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Conversion of Unsaturated Fatty Acids by Bacteria Isolated from Compost

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Abstract. A compost mixture amended with soybean oil was enriched in microorganisms that transformed unsaturated fatty acids (UFAs). When oleic acid or 10-ketostearic acid was the selective fatty acid, *Sphingobacterium thalpophilum* (NRRL B-23206, NRRL B-23208, NRRL B-23209, NRRL B-23210, NRRL B-23211, NRRL B-23212), *Acinetobacter* spp. (NRRL B-23207, NRRL B-23213), and *Enterobacter cloacae* (NRRL B-23264, NRRL B-23265, NRRL B-23266) represented isolates that produced either hydroxystearic acid, ketostearic acid, or incomplete decarboxylations. When ricinoleic (12-hydroxy-9-octadecenoic) acid was the selective UFA, *Enterobacter cloacae* (NRRL B-23257, NRRL B-23267) and *Escherichia* sp. (NRRL B-23259) produced 12-C and 14-C homologous compounds, and *Pseudomonas aeruginosa* (NRRL B-23256, NRRL B-23260) converted ricinoleate to a trihydroxyoctadecenoate product. Also, various *Enterobacter*, *Pseudomonas*, and *Serratia* spp. appeared to decarboxylate linoleate substrate incompletely. These saprophytic, compost bacteria were aerobic or facultative anaerobic Gram-negative and decomposed UFAs through decarboxylation, hydroxylation, and hydroperoxidation mechanisms.

Humus in soil is a dynamic organic residue resulting from extensive microbial activity on decaying plant and animal matter [19]. Taxonomy of bacteria isolated from humus, composts [7, 12], and bioremediations [3] suggests natural enrichments influenced by organic substrates. Aside from the isolates of bioremediated soils [2, 17], enriched bacterial populations of humus and compost formations have not been well characterized.

Various composts contain mono- and dihydroxystearic acids, the probable products of microbial action on plant and animal lipids [19]. Isolation of compost and sewage bacteria that convert oleic to 10-hydroxystearic acid (10-HSA) [12, 20] and 7,10-dihydroxy-8(E)-octadecenoic acid [11] supports this conjecture. Presumably, the oleic content in plant and animal lipids influences the enrichment of these oleic-converting bacteria. Accordingly, compost mixtures purposely amended with either vegetable or animal oils may serve as models for studying the dynamics of lipid decomposition and conversions.

The present work describes a model for lipid decomposition in compost by examining unsaturated fatty acid (UFA) conversion to oxygenated products and shorter-chain length (C-C units) fatty acids by diverse organisms isolated from lipid-amended compost. Enrichment and selective methods may distort the diversity of the saprophytic microbes involved [3] but enhance the likelihood of isolating UFA decomposers. Results indicate that mainly aerobic and facultatively anaerobic Gram-negative bacteria mediate UFA decomposition through decarboxylation, hydroxylation, and hydroperoxidation mechanisms.

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Materials and Methods

Compost. In July 1996, approximately 40 kg (80 pounds) of loose top soil was mixed with equal masses of horse manure–sawdust and decaying garden leaves–lawn grass mixtures. Three 300-ml applications of soybean cooking oil were mixed onto the top layer of this

Table 1. Compost bacterial isolates capable of ketofatty acid and unsaturated fatty acid (UFA) conversions[a]

Fatty acid[b] (total no. of isolates)	Reactive strains[c]		Products	Bacteria involved
	Number	Reaction type		
Oleic (74)	9	Hydroxylation	10-Ketostearic acid (10-KSA); 10-hydroxystearic acid	*Sphingobacterium thalpophilum; Acinetobacter* spp.
Oleic (35 of 74)	18	[β-Oxidation][d]	[CO_2; short-chain fatty acids]	
10-KSA (40)	15	Incomplete decarboxylation	6-Ketotetradecanoate; 4-ketododecanoate	*Enterobacter cloacae*
Ricinoleic (39)	18	Incomplete decarboxylation	12-C and 14-C homolog of ricinoleic acid	*Enterobacter cloacae; Escherichia* sp.; *Pseudomonas aeruginosa*
	2	Hydroxyperoxidation	7,10,12-Trihydroxyoctadecenoate	*Pseudomons aeruginosa*
Linoleic with hexane (42)	12	Incomplete decarboxylation	14-C and 16-C homologs of linoleic acid	*Pseudomonas putida; P. viridilivida; Serratia plymuthica*
	7	[Monooxygenation]	[Monooxygenated 18-C fatty acids]	
	2	[Multioxygenation]	[Multioxygenated fatty acid]	

[a] Compost consisting of about equal mass amounts of decaying plant residue, sawdust–horse manure, and topsoil, and amended with soybean oil.
[b] The specific fatty acid was added to enrichment broths, selection plates, and assay broths to evaluate bioconversion (see Methods).
[c] Colonies were picked at random from selection plates. Bioconversion products were detected by gas chromotography and mass spectrometry.
[d] [ ] Specifies undetermined reaction type or products.

outdoor compost heap at 2-week intervals. Thereafter, soybean oil was added at monthly intervals; the protocol was carried out for 11.5 months. Samples for enrichment cultures were taken from the top layer after 3, 10, and 12 months and stored in loosely capped jars at room temperature.

Enrichment broth and selection plates. Compost (1.5–2.0 g) and selective UFA (0.5 ml) were added to 100 ml enrichment medium (EM) containing (per L): 5 g glucose, 0.3 g yeast extract, 4.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 15 mg $FeSO_4 \cdot 7H_2O$, and 1 ml trace minerals [12]. The EM was adjusted to pH 7.3 before autoclaving. Because it was not readily miscible with water, linoleic acid (0.5 ml) was premixed with such dispersing agents as 0.1 ml *n*-octanol, 4 ml potassium stearate suspension (2.5% wt/vol), or 1.2 ml hexane. An oxygenated fatty acid, 10-ketostearic (KSA), was prepared also as a 7.5% substrate (wt/vol) in 90% ethanol (vol/vol) and added in 1.35-ml amounts per 50 ml preheated EM. The KSA-containing broth was cooled before adding a compost inoculum.

The inoculated broth was incubated aerobically (Gump rotary shaker at 200 rpm) at 28°C overnight and readjusted to pH above 7.0 with dilute NaOH. Daily microscopic examinations indicated that the neutral pH suppressed fast-growing yeasts and molds. After the fourth day, the regimen was repeated once or twice in fresh UFA-containing EM (2% inoculum) before dilution-plating onto a selection agar medium. The plates of EM nutrients contained (per L): 17 g agar, 25 mg bromo-cresol green indicator, and 2.0 ml UFA [12]. With the exception of *Pseudomonas aeruginosa* colonies which gave distinct, clear zones, other bacterial colonies on the agar surface were not distinctive. Consequently, discretely isolated colonies were randomly selected for activity screening. All cultures were maintained on tryptone-glucose-yeast extract (TGY) agar slants and stored at 4°C. Identity of the reactive cultures was established with the Biolog System (Biolog Inc., Hayward, CA).

Bioconversion assay. Cultures were transferred to fresh TGY broth (pH 7.0) and incubated aerobically for 2 days before transferring (0.3 ml inoculum) into 30 ml assay broths. The broth (pH 7.3) for assaying the decomposition or bioconversion of UFA contained (per L): 5.0 g yeast extract, 4.0 g glucose, 4.0 g K_2HPO_4, 250 mg $MgSO_4 \cdot 7H_2O$, and 10 mg $FeSO_4 \cdot 7H_2O$ [13]. The inoculated broth in an 125-ml Erlenmeyer flask was incubated overnight at 28°C and readjusted to pH > 7.0 before adding 0.3 ml UFA. After 2–3 days of incubation with fatty acid substrate, the fermentation broth was acidified and extracted twice in one volume of methanol:ethyl acetate (1:9, vol/vol). The total extracts containing residual fatty substrate and bioconversion products were concentrated with a rotary evaporator and dried in an evacuated desiccator before weighing.

GC-MS analysis. Dried lipid extracts (approximately 10 mg) were treated with diazomethane and analyzed by GC (Hewlett Packard 5890 Series II Gas Chromatograph instrument equipped with a SPB-1 capillary column and coupled to an HP7673 Auto Sampler and Chem Station accessories; Palo Alto, CA [15]. The GC samples diluted in solvent were chromatographed with a temperature program (190°–230°C) of 2°C/min for 7 min, 5°C/min for 5 min, and isothermal 230°C for 12 min. Emergent peaks were identified by retention times and area % of total peaks relative to standard compounds. Bioconversion products detected by GC were characterized further by an electron impact GC-MS equipment (HP 5890 Gas Chromatograph coupled to an HP-5 capillary column and 70eV HP 5972 Mass Selective Detector).

Biochemicals. Technical (>90% purity) and GC-analytic (99% purity) grade oleic, linoleic, and ricinoleic acids were purchased from Nu Chek Prep, Inc. (Elysian, MN) and used without further purification. All other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Analytic grade UFAs were used in assay broths to determine bioconversions. However, crude 10-KSA (20–90% estimated purity by GC peak areas) was prepared by oleate bioconversions using either *Nocardia* [14] or our current cultures.

Results

Enrichment cultures from commercial compost yielded *Sphingobacterium thalpophilum* (NRRL B-14797) and *Acinetobacter* sp. (NRRL B-14920, NRRL B-14921, NRRL B-14923) isolates that converted oleic acid to 10-HSA [12] and oleyl wax esters [5, 13], respectively. Under similar enrichment conditions, fresh compost produced from decaying plant residues and horse manure amended with soybean oil yielded similar and other

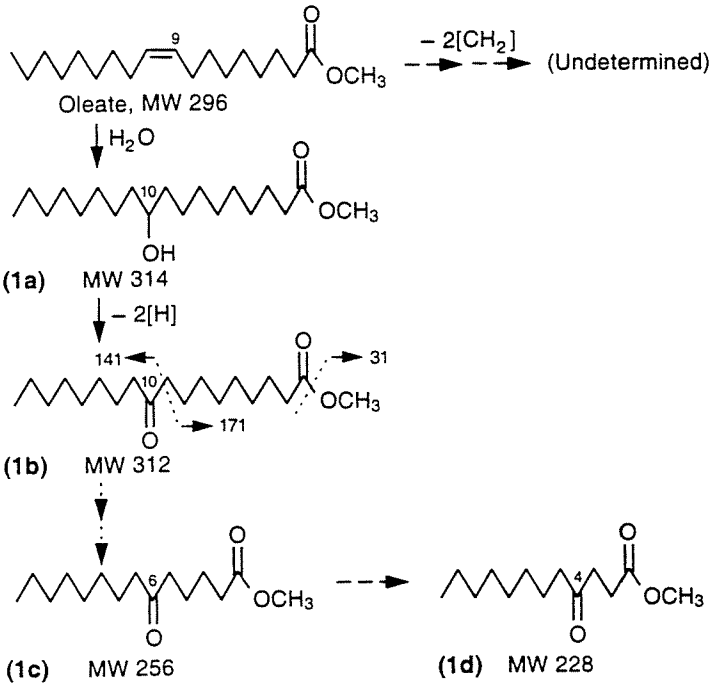

Fig. 1. Oleic acid bioconversion analyzed as methyl 9(Z)-octadecenoate, 10-hydroxystearate (a) and 10-ketostearate (KSA; b) when detected by gas chromatography–mass spectrometry (GC-MS). Proposed (---), incomplete decarboxylation of KSA substrate detectable as 14-C (c) and 12-C (d) methyl ester homologs. Bioconversions with solid-line arrows designate structures previously determined by GC-MS and NMR analyses [4, 12, 14].

coexisting microorganisms (summarized in Table 1) that could either convert or decarboxylate UFA.

Conversion products from oleic acid. Seventy-four isolates from fresh compost were screened for hydroxy- or ketofatty acids by a thin-layer chromatographic procedure utilized previously [12]. When oleic acid was the selective UFA of agar plates, 12% of the isolates (9 of 74) converted oleic acid to a 10-HSA and 10-KSA mixture (Fig. 1a and b). In comparison, approximately 8% of the isolates (14 of 165) from commercial compost produced a monohydroxy- or monoketofatty acid. Also, a subset (18 of 35) of the current isolates consumed large amounts of oleic acid (270 mg added per culture), leaving less than one half (70–130 mg recovery range) as total extractable lipids. These cultures, designated type i, presumably decomposed the 18-C fatty acids to produce CO_2 and short-chained fatty acids. However, 8 of the 35 (type ii cultures) yielded extractable lipids without significant decompositions (230 mg mean). Nine of the 35 cultures converted liquid oleic acid to solidified product(s), yielding a range of 110–250 mg extractable lipids. The GC analyses of these 9 lipid samples suggested a wide range of 0–12 area% HSA and 1–79 area% KSA products. Mass spectrometry of one GC-separated product gave mass ion fragments (m/z) 201, 171, 169, and 143 [12], the expected values for methyl 10-hydroxystearate (MW314; Fig. 1a). Also, MS of the other product resembling KSA gave prominent mass ion fragments of m/z 199, 171, and 141 [14] expected for methyl 10-ketostearate (MW312; Fig. 1b).

Eight of the nine hydroxylating cultures (Table 1) were identified by the Biolog System to be *Sphingobacterium thalpophilum* (NRRL B-23206, NRRL B-23208, NRRL B-23209, NRRL B-23210, NRRL B-23211, NRRL B-23212) and *Acinetobacter* spp. (NRRL B-23207, NRRL B-23213).

Because 10-KSA appeared to be a dehydrogenation product of 10-HSA [14], KSA was also used as a selective fatty acid to follow the chain-length (2-C unit) decomposition of oleic acid (Fig. 1c and d). Screening for active isolates was evaluated by a GC-MS procedure. Fifteen of 40 random isolates yielded incomplete or partly decarboxylated acids. These byproducts were characterized to be the 14-C (Fig. 1c; mean of 9 area%) and 12-C (Fig. 1d; 55 area%) homologs of KSA. In addition to identical GC retention times, the homologs gave the molecular ion and fragments expected for 6-ketotetradecanoate (m/z 256, 225, 158, 141) and 4-ketododecanoate (m/z 228, 197, 141, 130, 98). Three bacteria possessing these incomplete decarboxylation activities were identified as *Enterobacter cloacae* A (NRRL B-23265, NRRL B-23266) and *E. cloacae* B (NRRL B-23264).

Bioconversion of ricinoleic acid. With ricinoleic (12-hydroxy-9-octadecenoic) acid as a selective UFA (Fig. 2), 20 of 39 isolates gave detectable bioconversion or decomposition products. Two of them generated extractable lipids (170 mg each) with MS profile and a GC peak (approximately 85 area%) identical to 7,10,12-trihydroxy-

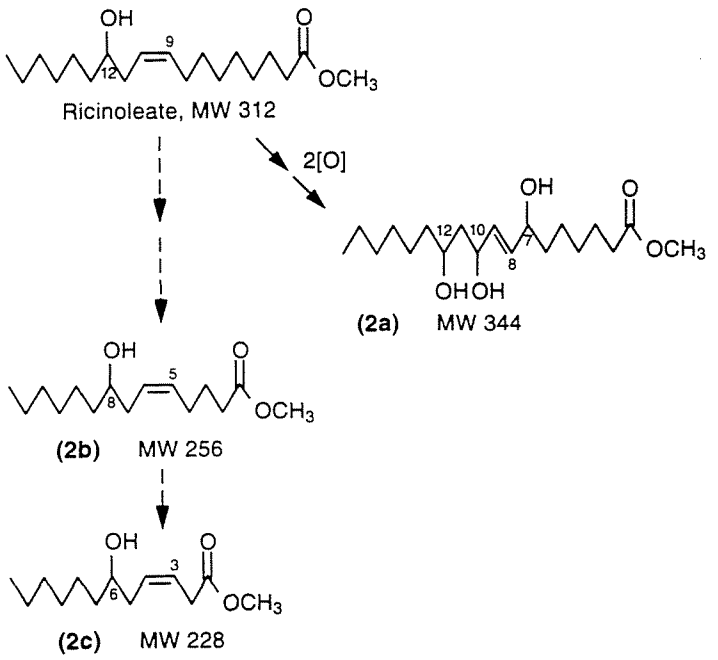

Fig. 2. Ricinoleate [9(*Z*)-12-hydroxyoctadecenoate] bioconversions to an 8(*E*)-7,10,12-trihydroxyoctadecenoate [15] (a) and incomplete decarboxylations (---) detectable as 14-C (b) and 12-C (c) homologs.

octadecenoate (Fig. 2a) [15]. These two isolates were similar to a *Pseudomonas* PR3 strain [11] and also identified as *Pseudomonas aeruginosa* (NRRL B-23256, NRRL B-23260). However, another oxidative (120 mg extractable lipids recovered) *P. aeruginosa* (NRRL B-23258) culture did not yield detectable amounts of the trihydroxyoctadecenoate product.

With the exception of NRRL B-23258, the remaining 17 positive cultures were type ii, giving more extractable lipids (140–350 mg range). Lipid extracts of the 18 positive cultures were identified by MS to contain residual ricinoleate (m/z 294, 198, and 166 ion fragments) substrate as well as homologous 14-C (Fig. 2b; m/z 238, 142, and 110 ion fragments) and 12-C (Fig. 2c; m/z 210, 114, and 82 ion fragments) compounds. A homologous 16-C (m/z 266, 170, and 138 ion fragments) compound was also detected in minor quantity (<4 area % of the total GC peaks). Three representatives with this biotype activity were identified as *Enterobacter cloacae* (NRRL NRRL B-23257, NRRL B-23267) and *Escherichia* sp. (NRRL B-23259).

Decomposition of linoleic acid. Soil bacteria have been found to convert oleate to ricinoleate-like compounds [18]. Because such activity was not found in our enrichment broths with oleate, approximately 300 mg linoleic acid was added either as the sole source of UFA (36 isolates selected) or as a mixture with *n*-octanol (33 isolates), stearate (32 isolates), or hexane (42 isolates) surfactants. None of the 143 isolates from compost produced ricinoleate-like compounds. Similar to the oleate-enriched cultures, however, the aerobic cultures gave two types of lipid extracts: type i producing 90 mg mean recovered lipid, and type ii producing 210 mg. When linoleate was added alone or mixed with surfactants (101 isolates not listed in Table 1), only 13 isolates gave significant amounts of partly decarboxylated compounds identified by GC-MS to be 7% (mean value) hexadecadienoate (Fig. 3a) with m/z 266 molecular ion and m/z 234 fragment, and 8% tetradecadienoate (Fig. 3b) with m/z 238 molecular ion and m/z 206 fragment. A representative with this biotype activity was identified to be an *Enterobacter agglomerans* biotype 5 (NRRL B-23214).

A hexane-containing subset of enrichment cultures (Table 1; 42 isolates) produced 21 linoleate-decomposing isolates. Twelve of the 21 cultures produced 14-C and 16-C homologs. In addition, seven gave lipid extracts containing triple GC peaks with retention times similar to monohydroxylated 18-C fatty acids. The MS analysis of each of the triple peaks suggested either an undetermined autoxidation or enzymic oxygenated product (m/z 310) originating from linoleate (MW 294). Two other active cultures produced 190 mg extractable lipid containing yet another unknown GC peak (approximately 1–7%) with retention times similar to trihydroxylated 18-C acids. Representatives of these linoleate-decomposing biotypes were identified to be *Pseudomonas putida* type A1 (NRRL B-23263), *Pseudomonas viridilivida*

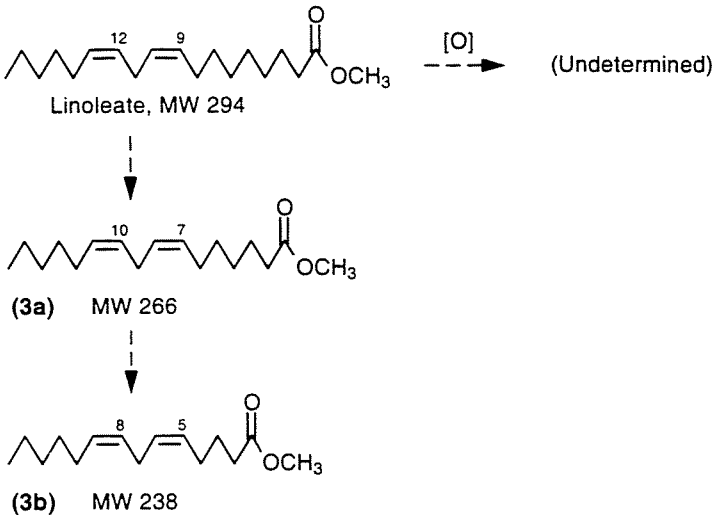

Fig. 3. Linoleic acid decomposition analyzed as methyl 9(*Z*), 12(*Z*)-octadecadienoate to 16-C homolog (a), 14-C homolog (b), and unexplained oxygenated product detectable by GC-MS.

(NRRL B-23261), and *Serratia plymuthica* (NRRL B-23262).

Discussion

Assessment of enrichment cultures suggests that the diversity of natural bacterial populations is distorted or underestimated by cultural procedures [3, 10] and that nutrient substrates and physical conditions can restrict growth to a few biotypes in humus [1] and bioremediated soils [2]. Notable studies on bioremediation demonstrate enrichment possibility in soils to enable the isolation of *Arthrobacter* strain KCC201 for degrading crude petroleum [1], *Pseudomonas aeruginosa* for utilizing gasoline [6], *Alcaligenes eutrophus* and *Burkholderia* sp. for decomposing, 2,4-dichlorophenoxyacetate [3], and *Sphingomonas* sp. RA2 for decomposing pentachlorophenol [2].

Studies indicate that only 0.1 to 10% of all soil bacteria [1] or a microbial community [3] has been identified. Our data suggest that 10% of the random selection convert oleate to hydroxy- or ketofatty acids. It follows that <1% of the bacterial compost population is our source of oleate-converting bacteria (Fig. 1). These consist of Gram-negative aerobic and facultative anaerobic bacteria. Also, many other oleate-decomposing species in compost bacteria remain likely to be determined, depending on enrichment conditions. Based on studies with *Corynebacterium*, *Mycobacterium* [4], and *Pseudomonas* [20], 10-HSA and 10-KSA syntheses from oleate require enzymatic intervention of a hydratase and dehydrogenase [14, 16], respectively. Presumably, *Sphingobacterium thalpophilum* and *Acinetobacter* sp. (Table 1) also synthesize 10-HSA and 10-KSA via the same mechanism.

Utilizing ricinoleate as a selective UFA substrate (Fig. 2), a *P. aeruginosa* strain produces trihydroxyoctadecenoate through an intermediate peroxidation mechanism. Similar to peroxidations of oleate [8, 9], an enzymatic peroxidation-hydroxylation at the C-10 position of 9(*Z*) isomer, a double bond shift from 9(*Z*) to the 8(*E*) position, and a third hydroxylation at the C-7 position may be coordinated events [15]. With linoleic acid substrate, undetermined mono- and multioxygenated compounds (Fig. 3) are produced sparingly through either autooxidations or enzymatic oxidations evident in *Enterobacter* sp. cultures.

Overall, the screening of bacterial isolates suggests that decarboxylations in 2-C units are the basic mechanism for microbial degradation of UFAs (Figs. 1–3). Although the decarboxylations were not monitored with oleate (Fig. 1), about one half of the isolates (18 of 35) may give type i cultures that yield significantly fewer extractable lipids. Incomplete 18-C decompositions evidently occur by removal of 2-C units from the carboxyl ends of UFAs. *Pseudomonas*, *Enterobacter*, *Serratia*, and *Escherichia* cultures may give incomplete decarboxylations, producing shorter-chained 16-C to 12-C homologs.

Our study suggests some of the possible enzymatic mechanisms used by compost bacteria to transform UFAs. Ideally, decomposition of insoluble fatty acids and lipid-like compounds [10] produces acetates and other water-soluble acids that become substrate for a highly diverse microbial population.

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