Hepoxilins (HXs) and trioxilins (TrXs) are involved in physiological processes such as inflammation, insulin secretion and pain perception in human. They are metabolites of polyunsaturated fatty acids (PUFAs), including arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid, formed by 12-lipoxygenase (LOX) and epoxide hydrolase (EH) expressed by mammalian cells. Here, we identify ten types of HXs and TrXs, produced by the prokaryote *Myxococcus xanthus*, of which six types are new, namely, HXB5, HXD3, HXE3, TrXB5, TrXD3 and TrXE3. We succeed in the biotransformation of PUFAs into eight types of HXs (>35% conversion) and TrXs (>10% conversion) by expressing *M. xanthus* 12-LOX or 11-LOX with or without EH in *Escherichia coli*. We determine 11-hydroxy-eicosatetraenoic acid, HXB3, HXB4, HXD3, TrXB3 and TrXD3 as potential peroxisome proliferator-activated receptor-γ partial agonists. These findings may facilitate physiological studies and drug development based on lipid mediators.
Lipid mediators are signalling transduction molecules essential for homeostasis and intracellular communication in humans. They are C20- and C22-polyunsaturated fatty acids (PUFAs) containing hydroxyl group(s) and/or an epoxide group with or without a C5 ring. Lipid mediators include leukotrienes (LTs), lipoxins (LXs), resolvin (RVs), protectins (PTs), hepoilins (HXs), trioxilins (TrXs) and prostaglandins (PGs), which are involved in the regulation of the immune and inflammatory responses of humans (see Supplementary Table 1 for abbreviations). Owing to their anti-inflammatory, anti-infective, antibacterial, anti-viral, anti-apoptotic, neuroprotective and tissue-healing properties, these lipid mediators have attracted much attention in recent years.

HXs and TrXs are C20- and C22-PUFAs, with the former having a hydroxyl group and an epoxide group at C11 and C12, and the latter having three hydroxyl groups. The classification and chemical names of all HXs and TrXs are presented in Supplementary Table 2. They are found in various types of organs, tissues and cells, including brain, aorta, insulinoma, epithelium, platelets and neutrophils. Compounds of HXA series contain a hydroxyl group at C8, whereas compounds of HXB series have a hydroxyl group at C10. In humans, arachidonic acid (ARA) is metabolized to HXA3 and HXB3 by ARA 12-lipoxygenase (LOX), which are converted to TrXA3 and TrXB3, through the hydrolysis of the epoxy group in HXA3 and HXB3, respectively, by HX epoxide hydrolase (EH) (Supplementary Fig. 1). HXs are involved in insulin secretion, calcium regulation, potassium regulation, platelet aggregation and vascular permeability. HXs are also chemotactic factors for human neutrophils like LTs and pathogen-elicited epithelial chemokactants. HXs and TrXs regulate vasorelaxation in the arteries, affect the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα) and are involved in regulating the life cycle of barnacles, e.g. in egg hatching and larval settlement. Thus, they are important lipid mediators for various organisms.

LOXs, cyclooxygenases (COXs) and the cytochrome P450 families are the starting enzymes for the biosynthesis of lipid mediators using PUFAs as substrates. Among these enzymes, LOXs, a family of non-heme-iron-containing dioxygenases, catalyse the dioxygenation of PUFAs containing one or more Z,Z,Z-3,4-pentadienyl structures to hydroperoxy fatty acids (HFPFAs). They also catalyse the epoxidation of HFPFAs to epoxy hydroxy fatty acids (EHFAs) such as LTs and HXs. LOXs are classified as 5-, 8-, 11-, 12- and 15-LOXs according to the number of oxygenated carbon site on ARA. LOXs have been mainly studied in mammals, COX converts ARA to PGH2, which can be converted to prostacyclin (PGI2), 6- and 12-epoxycicosatrienoic acid (12- and 6-HETE), 12-HETE, 15-HETE, HXB3, PGG2 and PGG2. However, four types of metabolites did not have matched compounds. Among them, metabolite numbers 9 and 10 were possibly new-type of HX and TrX, respectively, since they did not match with the compounds in available information databases, including the LIPID MAPS Database, PubChem, the Human Metabolome Database and KEGG. The other two types of metabolites were not suggested because they had many overlapping MS/MS fragments.

Identification of the biosynthetic genes and enzymes. Given that the M. xanthus genome has already been sequenced, the eight candidate biosynthetic genes of lipid mediators were selected by comparison with the sequences of human corresponding genes. The genes of MXAN_1744, MXAN_1745, MXAN_1644, MXAN_5137, MXAN_5217, MXAN_0683, MXAN_2304 and MXAN_3623 in M. xanthus were predicted to be the genes encoding LOX, LOX, EH, EH, COX, two thromboxane A (TXA) synthases and PGD synthase, respectively (Supplementary Table 4). Although the amino acid sequences of these enzymes showed 15–40% identities with human corresponding enzymes, the major residues affecting the activity were conserved (Supplementary Fig. 3). These candidate genes were cloned and expressed in E. coli in soluble forms (Supplementary Fig. 4). No activity was found for putative TXA synthases, putative PGD synthase or putative EH expressed from MXAN_5137. The protein from MXAN_5217 converted ARA to PGG2 (Supplementary Fig. 5), indicating that it is COX. The activity of COX towards ARA was 0.011 μmol min⁻¹ mg⁻¹. In animals, COX converts ARA to PGG2, which can be converted to diverse PGs by various types of PG synthases (Supplementary Fig. 1).

Results

LC-MS analysis for ARA-derived metabolites of M. xanthus. M. xanthus was cultivated in medium containing ARA for 24 h. After cultivation, the culture supernatant was analysed by high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS). M. xanthus consumed most of ARA and the peaks of some metabolites were detected (Supplementary Fig. 2). However, ARA was not consumed and there were no new peaks detected in the culture medium without M. xanthus. The molecular formulae of metabolites of M. xanthus were determined by MS/MS fragmentation analysis (Supplementary Table 3). Seven types of metabolites were suggested by comparison with the references in the LIPID metabolites and pathways strategy (MAPS) Database. They were eicosapentaenoic acid (EPA; C20:5), 11-hydroxy-5Z,8Z,12Z,14Z-eicosatetraenoic acid (11-HETE), 12-HETE, 15-HETE, HXB3, PGG2 and PGG2. However, four types of metabolites did not have matched compounds. Among them, metabolite numbers 9 and 10 were possibly new-type of HX and TrX, respectively, since they did not match with the compounds in available information databases, including the LIPID MAPS Database, PubChem, the Human Metabolome Database and KEGG. The other two types of metabolites were not suggested because they had many overlapping MS/MS fragments.

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hydroperoxyeicosatetraenoic acid (12-HpETE) and 11-HpETE, respectively, indicating that they are ARA 12-LOX and ARA 11-LOX, respectively. The enzyme expressed from MXAN_1644 converted HXB3 to TrXB3. Thus, it was identified as EH. The activities of ARA 12-LOX and ARA 11-LOX towards ARA, and EH towards HXB3 were 605, 489 and 1403 μmol min⁻¹ mg⁻¹, respectively, which were 55,000, 44,500 and 127,500-fold higher, respectively, than COX activity. COX from M. xanthus was not used for the biosynthesis of lipid mediators because of its low activity.

Establishment of biosynthetic pathways of PUFAs to TrXs. Although 12-LOX pathways for the conversion of PUFAs to TrXs in humans have already been reported, 11-LOX pathways are not yet known. Recombinant E. coli expressing 12-LOX or 11-LOX and EH from M. xanthus synthesized HXs and TrXs during cultivation with ARA for 120 min (Supplementary Fig. 6). However, non-enzymatic products were not found with only ARA and E. coli containing ARA in the absence of the plasmid under the same reaction conditions (Supplementary Fig. 7). E. coli expressing 12-LOX and EH produced 12-HpETE, 12-HETE, HXB3, and TrXB3, while E. coli expressing 11-LOX and EH produced 11-HpETE, 11-HETE, HXD3, and TrXD3. These results suggest that HXs and TrXs can be produced by not only 12-LOX pathways but also new 11-LOX pathways.

To investigate more exactly the biosynthetic pathways for the conversion of ARA to TrXs, the reactions were performed using purified enzymes, including 12-LOX, 11-LOX and EH. We found that 12-LOX and 11-LOX converted ARA to 12-HpETE and 11-HpETE, respectively, and further to HXB3 and HXD3, respectively, which were then converted to TrXB3 and TrXD3, respectively, by EH. 12-HpETE and 11-HpETE were also

Fig. 1 Pathways of polyunsaturated fatty acids converting to trioxilins established according to the genes of Myxococcus xanthus. PUFA polyunsaturated fatty acid, TrXs trioxilins. a Arachidonic acid (ARA) metabolism. b Eicosapentaenoic acid (EPA) metabolism. c Docosahexaenoic acid (DHA) metabolism.

* : These markings indicate the by-products in this pathway.
converted to 12-HETE and 11-HETE by natural reduction, respectively. In particular, 11-LOX produced two types of HXS, HXD3 and HXE3. Thus, the 11-LOX and 12-LOX pathways for the conversion of ARA to TrXs were identified (Fig. 1a). We also established the pathways of other eicosanoids, EPA (Fig. 1b) and docosahexaenoic acid (DHA; C22:6), to TrXs using the purified enzymes (Fig. 1c). 12-LOX and 11-LOX converted EPA to 12-hydroperoxypentadecanoic acid (HpEPE) and 11-HpEPE, respectively. 11-LOX did not convert 11-HpETE, whereas 12-LOX converted 12-HpETE to HXD3, which was converted to TrXB4 by EH. 11-LOX and 12-LOX catalysed the same reaction of DHA to 14-hydroperoxydocosahexaenoic acid (HpDoHE). However, only 12-LOX showed epoxidation activity for 14-HpDoHE to HXB5, because 11-LOX activity was significantly lower than 12-LOX activity. HXB5 was converted to TrXB5 by EH.

Identification of all compounds in the pathways. Compounds of HXA series (HXA3, HXA4 and HXA5) and TrXA series (TrXA3, TrXA4 and TrXA5) have already been identified. Although HXB and TrXB series have been reported, their chemical structures have not been identified by nuclear magnetic resonance (NMR). The chemical structures of all compounds involved in the established biosynthetic pathways were suggested by LC-MS/MS analysis (Supplementary Figs. 8–10). The suggested compounds HXB3, HXB4, HXB5, HXD3, TrXB3, TrXB4, TrXB5, TrXD3 and TrXE3 were purified by prep-HPLC (Supplementary Fig. 11). Only S-form of 12-HpETE has been used to convert to HX in nature34. The 12-HETE and 11-HETE products of M. xanthus LOXs were also S-forms (Supplementary Fig. 12). The stereoselectivity of HXSs and TrXSs was suggested and the chemical structures were accurately determined except for TrXE5 using NMR analysis (Supplementary Tables 6–14 and Supplementary Figs. 13–57). The determination of the structure of TrXE5 was difficult because the amount produced was very small. Therefore, we just suggested TrXE5 structure (Supplementary Fig. 53). HXB3, HXD3, HXE3, TrXB3, TrXD3 and TrXE3 were identified as new compounds, and HXB3, HXB4, HXB5, TrXB3, TrXB4 and TrXB5 were first identified by NMR. The detailed explanation for identification of all compounds in the present study was included in Supplementary Notes and Supplementary Methods.

Biotransformation of PUFAs to HXS and TrXSs. The time-course reactions for the production of HXSs and TrXSs were performed with 1 mM PUFA or HPFA by recombinant E. coli. E. coli expressing 12-LOX converted 1 mM of ARA, EPA and DHA to 0.68 mM HXB3, 0.61 mM HXD3 and 0.50 mM HXE3, respectively, in 90 min, with molar conversions of 68%, 61% and 50%, respectively (Fig. 2); converted 1 mM of 12-HpETE, 12-HpEPE and 14-HpDoHE as intermediates to 0.76 mM HXB3, 0.51 mM HXD3 and 0.53 mM HXE3, respectively, in 60 min (Supplementary Fig. 58); and produced 2.15 mM HXB3 from 6 mM ARA after 60 min, with a conversion of 36% (Supplementary Fig. 59). E. coli expressing 11-LOX converted 1 mM ARA to 0.15 mM HXD3 in 90 min and converted 1 mM 11-HpETE to 0.27 mM HXD3 in 60 min (Fig. 2d and Supplementary Fig. 58d). E. coli co-expressing 12-LOX and EH converted 1 mM of ARA, EPA and DHA to 0.23 mM TrXB3, 0.19 mM TrXB4 and 0.14 mM TrXB5, respectively, in 120 min (Fig. 2e–g), and E. coli co-expressing 11-LOX and EH converted 1 mM ARA to 0.13 mM TrXD3 in 120 min (Fig. 2h).

Determination on the transcriptional activity of PPARγ. PPARγ, a type II nuclear receptor, regulates fatty acid storage and glucose metabolism. Its agonists have been used in the treatment of hyperlipidaemia and hyperglycaemia. The effects of HETEs, HXSs and TrXSs on the transcriptional activity of PPARγ were investigated to find PPARγ agonists. The effects of HXB3, HXB4 and HXD3 on the transcriptional activity of PPARγ were similar to those of their corresponding TrXSs. HXB3 and HXD3 (TrXB3 and TrXD3) increased the transcriptional activity of PPARγ with increasing concentrations, although the increasing degrees of the
**Fig. 3** Transcriptional activity of peroxisome proliferator-activated receptor gamma for hepoxilins and trioxilins. HEK-293 cells were cultured in a 24-well plate (1.0×10⁵ cells per well). After 24 h, cells were transfected with plasmids expressing peroxisome proliferator-activated receptor gamma (PPARγ), PPAR response element (PPRE)×3-thymidine kinase-luciferase reporter constructs, and the Renilla luciferase control vector pRL. After another 24 h, cells were treated with HXs, TrXs and/or 3 μM troglitazone (TRO) for 24 h. Cells were harvested, and the transcriptional activity of PPARγ was determined by a luciferase assay. HXs hepoxilins, TrXs trioxilins. a HXB3. b HXB4. c HXB5. d HXD3. e TrXB3. f TrXB4. g TrXB5. h TrXD3. Data represent the means of three separate experiments, and error bars represent the standard deviations. *p-value are based on t-test. *p < 0.05, **p < 0.01. n.s. indicates not significant.
transcriptional activity were less than that of troglitazone (TRO), an antidiabetic and anti-inflammatory drug (Fig. 3). These compounds additively increased the transcriptional activity of PPARγ when TRO was supplemented. The increasing degree of the transcriptional activity for HXB4 was similar to that by TRO (Fig. 3b). HXB3, TrXB3 and TrXB5 did not affect the activity (Fig. 3c, f, g). However, HXB5 decreased the transcriptional activity of PPARγ when TRO was supplemented. 11-HETE and 12-HETE as intermediate products showed effects similar to those of HXB3 and HXB4, respectively, on the transcriptional activity of PPARγ (Supplementary Fig. 60).

Discussion
Lipid mediators regulate the immune and inflammatory responses of humans. LOXs are key enzymes involved in the formation of lipid mediators in animals and humans35. Recently, LOXs have been discovered in diverse organisms such as coral36, fungi37, and bacteria38. Among them, bacterial LOXs have only been found in the cyanobacteria Nostoc sp.39 and Acaryochloris marina40, and the prokaryote Pantoea agglomerans41 and Burkholderia thailandensis38 (Supplementary Fig. 61 and Supplementary Table 15). M. xanthus is also a proteobacterium. Interestingly, LOXs in other prokaryote bacteria have regiospecificity on C15 of ARA, whereas LOXs in M. xanthus has regiospecificity on C12 and C11 of ARA. Bacteria containing LOXs may produce lipid mediators. However, the formation of HXs and TrXs by bacterial LOXs has not been reported thus far.

In humans, HX is formed from ARA via 12-HpETE by dioxygenation and epoxidation reactions of 12-LOX42. HXA3, HXB3, HXA4 and HXB4, HXA4 and HXB3, and HXA3 and HXB3 are formed from ARA, EPA and DHA, respectively. Then, these HXs are converted to TrXA3, TrXB3, TrXA4, TrXB4 and TrXA5, in vivo by EH, respectively (Supplementary Fig. 62). 15-LOX also converts ARA, EPA and DHA to other lipid mediators such as LTs, LXs, RVs and PTs. However, recombinant cells in the present study converted 1 mM ARA, EPA and DHA to other lipid mediators such as LTs, LXs, RVs and PTs. We identified ten types of lipid mediators produced by M. xanthus enzymes was investigated (Fig. 3 and Supplementary Fig. 60) because fatty acids have played a role as modulators of PPARγ57. HXB3, HXB4, HXD3, TrXB3, TrXD3, and 11-HETE increased the transcriptional activity of PPARγ. These compounds were docked to LBD at human PPARγ using molecular models (Supplementary Fig. 63). Rosiglitazone, known as a full agonist, was interacted with Tyr473 (H12) (Supplementary Fig. 64a). HXB3, HXB4, HXD3, TrXB3, TrXD3 and 11-HETE were interacted with Ser289 (H3) and His323 (H5) (Supplementary Fig. 64b), suggesting that these compounds are partial agonists. However, this study does not demonstrate direct agonist activities of these products.

In conclusion, we discovered eukaryotic-like lipid mediator-biosynthetic enzymes, including 12-LOX, 11-LOX, COX and EH, from M. xanthus, a newly discovered bacterium that could produce HXs, TrXs and PGs. Owing to the high activities of microbial enzymes, we succeeded in the biotransformation of PUFAs to eight types of HXs and TrXs using recombinant cells expressing 12-LOX or 11-LOX with or without EH. The same strategy can be applied to the biotransformation processes of PUFAs to other lipid mediators such as LTs, LXs, RVs and PTs. We identified ten types of lipid mediators including six new types based on NMR analysis. We found that six types of lipid mediators were potential partial agonists of PPARγ. The identification of partial agonists of PPARγ has been required for development of the antidiabetic and anti-inflammatory drugs with reduced side effects. Thus, outcomes of this study may hold potential to stimulate physiological studies and drug development on lipid mediators.

Methods
Materials. The PUFA standards ARA, EPA and DHA, and the HFA standards 11-HETE, 12-HETE, 12-HEPE and 14-HDHE were purchased from Sigma (St. Louis, MO, USA) and Cayman Chemical (Ann Arbor, MI, USA), respectively. To prepare the lipid mediator standards HXB3, HXB4, HXB5, HXD3, TrXB3, TrXB4, TrXD3, and TrXD5, the reactions were performed at 30 °C in 50 mM 4-(2-hydroxyethyl)piperazinyl-1-propanesulfonic acid (EPPS) buffer (pH 8.5) containing 100 μM L-1 of ARA, EPA or DHA as a substrate, and 14.4 μL1 recombinant cells with shaking at 200 r.p.m. for 2 h. The reaction solution was extracted with an equal volume of ethyl acetate, and the solvent was removed using a rotary evaporator. The solvent-free solution was applied to a Prep-HPLC (Agilent 1260, Santa Clara, CA, USA) equipped with a Nucleosil C18 column (10×250 mm, 5-μm particle size; Phenomenex, Torrance, CA, USA) and a fraction collector. The column was eluted at 30 °C with a flow rate of 6 mL min−1, and the product fractions were collected by monitoring at 202 nm of absorbance. The collected samples showed >99% purity (Supplementary Fig. 11), and were used as the lipid mediator standards after identification by LC-MS/MS and NMR.
In silico docking studies. Metabolites were docked in the LBD of crystal structure of human PPARγ (PDB 2PGP.pdb) using the CDOCKER module of Discovery Studio 4.1 (Accelrys, San Diego, CA, USA). Substrate poses were refined by full-potential final minimization, and candidate poses were created using random rigid-body rotations followed by simulated annealing. The structure of protein–ligand complexes was subjected to energy minimization using the CHARMM force field in DS 4.5. The substrate orientation with the lowest interaction energy was selected for the subsequent rounds of docking. Candidate poses were created based on random rigid-body rotations followed by simulated annealing. The energy-docked conformation of the substrate was retrieved for post-docking analysis using the CDOCKER module.

Statistical analyses. The means and standard errors for all experiments were quantitatively calculated with t-test to evaluate significant differences between control and experimental groups. A p-value of <0.05, calculated using t-test, was considered statistically significant.

Data availability. Plasmins used in this article were deposited in Addgene. They were assigned to pET28a-mxLOX1 (ID 104975), pET28a-mxLOX2 (ID 104976), pET28a-mxEH (ID 104977), pACYCduet-mxLOX1-EH (ID 104978) and pACYCduet-mxLOX2-EH (ID 104979). All data that support the findings of this study are included in this article and in Supplementary Information. They are available from the corresponding author upon request.

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**Author contributions**

D.-K.O. supervised this study. J.-U.A. performed most experiments, including cloning, 
protein expression, enzyme purification, and enzyme and cell reactions. Y.-S.S. carried 
out the transcriptional activity assay, and Y.-J.K. analysed the NMR data. J.-U.A., Y.-S.S., 
K.-R.K., D.-Y.Y., and D.-K.O. designed the experiments and contributed to data analysis. 
J.-U.A. gathered and organised the results. J.-U.A., Y.-S.S., K.-R.K., Y.-J.K., D.-Y.Y., and 
D.-K.O. wrote the manuscript.

**Additional information**

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