Monooxygenase System of *Bacillus megaterium* ALA2: Studies on Palmitic Acid Oxidation Products

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ABSTRACT: We identified many novel oxygenated FA produced from linoleic acid by microbial strain ALA2: 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (12,13,17-THOA); 12,13,16-trihydroxy-9(*Z*)-octadecenoic acid (12,13,16-THOA); 12-hydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid; and 12,17;13,17-diepoxy-16-hydroxy-9(*Z*)-octadecenoic acid. 12,13,17-THOA, the main product, inhibits the growth of some plant pathogenic fungi. Recently, we reclassified strain ALA2 as *Bacillus megaterium* ALA2 NRRL B-21660 and opened a possible link with the well-studied catalytically self-sufficient P450 monooxygenase of *Bacillus megaterium* ATCC 14581 (NRRL B-3712) and *B. subtilis* strain 168 (NRRL B-4219). Now we have found that strain ALA2 also oxidizes palmitic acid into three oxygenated products: 13-, 14-, and 15-hydroxy palmitic acids. This indicates that strain ALA2 also possesses a monooxygenase system similar to the above-mentioned well-known strains. These data facilitate studies on the oxygenase system of strain ALA2.

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KEY WORDS: *Bacillus megaterium* ALA2, linoleic acid, monooxygenase, oxygenated fatty acids, palmitic acid.

It has been reported that microbial systems convert unsaturated FA to monohydroxy-, dihydroxy- and trihydroxy-FA (1–10). Production of various value-added hydroxy FA and their derivatives for industrial applications is made feasible by using the unique reaction specificities of microbial enzymes. Strain ALA2 is a unique microbe that produces a variety of hydroxy FA from linoleic acid (1), including: 12,13-dihydroxy-9(*Z*)-octadecenoic acid (12,13-DHOA); 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (12,13,17-THOA); 12,13,16-trihydroxy-9(*Z*)-octadecenoic acid (12,13,16-THOA); 12-hydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid (THFA); 7,12-dihydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid (HO-THFA); 12,17;13,17-diepoxy-16-hydroxy-9(*Z*)-octadecenoic acid (DEOA); 7-hydroxy-12,17;13,17-diepoxy-16-hydroxy-9(*Z*)-octadecenoic acid (HO-DEOA); and 12,17;13,17-diepoxy-16-hydroxy-9(*Z*)-octadecenoic acid. Oxygenated FA can be used not only as specialty chemicals but also as bioactive agents such as antifungal agents (11–13). The main product,12,13,17-THOA, was found to inhibit the growth of many plant pathogenic fungi (11). The structures of tetrahydrofuranyl fatty acids (THFA) resemble those of known anticancer agents (14,15). The diepoxy bicyclic FA are new chemical entities with many functional groups in their molecules. Their applications in biomedical and specialty chemical industries are expected. The biosynthetic pathways for these oxygenated FA were established. Linoleic acid is converted to 12,13-dihydroxy-9(*Z*)-octadecenoic acid (12,13-DHOA) first. 12,13-DHOA is the branch point for two biosynthetic pathways (16–19). We were interested in molecular engineering of the oxygenase system of strain ALA2 to produce specific products in large scale. However, too little was known about the oxygenase system of ALA2 to pursue the project. Strain ALA2 was recently reclassified as *Bacillus megaterium* based on DNA analysis (20). This opened a link to the well-studied *Bacillus* monooxygenase system.

Catalytically self-sufficient cytochrome P450 monooxygenase from *B. megaterium* (CYP102A1) is well studied (21,22). These monooxygenases oxidize palmitic acid to ω-1, ω-2, and ω-3 hydroxy palmitic acids. Two genes, CYP102A2 and CYP102A3, from *B. subtilis* code for single-peptide monooxygenases, comprising both a heme and a FAD/FMN (flavin adenine dinucleotide/flavin mononucleotide)-containing reductase domain, and demonstrate a notable sequence similarity to CYP102A1 (23,24). CYP102A3 is involved in the hydroxylation of unsaturated, saturated, and branched-chain FA (25,26). The ability of strain ALA2 to oxidize palmitic acid is not known. Therefore, it would be interesting to find out the palmitic acid-oxidizing ability of strain ALA2 and to compare the FA bioconversion products among these three well-studied *Bacillus* strains. If they all produce the same products from palmitic acid, then they might possess similar monooxygenase systems. In this paper, we compared the palmitic acid conversion products produced by strain ALA2 with the two above-mentioned, well-studied *Bacillus* species.

MATERIALS AND METHODS

Chemicals. Palmitic acid (purity 99%) was purchased from Sigma (St. Louis, MO). A mixture of trimethylsilylimidazole (TMSI) and pyridine (1:4 vol/vol) was purchased from Supelco Inc. (Bellefonte, PA). Diazomethane was prepared from Diazald (Sigma). Tri-Sil-Z for preparation of TMS ethers was obtained from Pierce Chemical Company (Rockford, IL). All other chemicals were reagent grade and used without further purification.

Microorganisms. Strain ALA2 (NRRL B-21660) was isolated from a dry soil sample collected from McCalla, Alabama

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(27,28). *Bacillus megaterium* ATCC 14581 (NRRL B-3712) and *B. subtilis* 168 (NRRL B-4219) were obtained from the ARS Culture Collection (Peoria, IL). Both strains B-3712 and B-4219 were cultivated on TGY medium as reported (21–23). Strain ALA2 was cultivated aerobically in 125-mL Erlenmeyer flasks containing 50 mL of medium with shaking at 200 rpm, 30°C. The medium (per liter) contained (29): dextrose, 5 g; K_2HPO_4, 5 g; yeast extract, 15 g; tryptone, 10 g; $MgSO_4{\cdot}7H_2O$, 2.0 mM; $FeSO_4{\cdot}7H_2O$, 0.5 mM; $ZnSO_4$, 0.1 mM; $NiCl_2{\cdot}6H_2O$, 0.01 mM; $CoCl_2{\cdot}6H_2O$, 0.05 mM; and nicotinic acid, 10 mg. The medium was adjusted to pH 6.8 with dilute phosphoric acid.

Production of oxygenated FA. Strain ALA2 was aerobically grown in 50 mL of the medium described above for 1 d at 30°C, 200 rpm. Ethanol (500 µL) containing 200 µL (160 mg) of palmitic acid was added into a 1-d-old culture, and the culture was then incubated for an additional 2 d. At the end of incubation, the culture was acidified to pH 2 with 6 N HCl, and the lipid fraction was extracted twice with 100 mL ethyl acetate followed by 100 mL ethyl ether. The solvent was evaporated from the combined extracts with a rotary evaporator.

Purification of products. The crude extract was further purified with preparative TLC. The solvent was diethyl ether/acetone = 4:1 vol/vol. The silica gel on the plate was scraped off in 1 cm fractions that were extracted with a mixture of methylene chloride/methanol 3:1 vol/vol. Each fraction was then methylated and subjected to GC–MS analyses.

Product analyses. The crude lipid extracts were methylated with diazomethane for GC analyses. Methyl ester derivatives were injected into an Agilent Technologies 6890N Network GC System equipped with an FID, a Supelco (Bellefonte, PA) SPB-1 capillary column (15 m × 0.32 mm i.d.; 0.25 µm thickness), a 7683 series auto sample injector, and a ChemStation A.10.02 [1757]. The column temperature was kept isothermal at 180°C. The injection and detector temperatures were 240 and 250°C, respectively. Methyl ester derivatives of products were also treated with Tri-Sil-Z to convert the products into TMS ethers before GC–MS analyses. The relative yield among the three products was calculated as the ratio of product peak area. GC–MS analyses were performed with a Hewlett-Packard Model 5890 gas chromatograph interfaced with a Model 5971 mass-selective detector operating at 70 eV. The capillary column used was a Hewlett-Packard HP-5-MS cross-linked 5% phenyl methyl silicone, 30 m × 0.25 mm i.d., film thickness 0.25 µm. The carrier gas was helium and its flow rate was 0.65 mL/min. The GC column was held at 190°C for 3 min and then ramped to 220°C at a rate of 2°C/min and then to 240°C at 5°C per min and then held at 240°C for 4 min.

RESULTS AND DISCUSSION

Control experiments with the same conditions but without a microorganism or without palmitic acid all showed no production of oxygenated FA products, indicating all products are produced by microbial action.

Comparison of palmitic acid bioconversion products among three Bacillus strains. Strains ALA2 and B-3712 produced three products [GC retention times (RT) 18, 18.5, and 19 min] from palmitic acid, whereas strain B-4219 produced two products (GC RT 19 and 19.5 min) under our reaction conditions. The yields, in mg oxygenated products/50 mL medium/48 h for strains ALA2, B-3712, and B-4219 were 14.0, 15.3, and 4.9, respectively. The ratios for each individual product are listed in Table 1. Among the three products produced from palmitic acid, strains ALA2 produced more GC RT 19 min product, and B-3712 produced more GC RT 18.5 min product. The yield ratio among the three hydroxyl FA products for strain B-3712 agrees well with those reported earlier (30). These crude extracts were subjected to preparative TLC for purification (see Materials and Methods section).

TABLE 1
Ratio of ω-1, ω-2, and ω-3 Hydroxy Palmitic Acid Products as Estimated from GC Peak Areas[a]

	Products		
Strains	ω-1	ω-2	ω-3
Bacillus megaterium ALA2	1.00	0.93	0.35
B. megaterium NRRL B-3712	0.42	1.00	0.35
B. subtilis NRRL B-4219	1.00	ND	ND

[a]ω-1 (15-hydroxy palmitic acid), ω-2 (14-hydroxy palmitic acid), and ω-3 (13-hydroxy palmitic acid) are products having GC retention times (RT) of 19, 18.5, and 18, respectively. The "ω-1" product of NRRL B-4219 includes both GC RT 19 and 19.5 products. ND, not detectable.

Chemical structure determination. The preparative TLC-purified fractions were subjected to GC–MS analyses. GC–MS analysis of the methylated fraction 8 products (R_f = 0.63–0.70) from palmitic acid and strain ALA2 showed product peaks at RT 18, 18.5, and 19 min. GC–MS analysis of these products showed all have a molecular ion of 286. The GC RT 18 min product has m/z 213 = $[(CH_2)_{11}COOCH_3]^+$, characteristic of 13-hydroxy methyl palmitate (Fig. 1A). The GC RT 18.5 min product has m/z 227 = $[(CH_2)_{12}COOCH_3]^+$, characteristic of 14-hydroxy methyl palmitate (Fig. 1B). GC RT 19 min product has m/z 241 = $[(CH_2)_{13}COOCH_3]^+$, characteristic of the known 15-hydroxy methyl palmitate (Fig. 1C). These products were silylated to TMS ether and then analyzed again by GC–MS. As shown in Figure 2A, the GC RT 18 min product showed characteristic m/z values of 317 and 145 and was confirmed to be the methyl ester, silyl ether of 13-hydroxy palmitate. The GC RT 18.5 min product showed (Fig. 2B) characteristic m/z values of 327 and 131 and was confirmed to be the methyl ester, silyl ether of 14-hydroxy palmitate. The MS spectra of the methyl ester, silylated ether of the GC RT 19 min product showed m/z 241 = $[(CH_2)_{13}COOCH_3]^+$ and mz 117 (Fig. 2C) indicating that this is a methyl ester, silyl ether of 15-hydroxy palmitate with cleavage at C_{14} and C_{15}. These data confirm that the three hydroxyl FA products derived from palmitic acid by strain ALA2 are indeed 13-, 14-, and 15-hydroxy palmitic acids.

GC analysis of methylated fraction 8 products obtained from palmitic acid and strain B-3712 also showed three product peaks at GC RT 18, 18.5, and 19 min. GC–MS analysis of the three methylated GC RT 18, 18.5, and 19 min products showed identical mass spectra to the corresponding products

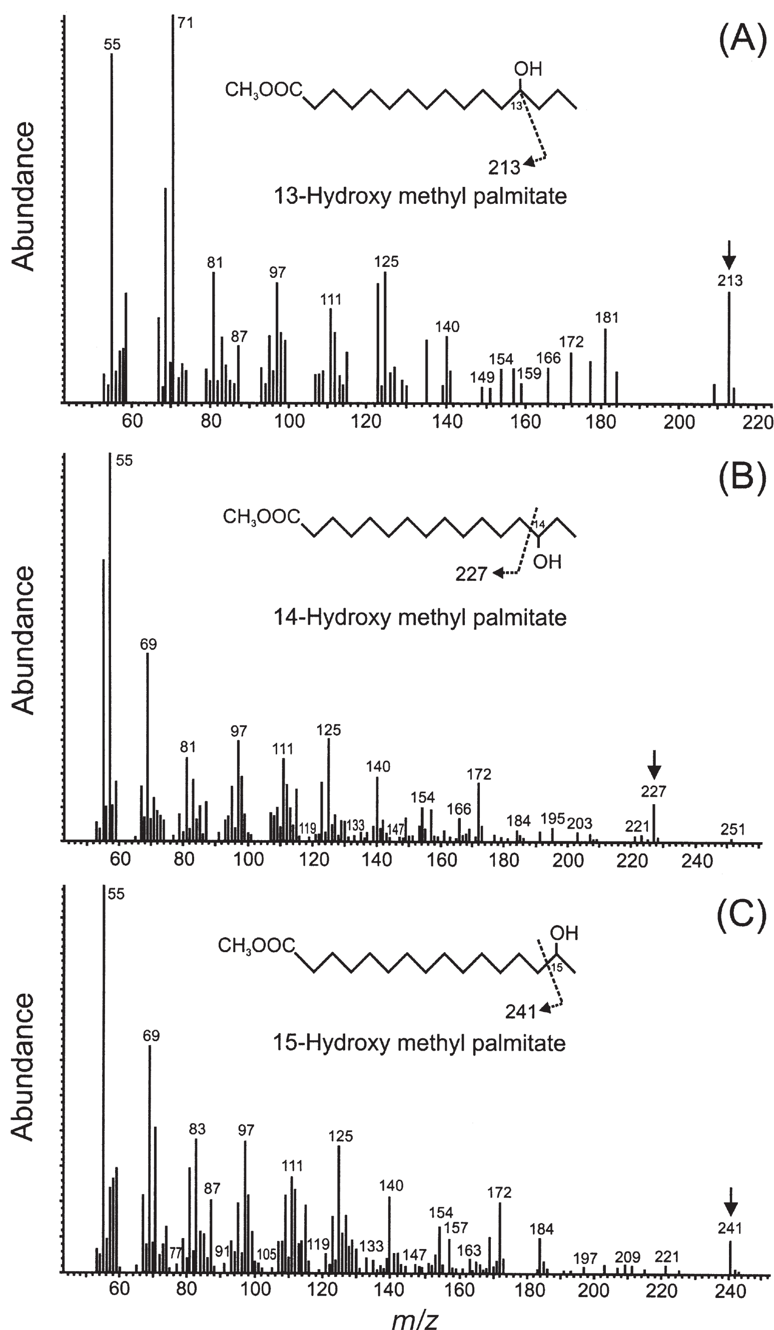

FIG. 1. Mass spectral analysis of methylated products having GC retention times (RT) of 18 (A), 18.5 (B), and 19 (C) min products from palmitic acid by strain ALA2.

obtained from strain ALA2 (Fig. 1A–C). GC–MS analysis of silylated methyl esters of these three products also showed identical mass spectra with those obtained from strain ALA2 (Fig. 2A–C). Therefore, the oxidation products from palmitic acid produced by strain B-3712 are also 13-, 14-, and 15-hydroxy palmitic acids.

The oxidation of palmitic acid by *B. subtilis* NRRL B-4219, however, showed only two products peaks (GC RT 19 and 19.5 min). GC–MS analyses of their methylated products showed *m/z* 241 = $[(CH_2)_{13}COOCH_3]^+$, characteristic of the known 15-hydroxy methyl palmitate (Fig. 1C). And the mass spectrum of GC RT 19.5 min showed *m/z* 255 = $[(CH_2)_{13}COOCH_2CH_3]^+$, characteristic of 15-hydroxy ethyl palmitate The ratio between these two products was 44.9 and 55.1%, for GC RT 19 and 19.5, respectively. Both of these products were ω-1 hydroxy product from palmitic acid. The formation of ethyl palmitate product may be due to the presence of ethanol used in dissolving the substrate palmitic acid. This also indicated that strain

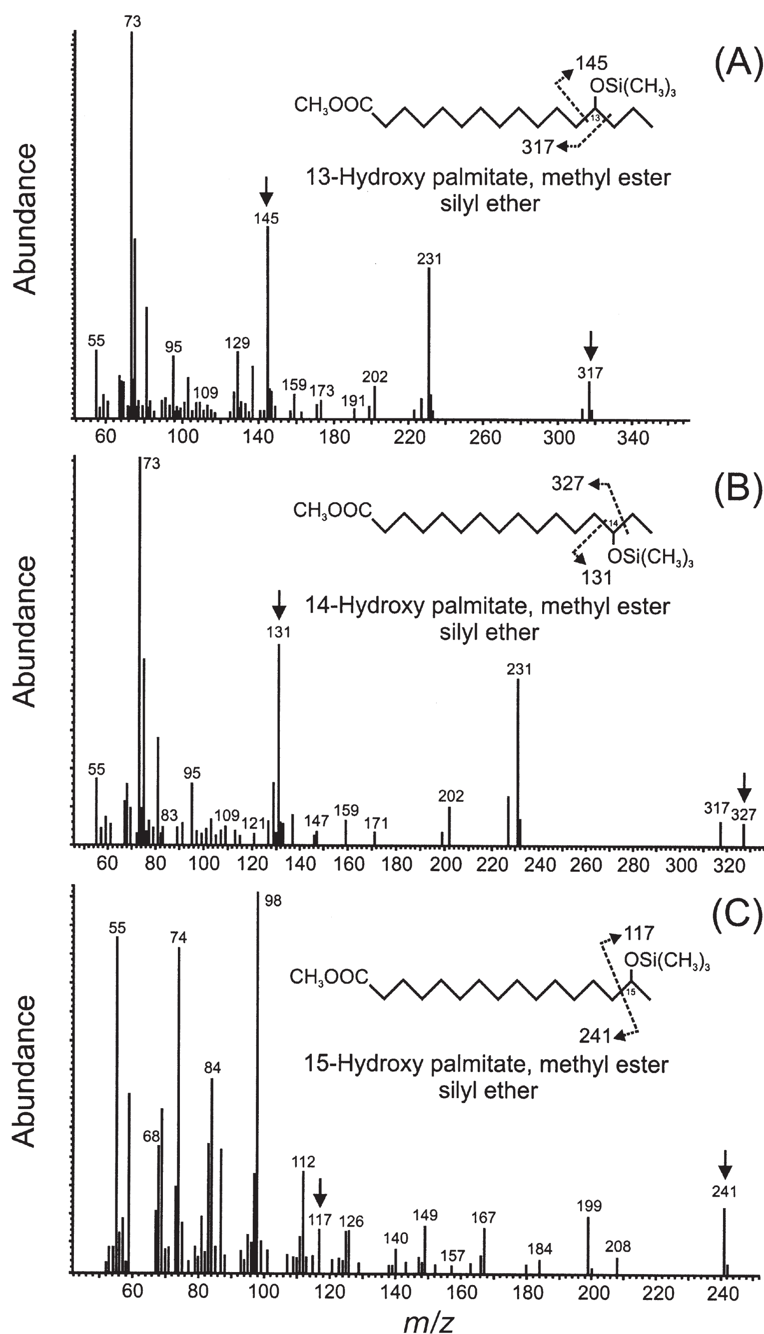

FIG. 2. Mass spectral analyses of methylated and silylated products from palmitic acid by strain ALA2. (A) GC RT 18 min product; (B) GC RT 18.5 min product; (C) GC RT 19 min product. For abbreviation see Figure 1.

B-4219 possesses strong lipase activity for the esterification reaction. Neither ω-2 nor ω-3 products were detected with strain B-4219 under our reaction conditions. In contrast, both strains ALA2 and B-3712 produced three oxygenated products, i.e., 13-, 14-, and 15-hydroxy palmitates. Although it was reported that both strains ATCC 14581 (NRRL B-3712) and strain168 (NRRL B-4219) produced ω-1, ω-2, and ω-3 hydroxy palmitates, we could only detect ω-1 hydroxy palmitate product from strain B-4219 under our reaction conditions. Nevertheless, these data indicated that strain ALA2 possesses a similar type of the well-known cytochrome P450 monooxygenase system (21–26).

Our comparison of reaction products from palmitic acid among strains ALA2, B-3712, and B-4219 indicated that they all possess a similar type of monooxygenase system. It is well-known that oxygenases carry out reactions such as hydroxylation, epoxidation, and hydroperoxylation. Our paper with substrate palmitic acid addresses only the hydroxylation of the FA

saturated hydrocarbon chain. It was known that both strains B-3712 and B-4219 also epoxidize unsaturated FA (25). Strain ALA2 also was known to produce many new oxygenated unsaturated FA from linoleic acid.

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