Monooxygenase System of *Bacillus megaterium* ALA2: Studies on Linoleic Acid Epoxidation Products

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ABSTRACT: *Bacillus megaterium* ALA2 produces many oxygenated FA from linoleic acid: 12,13-dihydroxy- 9(*Z*)-octadecenoic acid; 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid; 12,13,16-trihydroxy-9(*Z*)-octadecenoic acid; 12-hydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid; and 12,17;13,17-diepoxy-16-hydroxy-9(*Z*)-octadecenoic acid. Recently, we studied the monooxygenase system of *B. megaterium* ALA2 by comparing its palmitic acid oxidation products with those of the well-studied catalytically self-sufficient P450 monooxygenase of *B. megaterium* ATCC 14581 (NRRL B-3712) and of *B. subtilis* strain 168 (NRRL B-4219). We found that their oxidation products are identical, indicating that their monooxygenase systems (hydroxylation) are similar. Now, we report that strain ALA2 epoxidizes linoleic acid to 12,13-epoxy-9(*Z*)-octadecenoic acid and 9,10-epoxy-12(*Z*)-octadecenoic acid, the initial products in the linoleic acid oxidation. The epoxidation enzyme did not oxidize specific double bond of the linoleic acid. The epoxidation activity of strain ALA2 was compared with the above-mentioned two *Bacillus* strains. These two *Bacillus* strain also produced 12,13-epoxy-9(*Z*)-octadecenoic acid and 9,10-epoxy-12(*Z*)-octadecenoic acid, indicating that their epoxidation enzyme systems might be similar. The ratios of epoxy FA production by these three strains (ALA2, NRRL B-3712, and NRRL B-4219) were, respectively, 5.56:0.66:0.18 for 12,13-epoxy-9(*Z*)-octadecenoic acid and 2.43:0.41:0.57 for 9,10-epoxy-12(Z)-octadecenoic acid per 50 mL medium per 48 h.

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KEY WORDS: *Bacillus megaterium* ALA2, bioconversion, linoleic acid, 12,13-epoxy-9(Z)-octadecenoic acid, monooxygenase system.

Microbial systems convert unsaturated FA to monohydroxy-, dihydroxy-, and trihydroxy-FA (1). Strain ALA2 is a unique microbe that produces a variety of hydroxy FA from linoleic acid (2): 12,13-dihydroxy-9(*Z*)-octadecenoic acid; 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); and 7-hydroxy-12,17;13,17-diepoxy-16-hydroxy-9(*Z*)-octadecenoic acid (HO-DEOA). Oxygenated FA can be used not only as specialty chemicals but also as bioactive agents such as antifungals (3,4). The main product, 12,13,17-THOA, was found to inhibit the growth of many plant pathogenic fungi (3). The structure of tetrahydrofuranyl FA (THFA) resemble those of known anticancer agents (5). 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, and the biosynthetic pathways for these oxygenated FA are known (6–9).

Monooxygenases are known to hydroxylate alkanes and/or epoxidate alkenes including saturated and unsaturated FA, e.g., the *Pseudomonas oleovolans* monooxygenase system (10–12); the monooxygenase systems of methylotrophs (13); and *Bacillus megaterium* ATCC 14581 (14). The oxygenase system of ALA2 was not studied. Strain ALA2 was recently reclassified as *Bacillus megaterium* based on DNA analysis (15). This opened a link to the well-studied *Bacillus* monooxygenase system. Catalytically self-sufficient cytochrome P450 monooxygenase from *B. megaterium* ATCC 14581 (CYP102A1) has been well-studied (16). Two genes from *B. subtilis*, CYP102A2 and CYP102A3, code for single-peptide monooxygenases comprising both a heme and a FAD/FMN-containing reductase domain and demonstrate a notable sequence similarity to CYP102A1 (17). CYP102A3 is involved in the hydroxylation of unsaturated, saturated, and branched-chain FA (18). Recently, we compared the monoxygenase (hydroxylation) systems of strain ALA2 with the two just-mentioned well-studied *Bacillus* strains and found that their palmitic acid oxidation products were identical, indicating that their monooxygenase (hydroxylation) systems are similar (19).

The microbial enzyme (hydratase) attacks the double bond of unsaturated FA at a specific position (20). Microbial hydratases convert oleic, linoleic, and linolenic acids into their 10-hydroxy FA, indicating that the enzyme is carbon 10-positional specific. It would be interesting to know whether the epoxidation enzyme of *Bacillus* strains including strain ALA2 has positional specificity.

In our previous studies of the strain ALA2 and linoleic acid system, we focused on FA products having retention times greater than 10 min in GC analysis (GCRT). In this paper, we studied two product peaks having GCRT of 7.10 and 7.32 min and identified them as 12,13-epoxy-9(*Z*)-octadecenoic acid and 9,10-epoxy-12(*Z*)-octadecenoic acid, the initial epoxidation products of linoleic acid. We also compared the epoxidation activity of strain ALA2 and the two previously mentioned well-studied *Bacillus* strains: NRRL B-3712 and NRRL B-4219.

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MATERIALS AND METHODS

Chemicals. Oleic acid (purity 99%) and 12,13-epoxy-9(*Z*)-octadecenoic acid methyl ester were purchased from Sigma (St. Louis, MO). Diazomethane was prepared from Diazald (Sigma). 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 (21). *Bacillus megaterium* ATCC 14581 (NRRL B-3712) and *B. subtilis* 168 (NRRL B-4219) were obtained from the ARS Culture Collection (Peoria, IL). Strains B-3712 and B-4219 were cultivated on TGY medium as reported (16). Strain ALA2 was cultivated aerobically in a 125-mL Erlenmeyer flask containing 50 mL of medium with shaking at 200 rpm and 30°C. The medium (per liter) contained (22): 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 medium for 1 d at 30°C and 200 rpm. Two hundred microliters (190 mg) of linoleic 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 of ethyl acetate followed by 100 mL of ethyl ether. The solvent was evaporated from the combined extracts with a rotary evaporator.

(i) Purification of products. To prepare samples for GC–MS analysis, the crude extract was further purified by preparative TLC. The solvent was diethyl ether/acetone (4:1 vol/vol). The silica gel on the plate was scraped in 1-cm fractions, collected, and extracted with a mixture of methylene chloride/methanol (3:1 vol/vol). Each fraction was then methylated and subjected to GC–MS analyses.

(ii) Product analyses. The crude lipid extracts were methylated with diazomethane for GC analysis. 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 by 0.32 mm inner diameter; 0.25 mm thickness), a 7683 series auto sample injector, and a Chem Station A.10.02 [1757]. The column temperature was kept at 200°C isothermally. The injection and detector temperatures were 240 and 250°C, respectively. The relative yield of products was calculated as the ratio of product peak areas. 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 by 0.25 mm inner diameter, film thickness 0.25 μm. The carrier gas was helium and its flow rate was 0.65 mL/min. The GC column was programmed from 190°C for 3 min and then to 220°C at a rate of 2°C /min and then to 240°C at 5°C per min and then kept at 240°C for 4 min.

RESULTS AND DISCUSSION

Strain ALA2 produced many oxygenated FA products from linoleic acid such as trihydroxy unsaturated FA and diepoxy bicyclic unsaturated FA as well as tetrahydofuranyl unsaturated FA (2,6–8,23). These novel oxygenated unsaturated FA products are our primary interest because of their potential industrial applications. However, to improve their yields through molecular engineering, we need more information on the initial step of FA oxidation: hydroxylation and epoxidation. The epoxidation reaction should produce a maximum of two products from linoleic acid since there are only two double bonds on the substrate molecule. In this study, we found two products at GCRT 7.10 and 7.32 min that might be the products of the initial epoxidation reaction. We also compared these products with those produced by two known *Bacillus* strains: B-3712 and B-4219. Control experiments with the same condition but without a microorganism or without linoleic acid all showed no production of oxygenated FA products, indicating all products are produced by microbial action. Both strains B-3712 and B-4219 produced no product peaks having GCRT greater than 10 min.

Identification of GCRT 7.10 min product from linoleic acid and strain ALA2. The preparative TLC-purified fractions were subjected to GC–MS analyses. GC–MS analysis of the methylated TLC fraction 13 products with R_f of 0.6–0.75 from linoleic acid and strain ALA2 showed two peaks at GCRT 15.94 and 16.12 min, corresponding to the two products, GCRT 7.10 and 7.32 min, in isothermal GC analysis. MS analysis of the GCRT 15.94 min product showed a molecular ion of 310. It also had the following *m/z*: 279, 207 [i.e., $(C_{14}H_{23}O_3) - (OCH_3)$], and 164 [i.e., $(C_{14}H_{23}O_3) - (C_2O) - (OCH_3)$]. Both *m/z* 207 and 164 are characteristic for 12,13-epoxy-9(*Z*)-octadecenoic acid (Fig. 1). Other *m/z* values matched well with those of the authentic 12,13-epoxy-9(*Z*)-octadecenoic acid (24). Therefore, this product from linoleic acid and strain ALA2 is 12,13-epoxy-9(*Z*)-octadecenoic acid, the initial epoxidation product of linoleic acid. Based on this finding, we can add 12,13-epoxy-9(*Z*)-octadecenoic acid to our previously published linoleic acid bioconversion pathway (2) as shown in Figure 2.

Identification of GCRT 7.32 min product from strain ALA2. The mass spectrum of the GCRT 16.12 min product (corresponding to GCRT 7.32 min product in isothermal GC analysis) obtained from methylated TLC fraction with $R_f = 0.6–0.75$ was analyzed. It had a molecular ion of 310. Characteristic *m/z* were: 279, 168 [i.e., $(C_{11}H_{19}O_3) - (OCH_3)$], 153 ($C_{14}H_{23}O$), and 111 ($C_8H_{15}$) (Fig. 3). Other *m/z* values matched well with those of the authentic 9,10-epoxy-12(*Z*)-octadecenoic acid (24). Therefore, this product from linoleic acid and strain ALA2 was 9,10-epoxy-12(*Z*)-octadecenoic acid. This result indicated that the epoxidation enzyme system of strain ALA2 is not position-specific, as it acts on both double bonds at positions 9 and 12. However, our data could not rule out the possibility of two enzymes catalyzing the epoxidation reaction at

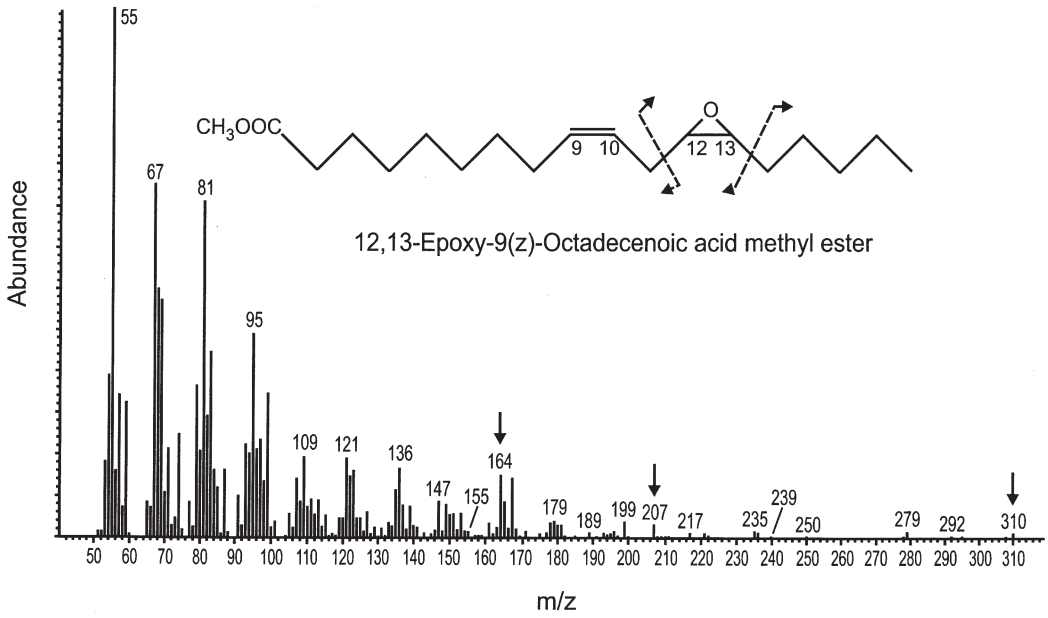

FIG. 1. Mass spectral analysis of methylated GCRT 7.10 min product from linoleic acid by *Bacillus megaterium* ALA2. GCRT, gas chromatographic retention time.

each double bond position. Since there are no further bioconversion products derived from 9,10-epoxy FA detectable on GC, it is not clear why strain ALA2 produced 9,10-epoxy-12(*Z*)-octadecenoic acid. It might be a dead-end product of linoleic acid bioconversion.

Linoleic acid epoxidation activities of strains NRRL B-3712 and NRRL B-4219. Strains NRRL B-3712 and NRRL B-4219 also produced GCRT 7.10 and 7.32 min products from linoleic acid. Their crude extracts were purified by preparative TLC as described in the Materials and Methods section. The methy-

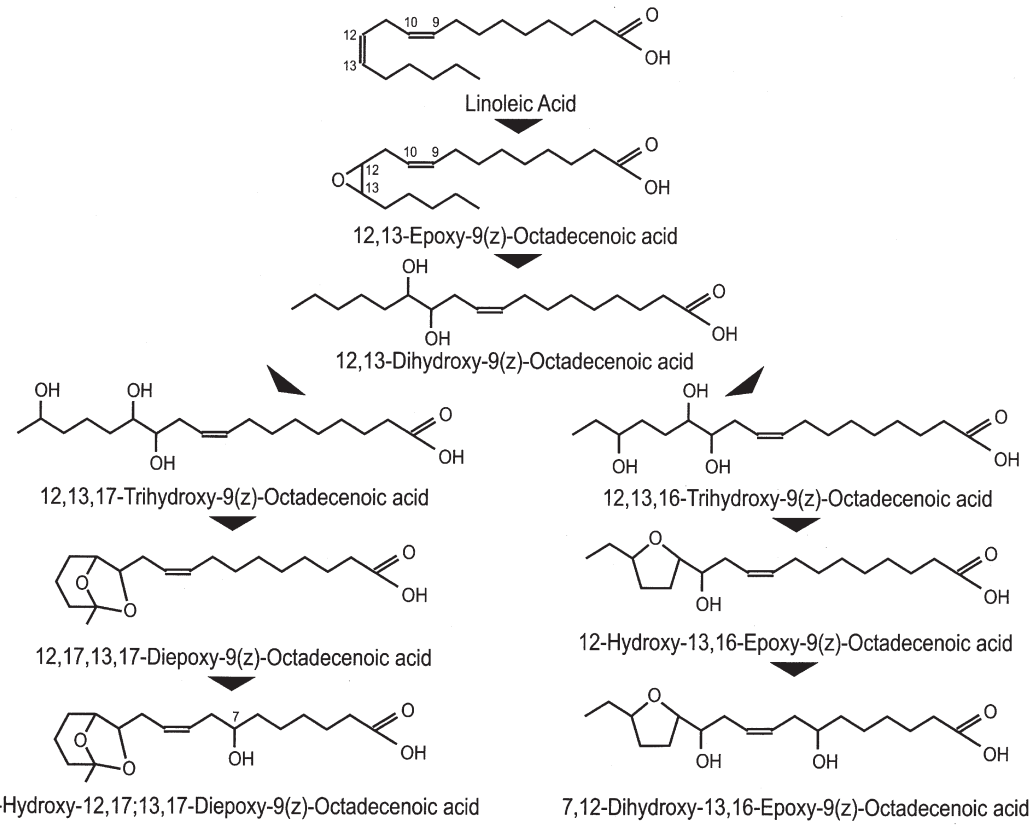

FIG. 2. Biosynthetic pathway of conversion of linoleic acid to its oxygenated FA by *Bacillus megaterium* ALA2.

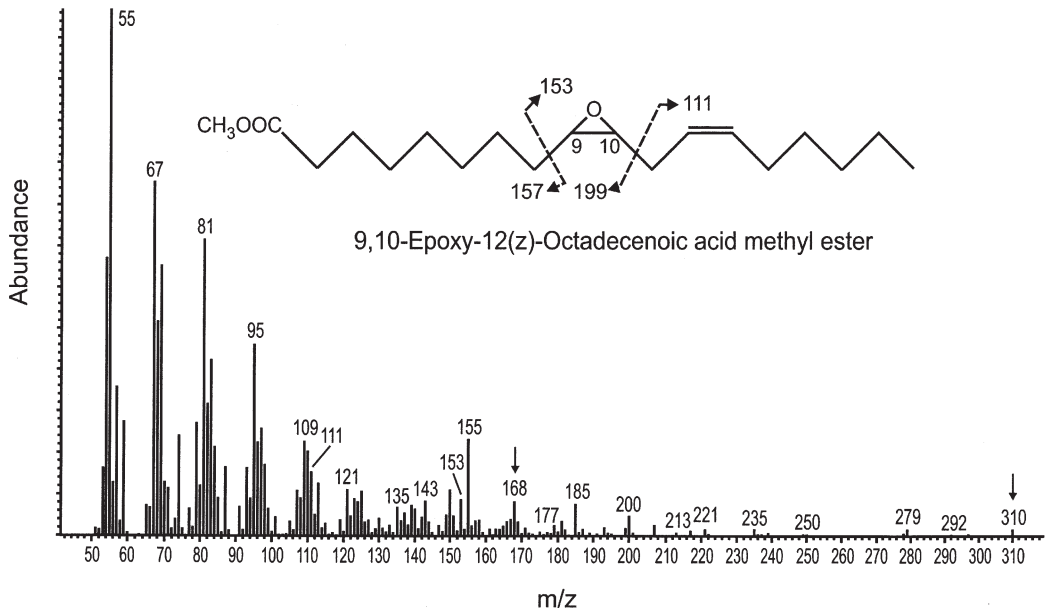

FIG. 3. Mass spectral analysis of methylated GCRT 7.32 min product from linoleic acid by *Bacillus megaterium* ALA2. For abbreviation see Figure 1.

lated TLC fraction products having R_f = 0.6–7.5 were then subjected to GC–MS analyses. GC–MS of the GCRT 7.10 min product from both strains NRRL B-3712 and NRRL B-4219 showed typical *m/z* of 310, 207, and 164 for 12,13-epoxy-9(*Z*)-octadecenoic acid. Other *m/z* values also matched well with those of the authentic 12,13-epoxy-9(*Z*)-octadecenoic acid. Therefore, both strains NRRL B-3712 and NRRL B-4219 also produced 12,13-epoxy-9(*Z*)-octadecenoic acid as their first epoxidation product from linoleic acid. The GC–MS spectra of the GCRT 7.32 min product obtained from the methylated TLC fraction having R_f = 0.6–0.75 from both strains NRRL B-3712 and NRRL B-4219 were analyzed. They had a molecular ion of 310. Characteristic *m/z* values were: 279, 168, 153, and 111, which were similar to those obtained by strain ALA2. Other *m/z* values matched well with those of the authentic 9,10-epoxy-12(*Z*)-octadecenoic acid (24). Therefore, the GCRT 7.32 min product from both strains NRRL B-3712 and NRRL B-4219 and from linoleic acid is 9,10-epoxy-12(*Z*)-octadecenoic acid. All three *Bacillus* strains produced two epoxy unsaturated FA products from linoleic acid, indicating that their epoxidation enzyme systems may be similar.

Comparison of linoleic acid epoxidation products among the three Bacillus strains. The ratio of epoxy products (GCRT 7.10 and 7.32 min) synthesized by the three *Bacillus* strains from linoleic acid was determined. The yields, in mg, for 12,13-epoxy-9(*Z*)-octadecenoic acid were 5.56, 0.66, and 0.18; and for 9,10-epoxy-12(*Z*)-octadecenoic acid were 2.43, 0.41, and 0.57 per 50 mL medium per 48 h for strains ALA2, NRRL B-3712, and NRRL B-4219, respectively. Strain ALA2 possessed the greatest linoleic acid expoxidation activity (combining both epoxidation products, the ratio of ALA2/ B-3712/B-4219 was 10.6:1.4:1).

Previously, we reported that the hydroxylation enzyme systems of strains ALA2 and NRRL B-3712 are quite similar; both produced ω-1, ω-2, and ω-3 hydroxy FA from palmitic acid (19). The hydroxylase of strain ALA2 was more active than that of B-3712 and produced equal amounts of ω-1, ω-2 hydroxy FA products whereas the strain B-4219 hydroxylase system was less active and produced only ω-1 hydroxy FA product. In this paper, we again showed that the epoxidation enzyme systems of these three well-studied *Bacillus* strains were similar, but strain ALA2 was the most active one.

Both strains B-3712 and B-4219 could not produce oxygenated FA products having GCRT greater than 10 min. This indicated that, unlike strain ALA2, both of these strains lack the enzyme systems for further conversion of the initial epoxidation product.

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