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ENZYMOLOGY AND PROTEIN ENGINEERING

Comparison of Biochemical Properties of the Original and Newly Identified Oleate Hydratases from *Stenotrophomonas maltophilia*

Woo-Ri Kang,[a] Min-Ju Seo,[a] Kyung-Chul Shin,[a] Jin-Byung Park,[b] Deok-Kun Oh[a]

Department of Bioscience and Biotechnology, Konkuk University, Seoul, Republic of Korea[a]; Department of Food Science and Engineering, Ewha Womans University, Seoul, Republic of Korea[b]

ABSTRACT Oleate hydratases (OhyAs) catalyze the conversion of unsaturated fatty acids to 10-hydroxy fatty acids, which are used as precursors of important industrial compounds, including lactones and ω-hydroxycarboxylic and α,ω-dicarboxylic acids. The genes encoding OhyA and a putative fatty acid hydratase in *Stenotrophomonas maltophilia* were identified by genomic analysis. The putative fatty acid hydratase was purified and identified as an oleate hydratase (OhyA2) based on its substrate specificity. The activity of OhyA2 as a holoenzyme was not affected by adding cofactors, whereas the activity of the original oleate hydratase (OhyA1) showed an increase. Thus, all characterized OhyAs were categorized as either OhyA1 or OhyA2 based on the activities of holoenzymes upon adding cofactors, which were determined by the type of the fourth conserved amino acid of flavin adenine dinucleotide (FAD)-binding motif. The hydration activities of *S. maltophilia* OhyA2 toward unsaturated fatty acids, including oleic acid, palmitoleic acid, linoleic acid, α-linolenic acid, and γ-linolenic acid, were greater than those of OhyA1. Moreover, the specific activity of *S. maltophilia* OhyA2 toward unsaturated fatty acids, with the exception of γ-linolenic acid, was the highest among all reported OhyAs.

IMPORTANCE All characterized OhyAs were categorized as OhyA1s or OhyA2s based on the different properties of the reported and newly identified holo-OhyAs in *S. maltophilia* upon the addition of cofactors. OhyA2s showed higher activities toward polyunsaturated fatty acids (PUFAs), including linoleic acid, α-linolenic acid, and γ-linolenic acid, than those of OhyA1s. This suggests that OhyA2s can be used more effectively to convert plant oils to 10-hydroxy fatty acids because plant oils contain not only oleic acid but also PUFAs. The hydration activity of the newly identified OhyA2 from *S. maltophilia* toward oleic acid was the highest among the activity levels reported so far. Therefore, this enzyme is an efficient biocatalyst for the conversion of plant oils to 10-hydroxy fatty acids, which can be further converted to important industrial materials.

KEYWORDS *Stenotrophomonas maltophilia*, enzyme characterization, oleate hydratase

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Address correspondence to Deok-Kun Oh, deokkun@konkuk.ac.kr.

Oleate hydratase (OhyA) converts unsaturated fatty acids to 10-hydroxy fatty acids, which are then converted to γ-lactones by the yeasts *Candida boidinii* and *Waltomyces lipofer* (1, 2). γ-Lactones are flavor compounds used in food and cosmetics (3). Multistep enzymatic reactions involving OhyA, alcohol dehydrogenase, Baeyer-Villiger monooxygenases, and esterase convert unsaturated fatty acids to ω-hydroxycarboxylic and α,ω-dicarboxylic acids (4), which are used in the preparation of resins, nylons, plastics, and lubricants (5). An effective OhyA is necessary for the efficient production of γ-lactones, ω-hydroxycarboxylic acids, and α,ω-dicarboxylic acids.

OhyAs catalyze regioselective hydration of 9-*cis* double bonds (C=C) in unsaturated fatty acids, for example, oleic acid, using water to produce 10-hydroxy fatty acids, like 10-hydroxystearic acid (10-HSA). The biochemical properties of OhyAs from *Elizabethkingia meningoseptica* (6, 7), *Bifidobacterium breve* (8, 9), *Streptococcus pyogenes* (10), *Lysinibacillus fusiformis* (11), *Macrococcus caseolyticus* (12), *Stenotrophomonas maltophilia* (13), and *Stenotrophomonas nitritireducens* (14) have been characterized to date. All OhyAs contain flavin adenine dinucleotide (FAD)-binding motifs. When FAD was not bound to OhyA, its catalytic activity was abolished (12) or significantly reduced (7). Thus, OhyAs are FAD-dependent enzymes belonging to the myosin cross-reactive antigen (MCRA) protein family, and they are widely distributed in bacteria, especially in pathogenic and intestinal bacteria. Many bacteria are sensitive to fatty acids. Unsaturated fatty acids, such as oleic acid, linoleic acid, and linolenic acid, are much more toxic than saturated fatty acids in bacteria (15, 16). Unsaturated fatty acids disrupt the membrane structure in cells because of their distorted structures (17) and inhibit fatty acid-synthesizing enzymes (18). For surviving in unsaturated fatty acid-rich environments, as a detoxification mechanism, bacteria harboring MCRA proteins hydrate unsaturated fatty acids and convert them to hydroxy fatty acids, which are degraded in cells (10).

OhyA from *S. maltophilia* showed the highest specific activity for oleic acid among the characterized OhyAs. However, it exhibited low activity for polyunsaturated fatty acids (PUFAs), including linoleic acid, α-linolenic acid, and γ-linolenic acid. An OhyA from *S. nitritireducens* has recently been reported to possess high activity toward PUFAs. The primary components of plant oils are oleic acid and PUFAs. To increase the conversion of plant oils to 10-hydroxy fatty acids, an OhyA with higher hydration activity for oleic acid and PUFAs is required.

In this study, we identified a putative fatty acid hydratase gene in *S. maltophilia* using genomic analysis. The biochemical properties of this enzyme, including molecular weight, substrate specificity, optimal pH, temperature, and solvent, cofactor effect, and unsaturated fatty acid biotransformation, were investigated and compared with those of the original OhyA from *S. maltophilia*. All characterized OhyAs were categorized as OhyA1 or OhyA2 based on the FAD-binding motif differences between these two enzymes in *S. maltophilia*. The role of residues of the FAD-binding motif on enzyme stability and different properties of holo-OhyAs upon the addition of cofactors were also investigated.

RESULTS

Purification and molecular weight determination of the original and putative fatty acid hydratases of *S. maltophilia*. Partial peptide sequences of the purified OhyA from *S. nitritireducens* (AVNDNDPN and ITAQF) were reported previously (14). We discovered another putative fatty acid hydratase from *S. maltophilia* by genomic analysis based on these available partial peptide sequences. The gene (1,983 bp) encoding the putative fatty acid hydratase of *S. maltophilia* was synthesized based on the available sequence (GenBank accession no. WP_017356052), and the synthesized gene was cloned and expressed in the *Escherichia coli* strain ER2566. The gene encoding the original OhyA of *S. maltophilia* was 1,770 bp (13). The enzyme was then expressed and purified as a soluble protein from the supernatant of lysed cells using HisTrap affinity chromatography. The specific level of the purified enzyme was determined to be 5.3 U/mg, which was 10-fold higher than that of the supernatant. The yield of the purified enzyme from the supernatant was 20.3%.

The subunit molecular mass of the putative fatty acid hydratase from *S. maltophilia*, as determined by SDS-PAGE, was approximately 75 kDa. This subunit molecular weight is almost consistent with the molecular weight calculated from the 661 amino acids with the hexahistidine tag (75,705 Da). The total molecular mass of the purified protein was 152 kDa, indicating that the protein exists as a dimer of 76 kDa. The original OhyA was a dimer of 68 kDa (see Fig. S1 in the supplemental material).

TABLE 1 Specific activities of bacterial OhyAs toward unsaturated fatty acids[a]

Type	Source	Specific activity (means ± SD) (μmol/min/mg)					Reference or source
		Oleic acid	Palmitoleic acid	Linoleic acid	α-Linolenic acid	γ-Linolenic acid	
OhyA1	*L. fusiformis*	1.58 ± 0.06	0.55 ± 0.03	0.11 ± 0.01	0.02 ± 0.00	0.16 ± 0.01	11
	M. caseolyticus	0.60 ± 0.04	0.35 ± 0.08	0.02 ± 0.01	0.01 ± 0.00	0.11 ± 0.02	14
	S. maltophilia	2.69 ± 0.02	1.02 ± 0.05	0.85 ± 0.06	0.26 ± 0.01	0.37 ± 0.08	This study
OhyA2	*S. nitritireducens*	1.70 ± 0.16	0.57 ± 0.08	1.51 ± 0.09	1.02 ± 0.02	1.00 ± 0.01	14
	E. meningoseptica[b]	0.21 ± 0.02	NR	NR	NR	NR	7
	S. maltophilia	5.36 ± 0.83	2.21 ± 0.01	1.67 ± 0.13	1.05 ± 0.12	0.78 ± 0.07	This study

[a]Data represent the results from three experiments. The reactions were performed in 50 mM citrate-phosphate buffer (pH 6.0) containing 0.01 mg/ml OhyA1 or 0.005 mg/ml OhyA2, 0.5 mM unsaturated fatty acid, and 5% (vol/vol) DMSO at 35°C for 10 min. NR, not reported.
[b]The reaction was performed in 50 mM HEPES (pH 6.0) containing 0.05 mg/ml enzyme, 2 mM oleic acid, and 2% ethanol at 25°C for 2 min.

Substrate specificities of the original and putative fatty acid hydratases of *S. maltophilia*. The specificity of the putative fatty acid hydratase (OhyA2) of *S. maltophilia* toward the substrates, including oleic acid, palmitoleic acid, linoleic acid, α-linolenic acid, and γ-linolenic acid, was evaluated, and the unsaturated fatty acids were converted to 10-HSA, 10-hydroxyhexanoic acid, 10-hydroxy-12(*Z*)-octadecenoic acid (10-HOD), 10-hydroxy-12,15(*Z*,*Z*)-octadecadienoic acid, and 10-hydroxy-6,12(*Z*,*Z*)-octadecadienoic acid, respectively, by the enzyme. The specific activity and catalytic efficiency (k_{cat}/K_m) of the putative fatty acid hydratase were, in descending order: oleic acid, palmitoleic acid, linoleic acid, α-linolenic acid, and γ-linolenic acid (Tables 1 and 2). We identified the putative fatty acid hydratase as an OhyA because the enzyme showed the highest catalytic efficiency for oleic acid among the unsaturated fatty acids tested. The new identified OhyA showed higher activity for unsaturated fatty acids than the reported OhyA in *S. maltophilia*. Thus, we designated the original and putative OhyAs OhyA1 and OhyA2, respectively.

Effects of temperature, pH, and solvent on the activities of OhyA1 and OhyA2 from *S. maltophilia*. The maximal activities of both OhyA1 and OhyA2 from *S. maltophilia* toward oleic acid were observed at 35°C and pH 6.0 (Fig. S2). Solvents were added in the reaction solution to increase the substrate solubility. The maximal

TABLE 2 Kinetic parameters of bacterial OhyAs toward unsaturated fatty acids

Organism by OhyA type	Kinetic parameter	Oleic acid	Palmitoleic acid	Linoleic acid	α-Linolenic acid	γ-Linolenic acid	Reference or source
OhyA1							
L. fusiformis	K_m (μM)	540	760	580	NR[a]	770	11
	k_{cat} (min^{-1})	850	430	80	NR	130	
	k_{cat}/K_m ($min^{-1} \cdot \mu M^{-1}$)	1.6	0.56	0.14	NR	0.17	
M. caseolyticus	K_m (μM)	340	570	390	320	590	12
	k_{cat} (min^{-1})	470	230	8.1	7.9	160	
	k_{cat}/K_m ($min^{-1} \cdot \mu M^{-1}$)	1.4	0.39	0.02	0.02	0.27	
S. pyogenes	K_m (μM)	49.0	NR	63.0	NR	NR	10
	k_{cat} (min^{-1})	101	NR	67.0	NR	NR	
	k_{cat}/K_m ($min^{-1} \cdot \mu M^{-1}$)	2.1	NR	1.1	NR	NR	
S. maltophilia	K_m (μM)	38.9	43.0	64.5	63.5	98.2	This study
	k_{cat} (min^{-1})	118	68.4	39.7	18.1	11.6	
	k_{cat}/K_m ($min^{-1} \cdot \mu M^{-1}$)	3.1	1.6	0.62	0.29	0.12	
OhyA2							
E. meningoseptica	K_m (μM)	110	NR	NR	NR	NR	7
	k_{cat} (min^{-1})	1.2	NR	NR	NR	NR	
	k_{cat}/K_m ($min^{-1} \cdot \mu M^{-1}$)	0.01	NR	NR	NR	NR	
S. nitritireducens	K_m (μM)	21.5	134	25.5	36.6	40.9	14
	k_{cat} (min^{-1})	78.2	31.0	62.7	60.8	58.9	
	k_{cat}/K_m ($min^{-1} \cdot \mu M^{-1}$)	3.6	0.2	2.5	1.7	1.4	
S. maltophilia	K_m (μM)	20.7	43.0	50.3	52.5	84.7	This study
	k_{cat} (min^{-1})	179	147	119	101	54.4	
	k_{cat}/K_m ($min^{-1} \cdot \mu M^{-1}$)	8.6	3.4	2.4	1.9	0.64	

[a]NR, not reported.

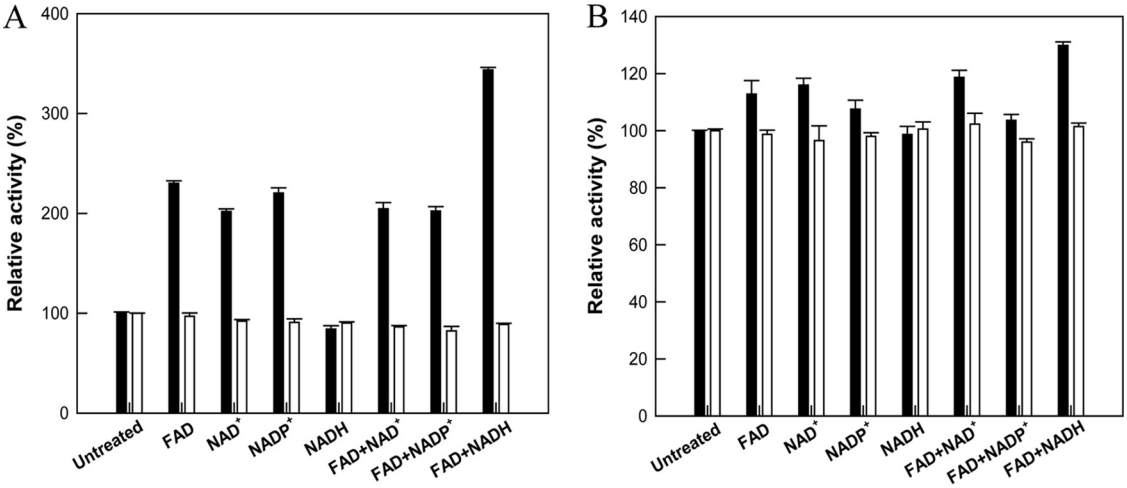

FIG 1 Effect of the addition of cofactors on the hydration activities of OhyA1 and OhyA2 from *S. maltophilia* toward oleic acid (A) and linoleic acid (B). The reactions were performed with the cofactors in 50 mM citrate-phosphate buffer (pH 6.0) containing 0.01 mg/ml OhyA1 (■) or 0.005 mg/ml OhyA2 (□), 0.5 mM oleic acid or linoleic acid, and 5% (vol/vol) DMSO at 35°C for 10 min. The cofactors used were 0.1 mM FAD, 5 mM NAD^+, 5 mM $NADP^+$, 5 mM NADH, and 0.1 mM FAD supplemented with 5 mM NAD^+, 5 mM $NADP^+$, and 5 mM NADH. Untreated indicates that the reaction was performed without the addition of cofactors. The relative activity of untreated was 100%. Data represent the means ± standard deviations of the results from three independent experiments.

activities of both OhyA1 and OhyA2 were observed when dimethyl sulfoxide (DMSO) was used as the solvent, at an optimal concentration of 5% (vol/vol) (Fig. S3). DMSO has been used for increasing the activity of diol synthase that converts unsaturated fatty acids to dihydroxy fatty acids (19).

Effect of cofactor on the activities of OhyA1 and OhyA2 from *S. maltophilia*. The absorption spectra of apo- and holo-OhyAs were measured for the detection of FAD (Fig. S4). The absorption spectra of halo-OhyAs and FAD showed peaks at 450 nm, whereas those of apo-OhyAs exhibited no peaks. Thus, the tested apo-OhyAs were not bound to the cofactor. The activity of apo-OhyA1 (enzyme without cofactors) was almost abolished, whereas the activity of apo-OhyA2 reduced to 7% (see Table S1 in the supplemental material). When FAD was added to apo-OhyA1 and apo-OhyA2, their activities were restored to 9% and 32%, respectively. Thus, the OhyAs were shown to be cofactor-dependent enzymes. The effect of the addition of various cofactors on the activities of holo-OhyA1 and holo-OhyA2 (enzymes with cofactor) toward oleic acid and linoleic acid was examined using FAD, NAD^+, $NADP^+$, NADH, and FAD supplemented with an additional cofactor, such as NAD^+, $NADP^+$, or NADH. With the exception of NADH, the activity of holo-OhyA1 toward oleic acid was 2.0- to 3.4-fold higher when supplemented with cofactors than that without the addition of cofactors (Fig. 1A). When FAD and NADH coexisted, the hydration activity of holo-OhyA1 was improved significantly. When holo-OhyA1 was treated with $FADH_2$, the hydration activity was 2.4-fold higher than that without the addition of cofactor (Fig. S5). When FAD and dithiothreitol (DTT) coexisted, the hydration activity of holo-OhyA1 was almost the same as that of FAD supplemented with NADH. The increased hydration activity of the linoleate hydratase was due to the formation of reduced FAD ($FADH_2$), the active cofactor, through the reduction of FAD by NADH. The activities of holo-OhyA1 toward linoleic acid were 113%, 116%, 119%, and 130% by FAD, NAD^+, and FAD supplemented with NAD^+ and NADH, respectively (Fig. 1B). The activity of holo-OhyA2 for oleic acid and linoleic acid, however, did not change significantly upon the addition of cofactors.

OhyAs contain the FAD-binding motifs $GXGXXS_{(A/G)}X_{15}E_{(D)}X_5E$ with five conserved residues, where X denotes any amino acid (12). However, the fourth conserved amino acids of OhyA1 and OhyA2 from *S. maltophilia* and OhyA2 from *S. nitritireducens* differed as Glu at position 50, Ala at position 86, and Gln at position 80, respectively. Site-directed mutagenesis was performed to obtain the E50A variant OhyA1 and A86E variant OhyA2 from *S. maltophilia* and the Q80E variant OhyA2 from *S. nitritireducens*

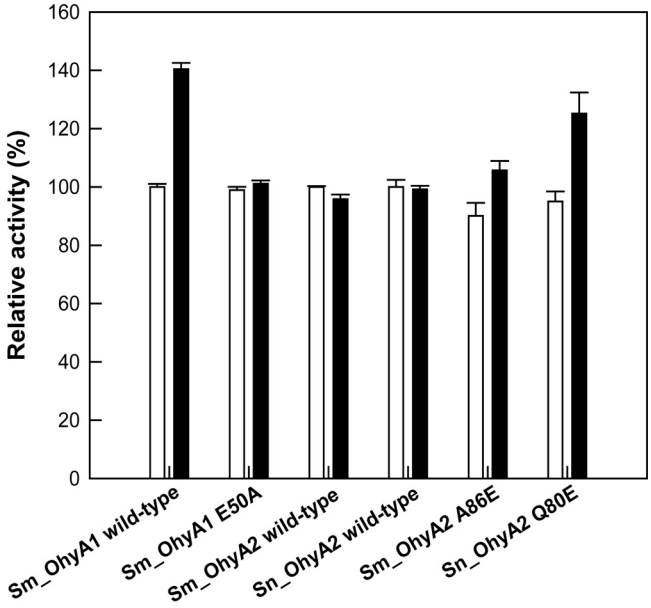

FIG 2 Effect of the addition of FAD on the hydration activities of the wild-type and variants of OhyA1 from *S. maltophilia* (Sm_OhyA1) or OhyA2 from *S. maltophilia* (Sm_OhyA2) and *S. nitritireducens* (Sn_OhyA2). The reactions were performed in 50 mM citrate-phosphate buffer (pH 6.0) containing 0.01 mg/ml Sm_OhyA1 or 0.005 mg/ml Sm_OhyA2 or 50 mM citrate-phosphate buffer (pH 6.5) containing 0.05 mg/ml Sn_OhyA2, 0.5 mM oleic acid, and 5% (vol/vol) DMSO at 35°C for 10 min. The reactions were performed without the addition of cofactors (□) and with 0.1 mM FAD (■). The relative activity of the wild-type OhyA1 or OhyA2 was 100%. Data represent the means ± standard deviations of the results from three independent experiments.

(Fig. 2). The activity of the wild-type OhyA1 from *S. maltophilia* increased significantly by adding FAD, whereas the activity of the E50A variant OhyA1 was not affected by adding FAD. The E50A variant OhyA1 became a cofactor-independent enzyme, like OhyA2. The activity of the wild-type OhyA2 from *S. maltophilia* was not affected by adding FAD, whereas the activity of the A86E variant OhyA2 increased by adding FAD. However, the increased amount was insignificant. To better investigate the cofactor dependence, we performed additional experiments with the wild-type and variant OhyA2s from *S. nitritireducens*. The wild-type OhyA2 from *S. nitritireducens* was not affected by adding FAD, whereas the Q80E variant OhyA2 distinctly increased by adding FAD. The Q80E variant OhyA2 from *S. nitritireducens* became a cofactor-dependent enzyme, like OhyA1, because the activity of the Q80E variant OhyA2 with the addition of FAD was 1.3-fold higher than that without FAD. Thus, the Q80E variant OhyA2 also became a cofactor-dependent enzyme, like OhyA1.

Biotransformations of unsaturated fatty acids to 10-hydroxy fatty acids by OhyA1 and OhyA2 from *S. maltophilia*. The biotransformations by OhyA1 and OhyA2 from *S. maltophilia* were performed at 35°C (pH 6.0) and 1.0 mM oleic acid or linoleic acid in the presence of 5% (vol/vol) DMSO for 120 min. The conversion rates of oleic acid to 10-HSA by OhyA1 and OhyA2 after 2 h were 75% and 86% (mol/mol), respectively (Fig. 3A). The conversion rates of linoleic acid to 10-HOD by OhyA1 and OhyA2 after 2 h were 65% and 77% (mol/mol), respectively (Fig. 3B).

DISCUSSION

The enzyme involved in the conversion of oleic acid to 10-HSA was first identified in *E. meningoseptica* and was named OhyA because of its highest activity being toward oleic acid among unsaturated fatty acids (6). The biochemical properties of OhyAs from other bacteria, including *S. pyogenes*, *M. caseolyticus* (12), *L. fusiformis* (11), *S. maltophilia* (13), and *B. breve* (8, 9), were subsequently characterized. These OhyAs showed low activity toward PUFAs. An OhyA from *S. nitritireducens* with high activity toward PUFAs was recently reported (14). The newly identified OhyA (OhyA2) from *S. maltophilia*

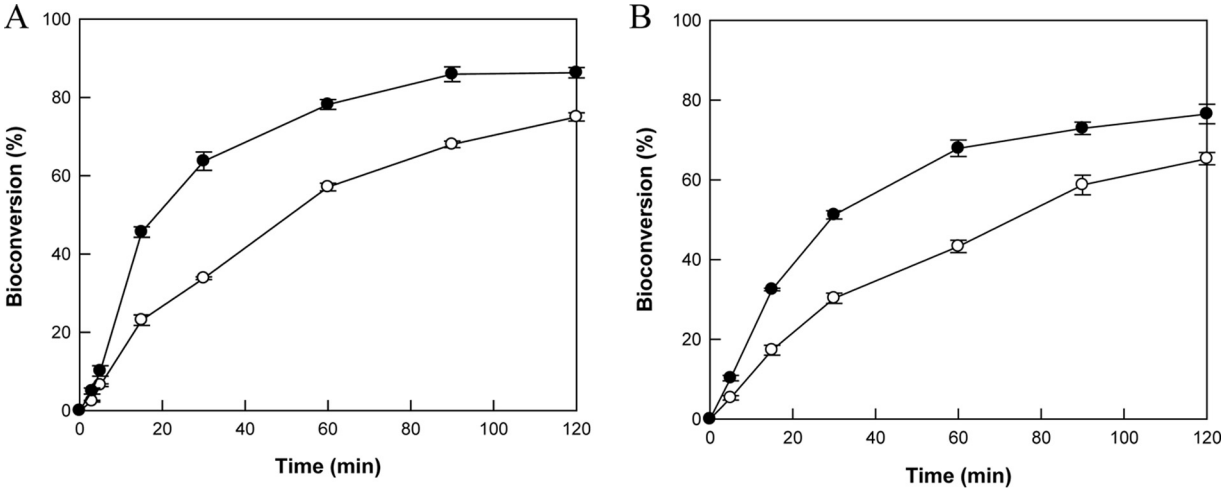

FIG 3 Biotransformations of unsaturated fatty acids to 10-hydroxy fatty acids by OhyA1 and OhyA2 from *S. maltophilia*. (A) Biotransformations of oleic acid to 10-HSA by OhyA1 and OhyA2. (B) Biotransformations of linoleic acid to 10-HOD by OhyA1 and OhyA2. The reactions were performed in 50 mM citrate-phosphate buffer (pH 6.0) containing 0.01 mg/ml OhyA1 (○) or 0.005 mg/ml OhyA2 (●), 1 mM oleic acid or linoleic acid, and 5% (vol/vol) DMSO at 35°C for 120 min. Data represent the means ± standard deviations of the results from three independent experiments.

showed higher hydration activity toward PUFAs than the original OhyA (OhyA1) (Table 1).

The activities of apo-OhyA1 and apo-OhyA2 from *S. maltophilia* toward oleic acid were increased upon the addition of FAD. The same results were reported in OhyAs from *S. pyogenes* (10) and *M. caseolyticus* (12). Thus, apo-OhyAs were shown to be cofactor-dependent enzymes. In contrast, the activity of holo-OhyA1 from *S. maltophilia* was increased by adding FAD, whereas the activity of holo-OhyA2 was not affected (Fig. 1). The activities of other OhyA1-type holoenzymes were also increased by adding FAD, whereas other OhyA2-type holoenzymes were not affected (Table S2). Thus, the influence of cofactor on the activities of holoenzymes is a critical factor for distinguishing OhyAs. Based on the different properties of holoenzymes for cofactor, all characterized OhyAs were classified into one of two distinct types, OhyA1 and OhyA2.

All members of the glutathione reductase family, including OhyAs, contain the FAD-binding motifs $GXGXXS_{(A/G)}X_{15}E_{(D)}X_5E$, with five conserved residues (12). The fourth conserved amino acid of the OhyA1 type is $E_{(D)}$, whereas this residue is not conserved in the OhyA2 type (Fig. 4A). Thus, the fourth conserved amino acid may be a critical factor for distinguishing OhyA types. According to the phylogenetic tree generated based on the FAD-binding motifs, OhyAs are distinctly classified into two types, OhyA1 and OhyA2 (Fig. 4B). OhyAs from *L. fusiformis*, *M. caseolyticus*, *S. pyogenes*, and *B. breve* and the original OhyA from *S. maltophilia* belonged to the OhyA1 type, whereas OhyAs from *S. nitritireducens* and *E. meningoseptica* and the newly identified OhyA from *S. maltophilia* belonged to the OhyA2 type. The amino acid sequence of the FAD-binding motif of OhyA2 from *S. maltophilia* was the closest to that of OhyA2 from *E. meningoseptica*. The amino acid sequence of OhyA2 from *S. maltophilia* exhibited the highest identity (67.6%) to that of *S. nitritireducens* (Table S3). The sequence identities within the OhyA1 or OhyA2 type were relatively high, ranging from 47.9% to 67.6%, or 54.9% to 66.9%, respectively. However, those between the OhyA1 and OhyA2 types were relatively low, ranging from 34.6% to 39.7%. Although the additional N-terminal amino acid sequences were only evident in members of the OhyA2 type, the active-site residues were completely conserved in all characterized OhyAs (Fig. S6). Although all characterized OhyAs are present as dimer forms, the total molecular masses of the OhyA2 type ranged from 146 kDa to 152 kDa, while those of the OhyA1 type ranged from 134 kDa to 140 kDa.

Based on the kinetic analysis of OhyA from *S. pyogenes*, FAD was involved in the

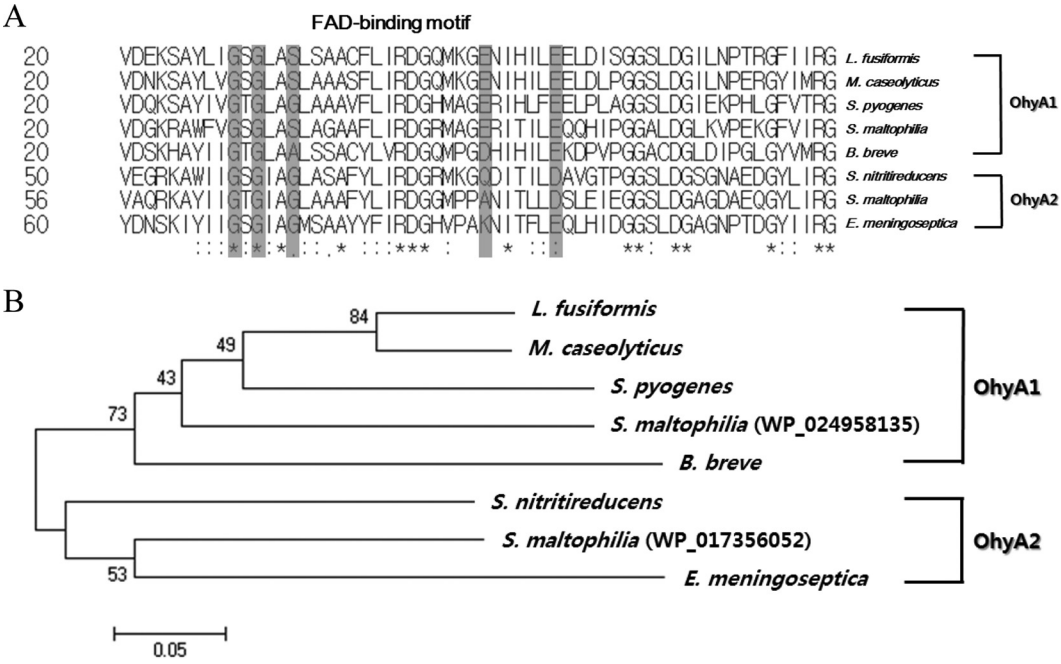

FIG 4 Amino acid sequence alignment and phylogenetic tree of FAD-binding motifs of OhyA1s from *L. fusiformis*, *M. caseolyticus*, *S. pyogenes*, *S. maltophilia* (GenBank accession no. WP_024958135), and *B. breve*, and OhyA2s from *S. nitritireducens*, *S. maltophilia* (GenBank accession no. WP_017356052), and *E. meningoseptica*. (A) Amino acid sequence alignment of the FAD-binding motifs of OhyAs. The FAD-binding residues are highlighted in gray. (B) Phylogenetic tree generated from the alignment of the amino acid sequences of FAD-binding motifs of OhyAs. The MEGA6 program software was used to generate the phylogenetic tree. Asterisks, colons, and periods represent identity, conserved residues, and semiconserved residues, respectively.

stabilization of the enzyme but not directly involved in catalysis (10). The reaction mechanism of OhyA from *E. meningoseptica* proposed that FAD was involved in the correct localization of the substrate and amino acids in the active site of OhyA (7). In the present study, the cofactor effect on the activities of holo-OhyAs and role of the residue of FAD-binding motif on the enzyme stability were investigated. We investigated the improved hydration activity of holo-OhyA1 with FAD and NADH together. The hydration activity of linoleate hydratase from *Lactobacillus plantarum* also increased significantly upon addition of FAD supplemented with NADH (20). Some oxidative enzymes, such as 2-haloacrylate hydratase (21) and 2-hydroxybiphenyl 3-monooxygenase (22), showed similar results for FAD supplemented with NADH. These enzymes are flavin-dependent enzymes that are influenced by NADH. NADH in these enzymes is an electron donor that reduces FAD. When FAD and a reducing agent, such as DTT, coexisted in holo-OhyA1, the increased hydration activity was almost the same as that of FAD supplemented with NADH. Thus, FAD can be reduced by adding NADH because it has reducing power. Reduced FAD ($FADH_2$) is an active cofactor of OhyAs, and the activity of OhyA from *E. meningoseptica* increased significantly upon treatment with reduced FAD (7).

The different fourth conserved amino acids in FAD-binding motifs of OhyA1 and OhyA2 from *S. maltophilia* and OhyA2 from *S. nitritireducens* were exchanged with OhyA2 and OhyA1 from *S. maltophilia* and OhyA1 from *S. maltophilia*, respectively (Fig. 3). The cofactor-dependent OhyA1 became a cofactor-independent enzyme (OhyA2 type) and variant by mutation of E50A, and the cofactor-independent OhyA2 became a cofactor-dependent enzyme (OhyA1 type) by mutations of A86E and Q80E. Therefore, the fourth conserved amino acid was the determinant residue involved in the cofactor dependence or independence of holo-OhyAs. Homology models for FAD-binding motifs of the wild-type and variant OhyAs were constructed with docked FAD (Fig. S7), and the binding energies ($\Delta E_{binding}$) were calculated. In the models, the determinant residue was far away from FAD and catalytic residues, suggesting that the OhyAs were

not directly involved in hydration activity and FAD binding. The $\Delta E_{binding}$ values of the wild-type and E50A variant OhyA1s were −316.5 and −386.9 kcal/mol, respectively, and those of the wild-type and A86E variant OhyA2s were −390.8 and −376.5 kcal/mol, respectively, suggesting that the fourth residue of the FAD-binding motif was involved in the stability. The enzymes with neutral Ala (OhyA2 type) as the fourth conversed residue in the FAD-binding motif were more stable than the enzymes with negatively charged Glu (OhyA1 type). The fourth conserved amino acid in the FAD-binding motifs of OhyA1s was Glu or Asp (negatively charged amino acid), whereas that of OhyA2s was Gln or Ala (neutral amino acid). The different activities of OhyA1 and OhyA2 with cofactors may be because the negatively charged fourth conversed residue in the OhyA1 interacts with FAD, but the neutral residue in the OhyA2 does not interact with FAD.

The specific activities of OhyA2 from *S. maltophilia* toward oleic acid, palmitoleic acid, linoleic acid, α-linolenic acid, and γ-linolenic acid were 2.0-, 2.2-, 2.0-, 4.0-, and 2.1-fold higher than those of OhyA1 from *S. maltophilia*, respectively (Table 1). Under the optimized conditions, OhyA2 from *S. maltophilia* converted 1.0 mM oleic acid and 1.0 mM linoleic acid to 0.86 mM 10-HSA and 0.77 mM 10-HOD in 120 min, respectively, which were 1.15- and 1.18-fold higher than those converted by OhyA1, respectively (Fig. 3). The binding energies of OhyA1 and OhyA2 from *S. maltophilia* were −37.3 and −72.1 kcal/mol, respectively. Thus, the higher activity of OhyA2 than that of OhyA1 may be due to a stronger binding interaction. The specific activities of the OhyA2 type toward PUFAs, including linoleic acid, α-linolenic acid, and γ-linolenic acid, were 1.8- to 84-, 3.9- to 105-, and 2.1- to 9.1-fold higher than those of the OhyA1 type, respectively (Table 1). The k_{cat}/K_m values of the OhyA2 type toward linoleic acid, α-linolenic acid, and γ-linolenic acid were 3.9- to 125-, 5.9- to 95-, and 2.4- to 12-fold higher than those of the OhyA1 type, respectively (Table 2). Therefore, OhyA2 is more efficient than OhyA1 in the biotransformation of plant oils, which contain oleic acid and PUFAs as primary components, to 10-hydroxy fatty acids.

The specific activity of OhyA2 from *S. maltophilia* for oleic acid was the highest among the reported bacterial OhyAs, and it was 2.0-fold higher than that of OhyA1 from *S. maltophilia*, which was the second highest (Table 1). The catalytic efficiency of OhyA2 from *S. maltophilia* for oleic acid was also the highest among the reported bacterial OhyAs, and it was 2.4-fold higher than that of OhyA2 from *S. nitritireducens*, which was the second highest (Table 2). The conversion rate of oleic acid to 10-HSA by OhyA2 from *S. maltophilia* after 2 h was 86% (mol/mol), which was 11% higher than that of OhyA1 from *S. maltophilia* (Fig. 3A). Therefore, *S. maltophilia* OhyA2 is an efficient biocatalyst for the production of 10-hydroxy fatty acids.

In conclusion, the cofactor-independent holo-OhyA2 from *S. maltophilia* was newly identified based on genetic analysis of the FAD-binding motif, its specificity for unsaturated fatty acids, and evaluation of the cofactor effect of holoenzymes. This enzyme was a cofactor-independent enzyme, differing from the original cofactor-dependent OhyA1. The different activities of the enzymes with cofactors may be due to the different fourth conserved amino acid of the FAD-binding motif. The specific activity of *S. maltophilia* OhyA2 toward not only oleic acid but also PUFAs, with the exception of γ-linolenic acid, was the highest among the OhyAs reported. Therefore, *S. maltophilia* OhyA2 is an efficient biocatalyst for the conversion of plant oils to 10-hydroxy fatty acids, which can be converted to important industrial compounds, including γ-lactones and ω-hydroxycarboxylic and α,ω-dicarboxylic acids via biotransformation.

MATERIALS AND METHODS

Materials. Oleic acid ($C_{18:1\Delta9Z}$), palmitoleic acid ($C_{16:1\Delta9Z}$), linoleic acid ($C_{18:2\Delta9Z,12Z}$), α-linolenic acid ($C_{18:3\Delta9Z,12Z,15Z}$), and γ-linolenic acid ($C_{18:3\Delta6Z,9Z,12Z}$) were purchased from Sigma-Aldrich (St. Louis, MO). The standards 10-hydroxyhexanoic acid, 10-HSA, 10-hydroxy-12(*Z*)-octadecenoic acid (10-HOD), 10-hydroxy-12,15(*Z*,*Z*)-octadecadienoic acid, and 10-hydroxy-6,12(*Z*,*Z*)-octadecadienoic acid were prepared as described previously (23). $FADH_2$ was prepared by chemical reduction using DTT as a reducing agent.

Gene cloning and site-directed mutagenesis. The gene encoding a putative fatty acid hydratase in *S. maltophilia* S028 (GenBank accession no. WP_017356052) was synthesized by Cosmo Genetech (Seoul, Republic of Korea). A one-step isothermal assembly method was used for cloning the synthesized gene of *S. maltophilia* (24). The primers were designed based on the DNA sequence of the putative fatty

acid hydratase. A forward primer (5′-AGC AGC GGC CTG GTG CCG CGC GGC AGC CAT ATG AGC CAG CCC ACC GCA CCG GGA CGC AAC-3′) and reverse primer (5′-GTT GCG TCC CGG TGC GGT GGG CTG GCT CAT ATG GCT GCC GCG CGG CAC CAG GCC GCT GCT-3′) were used to amplify the DNA fragment encoding the putative enzyme. A forward primer (5′-GTC TCA GTG GTG GTG GTG GTG GTG CTC GAG CTA GGG CGC GCG CCG CCT GCC CAG CGT TTC-3′) and reverse primer (5′-GAA ACG CTG GGC AGG CGG CGC GCG CCC TAG CTC GAG CAC CAC CAC CAC CAC CAC TGA GAC-3′) were used to amplify the pET-28a(+) plasmid (Novagen, Madison, WI). The primers were synthesized by Bioneer (Daejeon, Republic of Korea), and the putative fatty acid hydratase DNA fragment and linearized pET-28a(+) plasmid were amplified by PCR using the above-mentioned primers and Phusion high-fidelity DNA polymerase (New England BioLabs, Hertfordshire, UK). The amplified DNA fragment was ligated into the linearized plasmid, and the ligated plasmid was transformed into *E. coli* ER2566 cells (New England BioLabs). Mutations of the fourth conserved residues of FAD-binding motifs in OhyAs from *S. maltophilia* and *S. nitritireducens* were generated by site-directed mutagenesis using a QuikChange kit (Stratagene, Beverly, MA).

Enzyme expression and purification. Recombinant *E. coli* expressing the putative fatty acid hydratase was cultured in a 2,000-ml flask containing 500 ml of Luria-Bertani (LB) medium supplemented with 20 μg/ml kanamycin and incubated at 37°C with agitation at 200 rpm. When the optical density at 600 nm (OD_{600}) of the bacterial culture reached 0.6, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and the culture was incubated at 16°C for 16 h with agitation at 150 rpm to induce expression of the enzyme.

Recombinant *E. coli* cells were harvested by centrifugation at 8,000 $\times$ *g* and 4°C for 20 min. The harvested cells were washed twice with 0.85% NaCl and were resuspended in 50 mM phosphate buffer (pH 8.0) containing 10 mM imidazole, 300 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride, which is a protease inhibitor. The resuspended cells were lysed using sonication for 10 min on ice. The supernatant of the lysed cells was collected by centrifugation at 13,000 $\times$ *g* and 4°C for 20 min and filtered through a 0.45-μm-pore-size filter. A protein purification system (Profinia; Bio-Rad, Hercules, CA) equipped with an immobilized metal ion affinity chromatography (IMAC) cartridge (Bio-Rad) was used at 4°C to purify the enzyme. The filtrate was loaded onto the cartridge equilibrated with 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl, and the protein was eluted at a flow rate of 1 ml/min with a linear gradient of 10 to 250 mM imidazole. The collected active fractions were applied to a Bio-Gel P-6 desalting cartridge (Bio-Rad) equilibrated with 50 mM citrate-phosphate buffer (pH 6.0). The bound protein was eluted at a flow rate of 1 ml/min with 50 mM citrate-phosphate buffer (pH 6.0), and the eluate was used as the purified holoenzyme in subsequent experiments.

Molecular weight determination. The purified putative fatty acid hydratase from *S. maltophilia* was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the molecular weight was determined by comparing the migration of the hydratase with that of proteins of known sizes. The total molecular weight of the protein was estimated by applying the purified protein to a gel filtration chromatography column (Sephacryl S-300 HR 16/60; GE Healthcare, Piscataway, NJ). The protein was eluted from the column at a flow rate of 1 ml/min with 50 mM citrate-phosphate buffer (pH 6.0) containing 150 mM NaCl, and the total molecular weight was determined by comparing the retention time of the purified protein with those of reference proteins, including thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), and ovalbumin (43 kDa) (GE Healthcare).

Effects of temperature, pH, and solvent on enzyme activity. Unless otherwise stated, reactions were carried out under the standard conditions of 35°C and pH 6.0 (50 mM citrate-phosphate buffer) for 10 min with 0.5 mM unsaturated fatty acid, 0.01 mg/ml OhyA1 or 0.005 mg/ml OhyA2, and 5% (vol/vol) dimethyl sulfoxide (DMSO). The effects of temperature and pH on the activities of the two types of OhyA from *S. maltophilia* were examined by incubating the enzymes at different temperatures ranging from 25°C to 50°C and at different pH levels ranging from 5.0 to 8.0 using 50 mM citrate-phosphate buffer. To investigate the effect of solvent on the activities of the two types of OhyA, 5% and 10% (vol/vol) solutions of different solvents, including methanol, ethanol, isopropanol, DMSO, butanol, acetone, and toluene, were added to the reaction solution. To examine the effect of DMSO on the activities of the two types of OhyA, DMSO concentrations ranging from 1% to 12.5% (vol/vol) were added.

Preparation of apoenzyme. Urea was added to the purified enzyme at a final concentration of 3 M, and the enzyme was dialyzed at 4°C for 24 h against 50 mM citrate-phosphate buffer (pH 6.0). After dialysis, apoenzyme without cofactor was obtained. This procedure has negligible impact on the enzyme activity. The reconstituted apoenzyme was concentrated to 10 mg/ml by ultrafiltration using the Centricon and used for determining the enzyme activity without adding cofactors.

FAD assay. The UV-visible absorption spectra of the purified apo- and holoenzymes were measured from 280 nm to 600 nm using Cary 100 UV-vis spectrophotometer (Agilent, Santa Clara, CA). Spectral measurements were performed in 50 mM citrate-phosphate buffer (pH 6.0) containing 0.5 mM FAD or 1 mg/ml enzyme.

Effect of cofactor on enzyme activity. The effect of cofactors on the activities of the two types of holo-OhyAs from *S. maltophilia* for oleic acid and linoleic acid was studied using 0.1 mM FAD, 5 mM NAD^+, 5 mM $NADP^+$, 5 mM NADH, and 0.1 mM FAD supplemented with 5 mM additional cofactor, such as NAD^+, $NADP^+$, or NADH. Additionally, the effect of reduced cofactors on the activities was determined using 0.1 mM $FADH_2$ and 0.1 mM FAD supplemented with 5 mM DTT.

Determination of specific activity and kinetic parameters. One unit of OhyA activity was defined as the amount of OhyA required to produce 1 μmol 10-hydroxy fatty acid per min under the standard conditions described above. The specific activity was measured as initial velocity within a linear range of activity versus time under the standard reaction conditions. To determine the kinetic parameters of the two types of OhyAs from *S. maltophilia*, different amounts of unsaturated fatty acids, including palmit-

oleic acid, oleic acid, linoleic acid, α-linolenic acid, and γ-linolenic acid, at concentrations ranging from 0.004 mM to 1 mM, were incubated at 35°C in 50 mM citrate-phosphate buffer (pH 6.0) containing 0.01 mg/ml OhyA1 or 0.005 mg/ml OhyA2 in the presence of 5% (vol/vol) DMSO for 10 min. The K_m and k_{cat} for these substrates were calculated using the enzyme concentration and a Hanes-Woolf plot using a standard linear regression technique.

Determination of the change in binding energy by substrate docking in homology models. Homology models of OhyA1 and OhyA2 from *S. maltophilia* were constructed using the build homology models module of Discovery Studio (DS) 3.1 (Accelrys Software, San Diego, CA) using the crystal structure of OhyA from *E. meningoseptica* (Protein Data Bank [PDB] 4UIR) as the template. Assessing the compatibility of the amino acid sequences with the structure of OhyA from *E. meningoseptica* allowed refinement of the loop conformations of the generated structures (protein health module, DS 3.1). The geometry of the loop region was revised using the Refine Loop/MODELLER, and the best models of OhyA1 and OhyA2 were chosen. Model quality was analyzed with the PROCHECK software for additional validation (25). The substrate oleic acid and cofactor FAD were docked into the substrate-binding pocket of the models with the C-DOCKER module, and the homology models with the lowest interaction energies were chosen. The binding energies of OhyA1 and OhyA2 after docking oleic acid ($\Delta E_{Binding}$) were defined by the calculation $E_{complex} - E_{Ligand} - E_{Receptor}$ (26).

Analytical methods. Following the reactions, the solutions of fatty acids and hydroxy fatty acids were extracted by adding an equal volume of ethyl acetate, and the solvent in the extracted solutions was removed using an evaporator. The resultant fatty acids and hydroxy fatty acids were silylated by mixing pyridine and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide in a 3:1 ratio and incubated at 65°C for 30 min (27). The silylated fatty acids and hydroxy fatty acids were analyzed by a flame ionization detector and a Supelco SPB-1 capillary column (15 m by 0.32 mm internal diameter, 0.25-μm film thickness) in a gas chromatograph (Agilent 7890A). The temperature of the column was increased from 150°C to 210°C for 15 min, increased from 210°C to 280°C for 2.5 min, and finally held at 280°C until the analysis was complete. The temperatures in the injector and detector were 260°C and 250°C, respectively.

Statistical analyses. The means and standard errors for all experiments were quantitatively calculated with one-way analysis of variance (ANOVA) from triplicate experiments. ANOVA was carried out using Tukey's method, with a significance level of a P value of <0.05 using SigmaPlot 10.0 (Systat Software, Chicago, IL).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03351-16.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

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We declare no conflicts of interest.

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