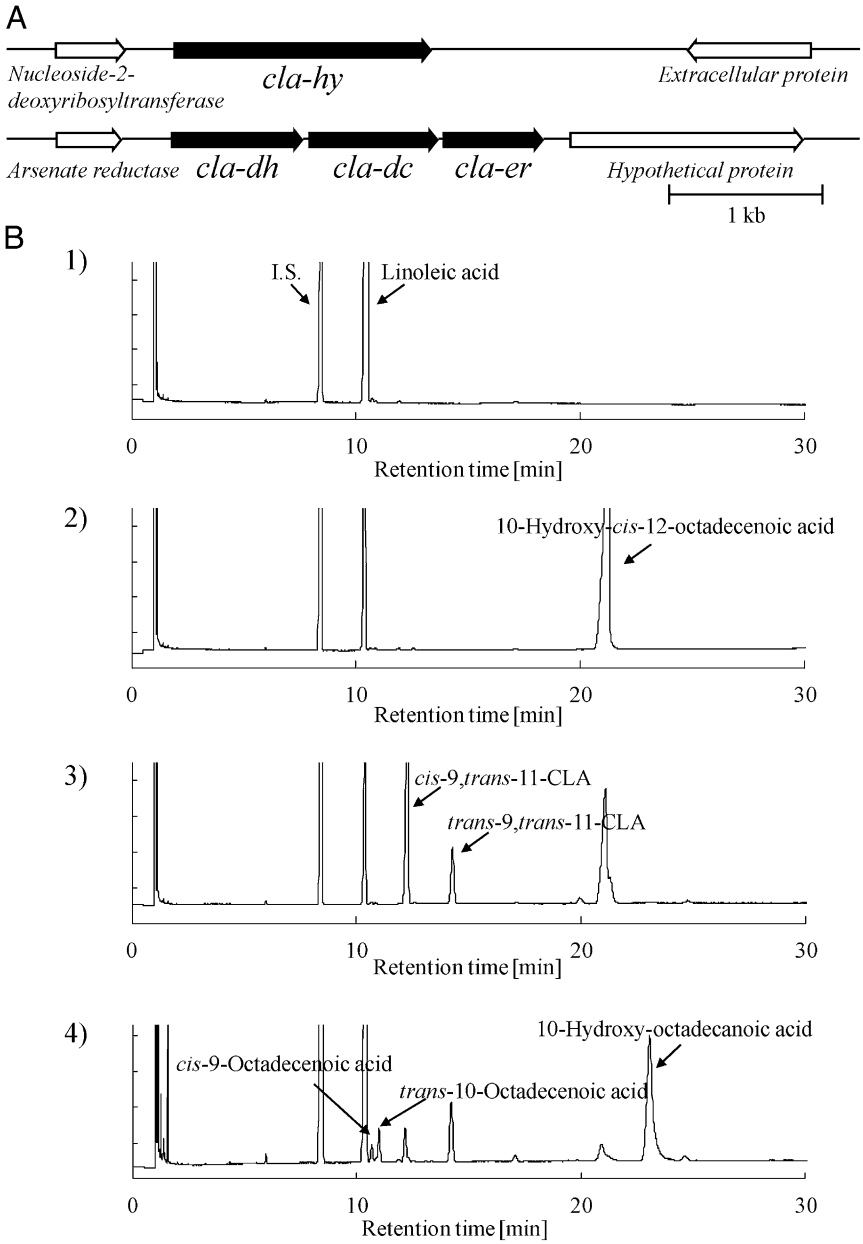

Fig. 1. Gene clusters for polyunsaturated fatty acid metabolism and GC chromatograms. (*A*) Gene clusters for fatty acid metabolic enzymes in *L. plantarum*. (*B*) GC chromatograms of substrate (*1*); reaction with CLA-HY (*2*); reaction with CLA-HY, CLA-DH, and CLA-DC together with FAD and NADH (*3*); and reaction with CLA-HY, CLA-DH, CLA-DC, and CLA-ER together with FAD and NADH (*4*). I.S., internal standard.

trans-10-octadecenoic acid, and *cis*-9-octadecenoic acid (oleic acid) were generated from linoleic acid (Fig. 1*B*, *4*). Thus, the combined action of these four enzymes generated saturated products of oleic acid and *trans*-10-octadecenoic acid from linoleic acid (i.e., these four enzymes catalyzed the saturation of an polyunsaturated fatty acid).

In our previous studies, we revealed that linoleic acid could be converted into 10-hydroxy-*cis*-12-octadecenoic acid by CLA-HY (Fig. 1*B*, *2*), as well as into CLA1 and CLA2 along with 10-hydroxy-*cis*-12-octadecenoic acid by CLA-HY, CLA-DH, and CLA-DC (Fig. 1*B*, *3*). We purified the resulting 10-hydroxy-*cis*-12-octadecenoic acid by HPLC and used it as a substrate for reactions containing each enzyme together with the oxidoreduction cofactors (i.e., FAD, NADH, or NADPH) that enhanced CLA synthesis by CLA-HY, CLA-DH, and CLA-DC (14). 10-Hydroxy-*cis*-12-octadecenoic acid was converted into linoleic acid and *trans*-10, *cis*-12-octadecadienoic acid by CLA-HY in the presence of FAD and NADH (*SI Appendix*, Fig. S1). The same substrate was converted into 10-oxo-*cis*-12-octadecenoic acid by CLA-DH in the presence of NAD^+ (*SI Appendix*, Fig. S2). As before, we purified the resulting *trans*-10,*cis*-12-octadecadienoic acid and 10-oxo-*cis*-12-octadecenoic acid by HPLC and used them as substrates in subsequent reactions.

None of the enzymes converted *trans*-10,*cis*-12-octadecadienoic acid under any conditions tested. By contrast, 10-oxo-*cis*-12-octadecenoic acid was converted into 10-oxo-*trans*-11-octadecenoic acid by CLA-DC in the absence of cofactors (*SI Appendix*, Fig. S3). HPLC-purified 10-oxo-*trans*-11-octadecenoic acid was converted into 10-oxo-octadecanoic acid by CLA-ER in the presence of FAD/FMN and NADH (*SI Appendix*, Fig. S4) and was converted into 10-hydroxy-*trans*-11-octadecenoic acid by CLA-DH in the presence of NADH (*SI Appendix*, Fig. S5). Purified 10-oxo-octadecanoic acid was converted into 10-hydroxy-octadecanoic acid by CLA-DH in the presence of NADH (*SI Appendix*, Fig. S6), and this product was, in turn, converted into *cis*-9-octadecenoic acid and *trans*-10-octadecenoic acid by CLA-HY in the presence of FAD and NADH (*SI Appendix*, Fig. S7). The 10-hydroxy-*trans*-11-octadecenoic acid could also be converted into *cis*-9,*trans*-11-CLA and *trans*-9,*trans*-11-CLA by CLA-HY in the presence of FAD and NADH (*SI Appendix*, Fig. S8).

These results demonstrate that the linoleic acid-saturation metabolism of *L. plantarum* consists of multiple reactions. The first reaction of linoleic acid metabolism is hydration of the carbon–carbon double bond at the Δ9 position, catalyzed by CLA-HY, to generate 10-hydroxy fatty acid. The second reaction is dehydrogenation of the hydroxy group at C10, catalyzed by CLA-DH, to generate 10-oxo fatty acid. The third reaction is isomerization of the carbon–carbon double bond at Δ12, catalyzed by CLA-DC, to generate the conjugated enone structure, 10-oxo-*trans*-11-fatty acid. The fourth reaction is hydrogenation of the carbon–carbon double bond at Δ11, catalyzed by CLA-ER, to generate the carbon–carbon single bond. The fifth reaction is hydrogenation of the oxo group at C10, catalyzed by CLA-DH, to generate 10-hydroxy fatty acid. The last reaction is dehydration of hydroxy group at C10, catalyzed by CLA-HY, to generate *cis*-9 and *trans*-10 monoenoic fatty acids. Through a branch of the saturation-metabolism pathway, conjugated fatty acids are generated by the combined actions of three of the enzymes, CLA-HY, CLA-DH, and CLA-DC. The branched pathway starts with hydrogenation of the oxo group at C10 in 10-oxo-*trans*-11-fatty acid, catalyzed by CLA-DH, to generate 10-hydroxy-*trans*-11-fatty acid; the final reaction is dehydration of hydroxy group at C10 in 10-hydroxy-*trans*-11-fatty acid, catalyzed by CLA-HY, to generate *cis*-9,*trans*-11– and *trans*-9,*trans*-11–conjugated fatty acids (Fig. 2). As we reported previously, C18 fatty acids with Δ9 and Δ12 diene systems such as α-linolenic acid, γ-linolenic acid, and stearidonic acid undergo the same transformations in *L. plantarum* AKU 1009a (20), indicating that the corresponding intermediates (hydroxy, oxo, conjugated, and partially saturated *trans*-fatty acids) are produced by the combined actions of these enzymes. The revealed fatty acid-saturation metabolism consists of similar reactions in known fatty acid biosynthesis and degradation pathway; however, it is a pathway that uses only free fatty acids as substrates but not CoA- or acyl carrier protein-activated fatty acids.

In the experiments described above, we revealed the pathway of unsaturated fatty acid metabolism in *L. plantarum* and the enzymes involved in this metabolism. These enzymes function as catalysts of hydration/dehydration (CLA-HY), oxidation of hydroxy groups/reduction of oxo groups (CLA-DH), migration of carbon–carbon double bonds (CLA-DC), and saturation of carbon–carbon double bonds (CLA-ER). The genes that encode CLA-DH, CLA-DC, and CLA-ER form a gene cluster in *L. plantarum.* When we searched for gene clusters containing *cla-dh*, *cla-dc*, and *cla-er* in other microorganisms using the Kyoto Encyclopedia of Genes and Genomes database (www.genome.jp/kegg), we found that *Lactobacillus casei* and *Lactobacillus rhamnosus* have the same gene cluster, as well as the CLA-HY gene. Furthermore, many species of lactic acid bacteria have one or more of these four genes. For example, *Lactobacillus salivarius* has *cla-dc*, *cla-er*, and *cla-hy*; and *Lactobacillus amylovorus*, *Lactobacillus johnsonii*, *Lactobacillus helveticus*, and *Lactobacillus crispatus* have *cla-dh*, *cla-er*, and *cla-hy.* Therefore, acting in concert, these species may mediate the polyunsaturated fatty acid-saturation metabolism in gastrointestinal tract. The in vivo distributions of these strains in relation to the fatty acid profiles and the health conditions of host organisms are also of interest.

The reactions and enzymes we identified will be useful for modifying the properties of fatty acids in foods. The apparent isomerization reaction catalyzed by the combined activities of CLA-HY, CLA-DH, and CLA-DC will be useful for production of *cis*-9,*trans*-11– and *trans*-9,*trans*-11–conjugated fatty acids. The other isomerization reaction catalyzed by CLA-HY will be useful for production of *trans*-10,*cis*-12–conjugated fatty acids. Furthermore, the dehydration reaction catalyzed by CLA-HY might determine the ratio of *trans* to *cis*-fatty acids. In other words, enhancing *cis*-dehydration by CLA-HY could be useful for reducing the amounts of *trans*-fatty acid in foods. This might be possible as we reported previously that the CLA1/CLA2 ratio (*cis*/*trans* ratio) can be controlled by optimizing the reaction conditions (14, 16). Furthermore, not only in the food industry but also in the chemical industry, the reactions found in the fatty acid-saturation metabolism are useful (e.g., enzymatic production of hydroxy fatty acids for polymer industry).

To evaluate the effects of gastrointestinal bacteria on the profile of fatty acids in host tissues, we monitored endogenous formation of the fatty acid intermediates of polyunsaturated fatty acid-saturation metabolism (i.e., hydroxy and oxo fatty acids) in mice bred in either germ-free or specific pathogen-free (SPF) conditions. In the colon, small intestine, and plasma of both groups of mice, we detected 10-hydroxy-*cis*-12-octadecenoic acid, 10-hydroxyoctadecanoic acid, and 10-oxooctadecanoic acid. By contrast, we did not detect 10-oxo-*cis*-12-octadecenoic acid or 10-oxo-*trans*-11-octadecenoic acid derived from linoleic acid or metabolites derived from α-linolenic acid and γ-linolenic acid. There were significant differences in the levels of hydroxy fatty acids between SPF and germ-free mice: in particular, we observed higher levels of 10-hydroxy fatty acids derived from linoleic acid, oleic acid, or both in SPF mice than in germ-free mice (Fig. 3). We also detected a structurally related hydroxy fatty acid, 13-hydroxy-9-*cis*-octadecenoic acid, which is produced by lactic acid bacteria (27), at higher levels in SPF mice than in germ-free mice (Fig. 3). These differences in the levels of hydroxy fatty acids could be clearly observed in the small intestine, the primary site of fatty acid absorption. These results indicate that gastrointestinal microbes play roles in modifying the fatty acid profiles of their host mice,

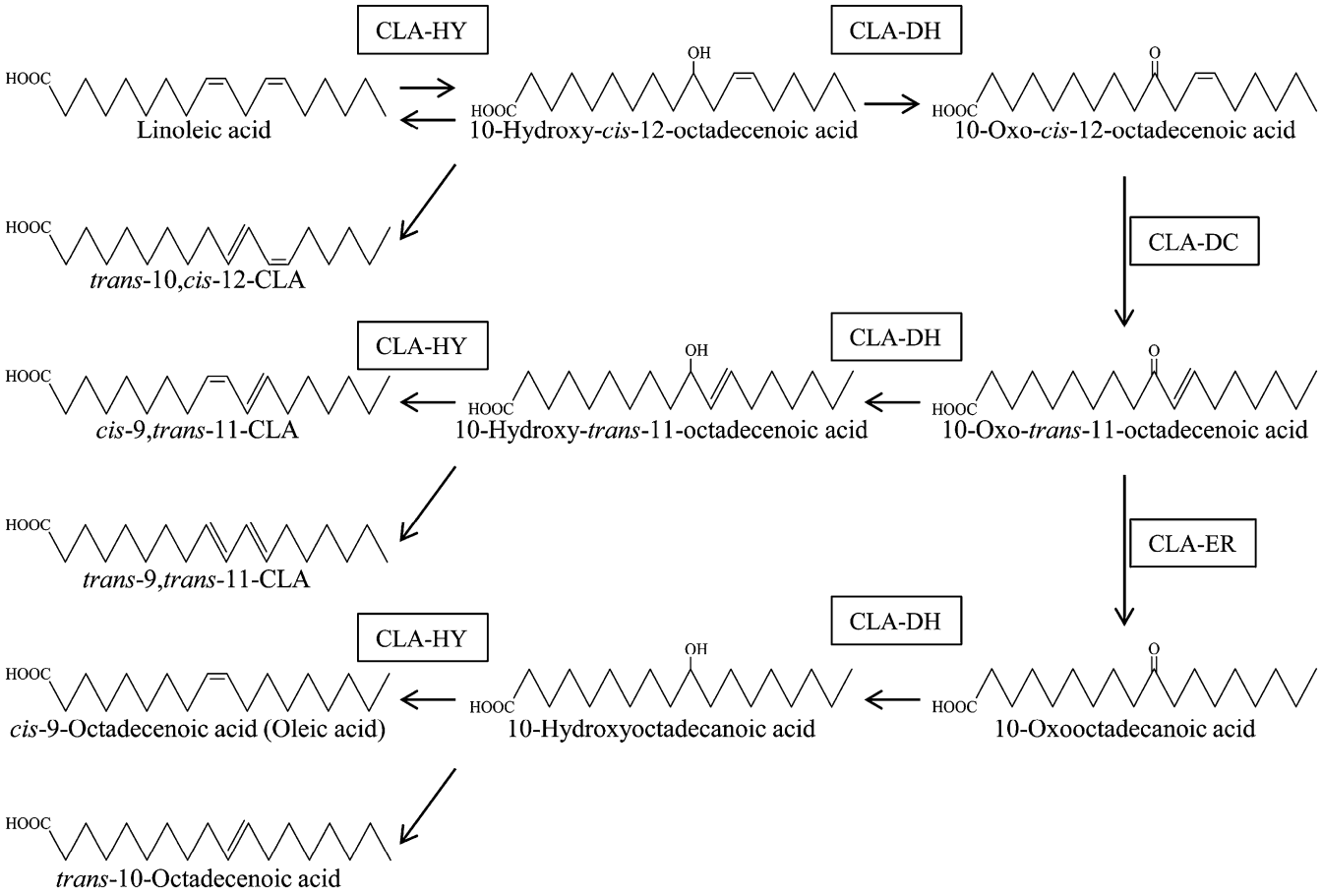

Fig. 2. Polyunsaturated fatty acid-metabolism pathway.

in particular, by increasing the levels of hydroxy fatty acids that are initial intermediates of polyunsaturated fatty acid-saturation metabolism. The investigations using monocolonize germ-free mice with *L. plantarum* or a strain deficient in one or more of the fatty acid saturating genes to see whether the host accumulates intermediates are also future interests.

The intermediates found in the polyunsaturated fatty acid-saturation metabolism described here are predicted to exert specific physiological functions. For example, hydroxy and oxo fatty acids act as ligands for PPARγ (28); oxo-fatty acids found in tomato juice are potent PPARα activators and decrease the amount of triacylglycerol in obese diabetic mice (29). Therefore, functional investigations of these fatty acid intermediates of the polyunsaturated fatty acid-saturation metabolism may provide new methods for improving our health by altering lipid metabolism related to the onset of the metabolic syndrome. Exploration of the lipid metabolism of gastrointestinal microorganisms at the enzymatic and genetic levels, and integration of these findings with metagenomic information, might enable us to promote health by controlling intestinal lipid metabolism.

Materials and Methods

Chemicals. Standard samples of *cis*-9,*trans*-11-CLA, *trans*-9,*trans*-11-CLA, and 10-hydroxy-*cis*-12-octadecenoic acid were prepared as described previously (11, 13). Linoleic acid and fatty acid-free (<0.02%) BSA were purchased from Sigma. All other chemicals were of analytical grade and were obtained commercially.

Cloning and Expression of Recombinant CLA-ER Proteins in *E. coli*. Primers were designed to amplify the *cla-er* sequence from *L. plantarum* AKU 1009a genomic DNA. The PCR-amplified product was ligated into the pET101/D-TOPO expression vector (Invitrogen), according to the manufacturer's instructions. The resulting plasmid, pCLA-ER, was transformed into *E. coli* Rosetta2 (DE3) (Novagen) to generate *E. coli* Rosetta/pCLA-ER. The integrity of the cloned gene was verified by DNA sequencing using a Beckman-Coulter CEQ8000.

Expression of Recombinant Proteins in *E. coli*. Transformants (*E. coli* Rosetta/pCLA-HY, *E. coli* Rosetta/pCLA-DH, *E. coli* Rosetta/pCLA-DC, and *E. coli* Rosetta/pCLA-ER) were cultured in Luria–Bertani medium at 37 °C for 1 h with shaking at 300 rpm, and then isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM. After addition of IPTG, the transformed cells were cultivated at 20 °C for 6 h with shaking at 300 rpm. The cells were harvested by centrifugation (12,000 × *g*; 10 min), washed twice with 0.85% NaCl, and stored at −20 °C until further use.

Purification of Enzymes from Transformants. To determine the approximate concentration of protein eluted in chromatography, effluents were monitored by UV detection at 280 nm. Enzymes were purified using a fast protein liquid chromatography system (Amersham Pharmacia Biotech) equilibrated with binding buffer [20 mM potassium phosphate buffer (KPB), 50 mM imidazole; pH 7.4] or standard buffer (20 mM KPB, 1 mM DTT, 10% (vol/vol) ethylene glycol; pH 6.5). Fractions with enzymatic activity were collected and concentrated using an Amicon Ultra YM-10 (Millipore). All procedures were carried out at 4 °C.

CLA-HY and CLA-ER were purified as His-tagged proteins. *E. coli* Rosetta/pCLA-HY and *E. coli* Rosetta/pCLA-ER cells (8 g) in 1.5 L of culture broth were suspended in binding buffer and disrupted with an Insonator 201M ultrasonic oscillator (Kubota). After ultracentrifugation (100,000 × *g*; 60 min) of the cell lysate, the resulting supernatant containing His-tagged CLA-HY or His-tagged CLA-ER was loaded onto a Ni-Sepharose column (His Trap HP; GE Healthcare) preequilibrated with binding buffer. After washing, the bound proteins were eluted with elution buffer (20 mM KPB, 250 mM imidazole; pH 7.4). Active fractions were collected and concentrated with a Centriprep

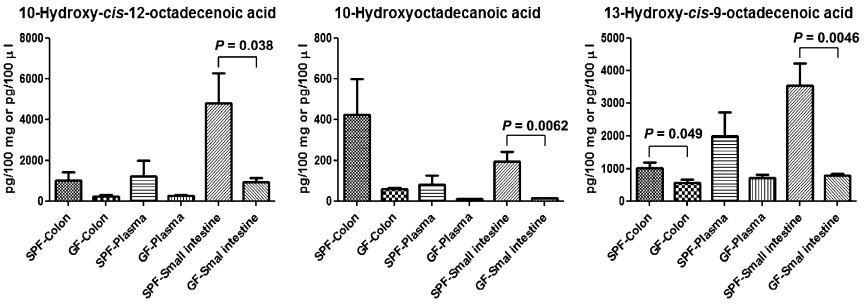

Fig. 3. Detection and quantitative analyses of polyunsaturated fatty acid-saturation metabolism intermediates in mice. Lipids extracted from colon (100 mg), intestine (100 mg), or plasma (100 μL) of SPF or germ-free (GF) mice were analyzed by LC-MS/MS-based lipidomics as described in *Materials and Methods*. Data are presented as means ± SEM ($n = 8$). The statistical significance between mean values was determined by unpaired *t* test with Welch's correction.

YM-3 (Millipore) and applied to a Hi-load 26/60 Superdex 200 prep-grade column (GE Healthcare) equilibrated with 50 mM KPB (pH 6.5). Active fractions were collected, dialyzed with 50 mM KPB (pH 6.5) including 50% (vol/vol) glycerol, and stored at −20 °C until use.

For purification of CLA-DH, *E. coli* Rosetta/pCLA-DH cells (8 g) in 1.5 L of culture broth were suspended in BugBuster Master Mix (Merck) (30 mL) and incubated for 20 min at room temperature. After ultracentrifugation (100,000 × *g*; 60 min) of the cell lysate, the resulting supernatant was concentrated with a Centriprep YM-3 and applied to a HiLoad 26/60 Superdex 200 prep-grade column that had been equilibrated with standard buffer and eluted. CLA-DH was purified further using a Mono Q 10/100 GL column and a Superdex 200 10/300 GL column (GE Healthcare). The purified CLA-DH was dialyzed with 50 mM KPB (pH 6.5) including 50% (vol/vol) glycerol and stored at −20 °C until use.

For purification of CLA-DC, *E. coli* Rosetta/pCLA-DC cells (8 g) in 1.5 L of culture broth were suspended in standard buffer and disrupted with an Insonator 201M ultrasonic oscillator. After centrifugation (20,000 × *g*; 30 min) of the cell lysate, solid sulfate was added to the resulting supernatant to 50–80% saturation. The precipitate was recovered by centrifugation, dissolved in 10 mL of standard buffer, and then dialyzed three times against 2 L of standard buffer for 8 h. CLA-DC was purified further using a Phenyl Superose HR 10/10 column and a Mono Q 5/50 GL column (GE Healthcare). Purified CLA-DC was dialyzed with 50 mM KPB (pH 6.5) including 50% (vol/vol) glycerol and stored at −20 °C until use.

Reaction Conditions. Reactions were performed in test tubes (16.5 × 125 mm) that contained 1 mL of reaction mixture (20 mM KPB; pH 6.5) with 0.1% (wt/vol) fatty acid complexed with BSA [0.02% (wt/vol)] as the substrate and purified enzymes (CLA-HY, CLA-DH, CLA-DC, and CLA-ER) in various combinations. The reactions were performed with 5 mM NADH, 5 mM NAD^+, 0.1 mM FMN, or 0.1 mM FAD under microaerobic conditions in a sealed chamber with an O_2 absorber (Anaeropack Kenki; Mitsubishi Gas Chemical) and gently shaken (120 strokes per minute) at 37 °C for 12 h. The oxygen concentration under microaerobic conditions was maintained below 0.1% (<1,000 ppm) and monitored with an oxygen indicator (Mitsubishi Gas Chemical). All experiments were performed in triplicate, and the averages of three separate experiments that were reproducible within ±10% are presented in the figures.

Lipid Analyses. Before lipid extraction, *n*-heptadecanoic acid was added to the reaction mixture as an internal standard. Lipids were extracted from 1 mL of the reaction mixture with 5 mL of chloroform/methanol/1.5% KCl in H_2O (2:2:1, by volume), according to the procedure of Bligh-Dyer, and concentrated by evaporation under reduced pressure (30). The resulting lipids were dissolved in 2 mL of methanol and 3 mL of benzene and then methylated with 300 μL of 1% trimethylsilyldiazomethane at 28 °C for 30 min. After methylation, the resulting fatty acid methyl esters were concentrated by evaporation under reduced pressure. The resulting fatty acid methyl esters were analyzed by gas–liquid chromatography (GC) using a Shimadzu GC-1700 gas chromatograph equipped with a flame-ionization detector and split-injection system, fitted with a capillary column (SPB-1; 30 m length × 0.25 mm i.d.; Supelco). The initial column temperature was 180 °C for 30 min but was subsequently increased to 210 °C at a rate of 60 °C/min and then maintained at that temperature for 29.5 min. The injector and detector were operated at 250 °C. Helium was used as a carrier gas at a flow rate of 1.4 mL/min. The fatty acid peaks were identified by comparing retention times to known standards.

Isolation and Identification of Reaction Products. Reaction products were separated by reverse-phase HPLC using a Shimadzu LC-10A system equipped with a Cosmosil column (5C18-AR; 20 × 250 mm; Nacalai Tesque). The mobile phase was acetonitrile-H_2O (8:2, by volume) at a flow rate of 3.0 mL/min, and the effluent was monitored by UV detection (205 and 233 nm). The methyl esters of purified fatty acids were transformed to the pyrrolidide and trimethylsilyl (TMS) derivatives. Pyrrolidide derivatives were prepared by direct treatment of the purified fatty acid methyl esters with pyrrolidine-acetic acid [10:1 (vol/vol)] in screw-cap tubes for 1 h at 115 °C, followed by extraction with dichloromethane. The organic extract was washed with water and dried over anhydrous Na_2SO_4, and then the solvent was removed under vacuum in a rotary evaporator. The TMS derivatives were prepared by direct treatment of the purified fatty acid methyl esters with a mixture of TMS agent (pyridine/hexamethyldisilazane/trimethylchlorosilane; 9:3:1, by volume) in screw-cap tubes for 30 min at 60 °C, followed by extraction with chloroform. The chemical structures of purified fatty acid methyl esters, pyrrolidide derivatives, and TMS derivatives were determined by mass spectroscopy (MS), and the chemical structures of purified free fatty acids were determined by 2D proton NMR (^{1}H-NMR) techniques including ^{1}H-^{1}H double-quantum–filtered chemical-shift correlation spectroscopy and 2D nuclear Overhauser effect spectroscopy, as described previously (20).

Mice. SPF and germ-free BALB/c mice (9 wk; female) were obtained from CLEA Japan and maintained under SPF and germ-free conditions with a sterile diet (CL-2; CLEA Japan), respectively, at the Experimental Animal Facility, Institute of Medical Science, The University of Tokyo. Isolated tissues were immediately frozen by liquid nitrogen and always kept at –80 °C before fatty acid analysis. All experiments were approved by the Animal Care and Use Committee of the University of Tokyo and conducted in accordance with their guidelines.

Fatty Acid Analysis in Mice. Liquid chromatography–tandem MS (LC-MS/MS)-based lipidomics was performed as described (31). Briefly, samples were subjected to solid-phase extraction using a Sep-Pak C18 cartridge (Waters) with a deuterium-labeled internal standard (arachidonic acid-d8, leukotriene B_4-d4, 15-hydroxyeicosatetraenoic acid-d8, prostaglandin E2-d4). Lipidomic analyses were performed using an HPLC system (Waters UPLC) with a linear ion-trap quadrupole mass spectrometer (QTRAP 5500; AB SCIEX) equipped with an Acquity UPLC BEH C18 column (1.0 mm × 150 mm × 1.7 μm; Waters). Samples were eluted with a mobile phase consisting of water/acetate [100:0.1 (vol/vol)] and acetonitrile/methanol [4:1 (vol/vol)] (73:27) for 5 min; increased to 30:70 after 15 min, increased to 20:80 after 25 min, and held for 8 min; and then increased to 0:100 after 35 min and held for 10 min with flow rates of 70 μL/min (0–30 min), 80 μL/min (30–33 min), and 100 μL/min (33–45 min). MS/MS analyses were conducted in negative-ion mode, and fatty acid metabolites were identified and quantified by multiple-reaction monitoring. Quantitation was performed using calibration curves constructed for each compound, and recoveries were monitored using added deuterated internal standards.

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