Bioconversion of Oleic Acid to a Series of Keto-Hydroxy and Dihydroxy Acids by *Bacillus* Species NRRL BD-447: Identification of 7-Hydroxy-16-oxo-9-*cis*-octadecenoic Acid

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ABSTRACT: Fifty-eight strains of eight *Bacillus* species were selected from the ARS Culture Collection to investigate their ability to bioconvert oleic acid to unique hydroxy or keto derivatives. Addition of 0.5% oleic acid substrate to a whole-cell culture of *Bacillus* strain NRRL BD-447 produced mixtures of mono-, di-, and trisubstituted compounds. These products appear to contain combinations of keto and hydroxy groups. One of the major products was isolated by high-performance liquid chromatography and its structure was determined by gas chromatography–mass spectrometry and nuclear magnetic resonance to be 7-hydroxy-16-oxo-9-*cis*-octadecenoic acid.
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KEY WORDS: *Bacillus*, bioconversion, dihydroxy, fatty acid, hydroxy, ketone, octadecenoic acid, oleic acid.

Vegetable oil, particularly soybean oil, has been an annual surplus commodity of up to 1.2 billion pounds in the United States for many years. It would be economically beneficial to convert these surplus oils to new value-added products (1). To do this, the structure of soybean oil must be modified to change its physical and chemical properties so that it might find new nonfood industrial uses. Ricinoleic acid (12-hydroxy-9-octadecenoic acid), for example, makes up 90% of the fatty acids in castor oil and is the major hydroxy fatty acid used in industry. Castor oil is a U.S. import. Therefore, the discovery and development of new sources or methods to convert existing surplus oils into unique fatty acids would be of great interest.

Industrially useful fatty chemicals, e.g., hydroxy and keto acids for use in surfactants, lubricants, detergents, cosmetics and fragrances, are often manufactured from fats and oils by chemical reactions. Direct synthesis of only one isomer is usually not possible with chemical synthesis, whereas microorganisms often are very specific in their syntheses. Enzymatic modification of surplus soybean oil or other vegetable oils or their fatty acids may provide new products with greater specificity.

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Wallen *et al.* (2) first reported the microbial conversion of oleic acid to 10-hydroxystearic acid. Oleic acid was converted to 10-hydroxystearic acid by several *Nocardia* strains at greater than 90% yield (3), to 10-hydroxy- and 10-ketostearic acid by *Flavobacterium* sp. DS5 (4), to 10-ketostearic acid by *Staphylococcus warneri* (5), and to 7,10-dihydroxy-8(*E*)-octadecenoic acid by *Pseudomonas* sp. PR3 (6). Strains of *Nocardia* (7) and *Flavobacterium* (8) produced 10-hydroxy-12-octadecenoic acid from linoleic acid. α- and γ-Linolenic acids were converted to 10-hydroxy-6,12- and 10-hydroxy-12,15-octadecadienoic acids, respectively, by a *Flavobacterium* (9). The production of hydroxy fatty acids was recently reviewed by Hou (10). Since then, the novel 12,13,17-trihydroxy-9-octadecenoic acid produced from linoleic acid by *Clavibacter* sp. ALA2 has been reported (11).

Previously we reported that two strains of *Bacillus pumilus* (NRRL BD-174 and NRRL BD-226) produced the unique 15-, 16-, and 17-hydroxyoctadecenoic acids from oleic acid (12). In our continuing effort to produce value-added products from soybean oil, 58 *Bacillus* strains were screened for ability to convert oleic acid into novel hydroxy derivatives. The present study reports on the bioconversion of oleic acid by strain NRRL BD-447 and on the isolation and identification of one of the bioconversion products as 7-hydroxy-16-oxo-9-*cis*-octadecenoic acid.

MATERIALS AND METHODS

Microorganisms. Fifty-eight *Bacillus* strains from the ARS Culture Collection were screened for their ability to bioconvert oleic acid. Cultures were grown aerobically at 30°C in 50 mL of media in 125-mL Erlenmeyer flasks shaken at 150 rpm. Bioconversion was assessed in both TGY and BD media. Media composition was (per liter): tryptone, 5.0 g; yeast extract, 5.0 g; dextrose, 1.0 g; and K_2HPO_4, 1.0 g. The pH was adjusted to 7. BD composition was (per liter): proteose peptone No. 3 (Difco Laboratories, Detroit, MI), 7.5 g; tryptone, 7.5 g; KH_2PO_4, 2 g; K_2HPO_4, 2 g; NaCl, 5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; dextrose, 1 g; and yeast extract, 0.1 g. The pH was adjusted to 7.2.

Bioconversion reaction. Oleic acid (0.28 mL, 0.5%) (Nu-

Chek-Prep, Inc., Elysian, MN; 99+% purity) was added to 18- to 24-h-old cultures and incubated at 30°C and 150 rpm. Aliquots were taken 3 d after oleic acid addition to assess the extent of conversion; however, incubations were allowed to continue for 6 d. At the end of incubation, cultures were acidified to pH 2 with 9 N sulfuric acid and extracted twice with equal volumes of ethyl ether. The combined ether fractions were washed with water and dried over anhydrous sodium sulfate. After filtering to remove the sodium sulfate, the solvent was removed with a rotary evaporator.

Bioconversions were scaled up to 600 mL of TGY or BD media in Fernbach flasks. After microorganism growth, 3 mL (0.5%) oleic acid substrate was added and incubation continued for 6 d at 28°C and 200 rpm.

Detection of products. Bioconversion was monitored by gas chromatography (GC) and thin-layer chromatography (TLC). Extracted lipids were esterified with diazomethane and then injected into a Hewlett-Packard (HP) model 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector, a Supelco SPB-1 capillary column (15 m × 0.32 mm i.d., 1 μm film thickness; 1 mL/min helium flow; Supelco, Inc., Bellefonte, PA), and an HP 3396A integrator. GC runs were isothermal at 230°C. For yield quantitation, palmitic acid was added as an internal standard and linear response curves for known monohydroxy-(ricinoleic acid) and dihydroxy-octadecenoic acids were established.

TLC analyses were performed on either 5 × 20 cm or 20 × 20 cm Silica Gel 60 (0.25 mm thickness) plates (EM Science, Gibbstown, NJ) developed in hexane/2-propanol/acetic acid (80:20:0.1, vol/vol/vol). The chromatograms were visualized with 50% sulfuric acid/water spray and charring by means of a heat gun followed by vanillin spray and heating (13).

Product isolation. One gram of crude lipid extract was initially fractionated on a 30 × 2 cm column packed with 20 g of Silica Gel 60 (230–400 mesh). The column was eluted and fractions collected with 250 mL of hexane, then 50 mL of hexane/2-propanol (50:50, vol/vol), 100 mL hexane/2-propanol (20:80) and 100 mL and 50 mL 2-propanol. Products (992 mg) were recovered in the final three fractions.

One major product has been isolated from one of the fractions by silica high-performance liquid chromatography (HPLC) using the solvent system reported by Gérard *et al.* (14) and a modified gradient. The HPLC system consisted of a Spectra Physics (San Jose, CA) 8800 solvent delivery system, a Dynamax 60A 250 × 21.4 mm i.d. silica column (8 μ), and a Varex Evaporative Light-Scattering Detector (ELSD II; Varex Corporation, Rockville, MD). Solvent flow was 4 mL/min and was split after the column to send 5% to the detector. Fractions were collected manually as peaks eluted.

Product identification. This preliminary product identification was assessed through GC retention times, TLC, and GC–mass spectrometry (GC–MS) as compared to known compounds. Electron impact (EI) GC–MS was run on an HP Model 5972 Mass Selective Detector coupled to an HP 5890 Series II gas chromatograph. Separation of components was achieved with an HP-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness) with a temperature program starting at 70°C, increasing at 20°C/min to 170°C with a 1-min hold, and then to 250°C at 5°C/min with a 15 min hold. Samples and known compounds were also run in EI, methane chemical ionization (CI), and isobutane CI as either methyl esters prepared with diazomethane or as trimethylsilyl (TMS) derivatives prepared by standard techniques with Sylon BTZ (Supelco, Inc.) on a Finnigan MAT TSQ 700 mass spectrometer (Finnigan Corp., San Jose, CA) equipped with a Varian GC and an A200S Autoinjector (Varian Associates, Inc., Sugarland, TX). A DB-1 capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness) was held at 80°C for 1 min after injection, programmed to 200°C at 40°C/min, then to 250°C at 5°C/min where it was held for 10 min.

Proton, ^{13}C NMR spectra, and heteronuclear correlations were determined on the fatty acid dissolved in deuterated chloroform with a Bruker ARX 400 spectrometer (Billerica, MA) operating at 400 MHz and 100 MHz, respectively.

RESULTS AND DISCUSSION

During a previous screening of *Bacillus* for the ability to hydroxylate oleic acid, two strains—NRRL BD-174 and BD-226—were reported to possess the capability to convert oleic acid to the unique 15-, 16-, and 17-hydroxyoctadecenoic acids in more than trace amounts (12). In our program to discover novel bioconversion pathways and products, an additional 58 *Bacillus* strains selected from the ARS Culture Collection were examined. One organism—NRRL BD-447—converted oleic acid to three groups of products with GC retention times previously observed for mono-, di-, and trisubstituted oleic acid derivatives (Fig. 1). Group identifications were confirmed by TLC by comparison with the R_f of known compounds.

Preliminary structure determination was performed by EI and CI GC–MS of the methyl esters. Products were often not completely separated by GC, and it was obvious in some cases that mass spectra of compounds overlapped. Some useful data was derived from these spectra, however. EI spectra of the methyl esters of the major products in the "disubstituted region" (Fig. 1D) have the two largest fragment ions at m/z 159 [-CHOH-$(CH_2)_5$-$COOCH_3$] and 127. No molecular ions were seen. TMS derivatization of the methyl ester and of the free fatty acid shifted the base peak at *m/z* 159 to *m/z* 231 and 289, respectively. The fragment ion at *m/z* 127 remains but is much smaller. These MS data are consistent with $C_{7,8}$ bond cleavage of a fatty acid with hydroxy substitution on the C_7 carbon and subsequent loss of methanol (15). Production of C_7-hydroxy substituted fatty acids has been reported in fungi (16) and in *Pseudomonas* (6).

While these same base peaks are in all the major products, small differences, such as *m/z* of 2 or 14, are seen in fragment ions. These could be attributed to unsaturation, substitution on an adjacent methylene group, or addition of an oxygen atom as a keto- or epoxy-moiety. In addition, prominent ions at *m/z* 145, 131, and 117 were observed in successive peaks.

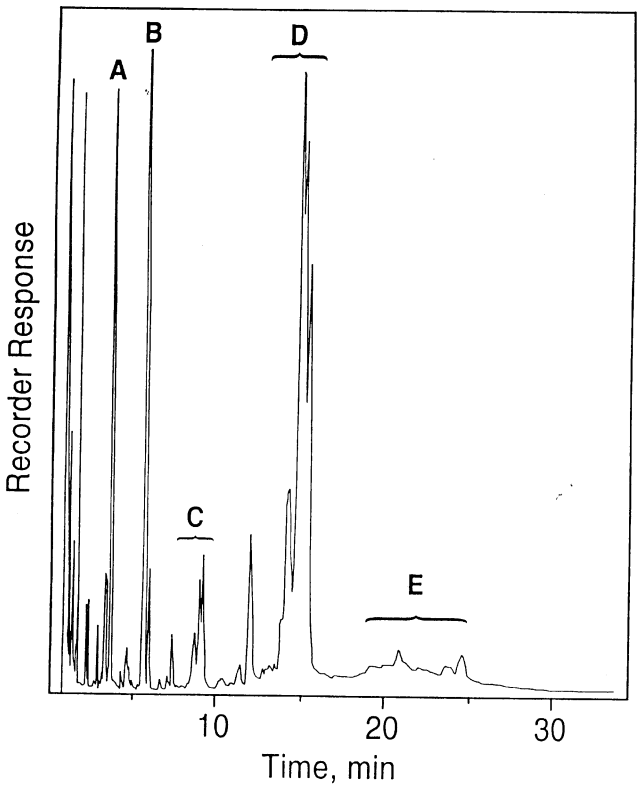

FIG. 1. Gas chromatogram of methyl esters recovered after bioconversion of oleic acid by *Bacillus* sp. NRRL BD-447. Peaks were identified as (A) methyl palmitate (internal standard); (B) methyl oleate, (C) monosubstituted products, (D) disubstituted products, and (E) trisubstituted products.

These are the same ions which were identified previously in the 15-, 16-, and 17-hydroxyoctadecenoic acids (12).

Methane CI provided evidence for mono-hydroxy octadecenoate derivatives [*m/z* 313, MH^+; base peak at *m/z* 281 $(MH - 32)^+$], as well as compounds with base peaks at *m/z* 279 and 277 for the methyl esters. With isobutane CI of the TMS esters and ethers, several *m/z* fragments repeatedly appeared: 441, 457, 531, 545, 547, and 619. Plotting these as extracted ion chromatograms suggests there may be two or three series of compounds that contain multiple functional groups (Fig. 2). In this procedure the ion abundance in all scans containing the specified *m/z* is plotted as normalized abundance. RIC (reconstructed ion chromatogram) shows the total ion abundance in each scan. Disubstituted products gave base peaks at $[MH - 90]^+$ and smaller ions at *m/z* 531 (dihydroxyoctadecenoate) and at *m/z* 457, possibly a keto-hydroxyoctadecenoate. Trifunctionality products had MH^+ ions at *m/z* 619 and 545, which could be trihydroxyoctadecenoates and dihydroxy-keto-octadecenoates, respectively. None of the monohydroxy, dihydroxy, or trifunctionality products has been purified yet.

One of the bioconversion products with GC (Fig. 1D) and TLC retention times similar to known disubstituted octadecenoates has been isolated by HPLC to greater than 95% purity. Based on the palmitic acid internal standard, this compound represents 36% of the "disubstituted" products and 7% of the oleic acid added to the culture. Structure analysis was performed on this product with GC–MS and NMR. The EI spectrum of the methyl ester had a base peak at *m/z* 159. As seen before, this fragment was indicative of a C_7 hydroxy substitution. The fragment ion at 127 could result both from a loss of methanol from the base peak (159–32) and from $C_{10,11}$ cleavage to yield an eight-carbon fragment with an oxo substitution toward the CH_3 terminal end of the molecule [e.g., CH_3-CH_2-CO-$(CH_2)_5$-]. This would account for the large fragment ion at *m/z* 127 seen previously for the methyl ester and the smaller ion peak after TMS derivatization. No molecular ion was seen. Methane CI provided proof of a molecular weight of 326 with a base peak at *m/z* 309 $[MH - 18]^+$, *m/z* 277 $[MH - (18 + 32)]^+$, small fragments at *m/z* 325 and 327 $[(M - H)^+$ and $MH^+)]$ and at 355 [M + 29]. Isobutane CI of the TMS derivative of the fatty acid gave a molecular ion $[MH^+]$ at *m/z* 457 and a base peak at *m/z* 367

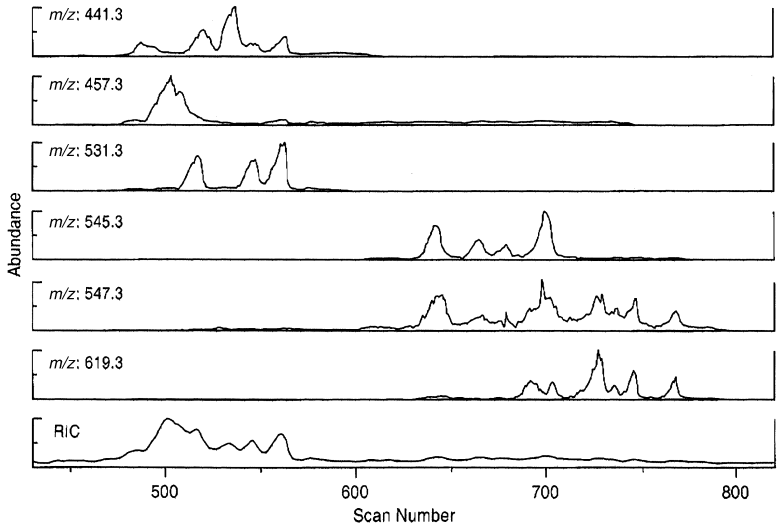

FIG. 2. Extracted ion chromatograms of repeating ion fragments in the isobutane chemical ionization mass spectra of di- and trisubstituted bioconversion products. RIC = reconstructed ion chromatogram.

$[MH - HO\text{-}TMS]^+$ which indicates a molecular weight of 456, consistent with a hydroxy-keto-octadecenoic compound.

The position of the keto group was determined to be at C_{16} by comparison of the EI mass spectra of this isolated compound before and after $NaBH_4$ reduction of the oxo functionality to hydroxyl (17) followed by TMS derivatization. The fragment ion at *m/z* 57 [CH_3-CH_2-CO-] is replaced by a fragment ion at *m/z* 131 [CH_3-CH_2-CH-OSi$(CH_3)_3$] confirming the oxo substitution at C_{16}.

Proton NMR analysis of this isolated bioconversion product provided evidence for unsaturation, keto and hydroxy moieties, and for one methylene group between the hydroxy and double bond. The configuration of the double bond was determined to be *cis* because the observed coupling of the 9,10 protons is 10.8 Hz (Table 1). The hydroxyl-bearing carbon is separated from the double bond by one methylene, because the methylene protons observed at 2.18 ppm are coupled to a single proton at 3.55 ppm and an olefinic proton at 5.38 ppm. ^{13}C NMR spectra showed a single peak at 71.3 ppm, characteristic of a hydroxyl attachment, and the distinctive peak at 212.1 ppm confirmed keto substitution (Table 1). The following resonance signals were also present: two olefinic carbons at 125.3 and 133.2, methylene carbons from 23.7 to 42.3 ppm, and the terminal methyl carbon at 7.8. Tulloch (18) has tabulated the ^{13}C chemical shifts in spectra of methyl oxooctadecanoates. The signal for the terminal methyl carbon (C_{18}) was found at 7.82 ppm when the oxo-group was at the C_{16} position. These data, together with the GC–MS data, confirm that this bioconversion product is 7-hydroxy-16-oxo-9-*cis*-octadecenoic acid.

To my knowledge, this is the first report of the unique combination of keto, hydroxy, and unsaturation in the same molecule produced by a *Bacillus* species. Apparently, this microorganism produces a series of compounds which could be positional isomers containing multiple hydroxy and keto substitutions. Further purification and identification studies are underway.

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TABLE 1
Proton and ^{13}C Nuclear Magnetic Resonance Signals for the Isolated Bioconversion Product

Carbon number	Proton resonance (ppm)	Hydrogens	Coupling constant, J (Hz)	^{13}C resonance (ppm)
1	—-			178.9
2	2.32	2		35.4
3	1.62	2		24.7
4	1.32	2		29.4
5	1.46	2		25.3
6	1.46	2	6.5	36.5
7	3.55	1	6.5	71.3
8	2.18	2	7.4	35.4
9	5.38	1	10.8, 7.4	125.3
10	5.52	1	6.7	133.2
11	2.02	2	7	27.2
12	1.30	2		29.0
13	1.30	2		28.6
14	1.55	2		23.7
15	2.38	2		42.3
16	—-			212.1
17	2.39	2		35.9
18	1.03	3		7.8

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