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10(*R*)-Hydroxystearic acid production by a novel microbe, NRRL B-14797, isolated from compost

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SUMMARY

A bacterium, NRRL B-14797, isolated from composted manure, converted oleic acid exclusively to 10(*R*)-hydroxystearic acid in 3-day batch cultures. 9(*Z*)-Unsaturated fatty acids in a lipid extract from soybean soapstock were also hydrated effectively. Aerobic bioconversions by isolate B-14797 were compared with those by *Pseudomonas* B-2994 and *Nocardia* 5767, which produce mixtures of 10-hydroxy- and 10-ketostearic acids. The results of studies with resting cells and cell-free extracts were consistent with action of a hydratase and absence of secondary alcohol dehydrogenase in strain B-14797.

INTRODUCTION

Microbial conversions of oleic acid to 10-hydroxystearic (HSA) and 10-ketostearic (KSA) acids are being investigated in order to develop a basis for industrial applications of the products [2,5,6]. Previous assessments of *Nocardia cholesterolicum* NRRL 5767 [5] and *Pseudomonas* sp. NRRL B-2994 [1,2] demonstrated that these strains convert oleic acid to a mixture of 10-hydroxy and 10-keto compounds. Anaerobiosis favors bioconversion to HSA [1,8]. Other microorganisms, i.e. *Staphylococcus* [6] and *Aspergillus terreus* strains UI 58 and ATCC 11156 [2], produce predominantly KSA. A third group, *Saccharomyces cerevisiae* NRRL Y-2034 and *Candida intermedia* UI 5159 [2] produce HSA exclusively. We have now studied a novel eubacterial isolate, NRRL B-14797, that produces only HSA aerobically.

Pseudomonas B-2994, *Nocardia* 5767 and isolate B-14797 were compared for their biotransformation capabilities by both one-step fermentation (growth/bioconversion) and resting cell procedures. Stereospecific hydratase activity [2,5,10] in cell-free extracts was determined to underlie hydration of unsaturated fatty acids by isolate B-14797.

MATERIALS AND METHODS

Pseudomonas sp. B-2994 and *Nocardia cholesterolicum* 5767, obtained from the NRRL Culture Collection (Agricultural Research Service, Peoria, IL, USA), were maintained on TGY agar slants containing (g L^{-1}): tryptone, 5: glucose, 1: yeast extract, 5; K_2HPO_4, 1. The medium was adjusted to pH 7.0 before autoclaving it. A colony designated NRRL B-14797, was isolated from an agar plate containing 0.2 ml oleic acid per 100 ml nutrient solution (pH 7.0–7.2). Isolate B-14797 originated from composted manure.

Morphologically, strain B-14797 is a non-motile, plump rod 1–3 micrometers in length coated with a capsule-like substance which often forms strands of 2–8 cells. The bacterium was tentatively identified by L. K. Nakamura (ARS Culture Collection) to be a *Sphingobacterium*, using an automated Biolog System (Biolog, Inc., Heyward, CA, USA).

Media, growth/bioconversion conditions and extraction

For one-step bioconversions, maintenance cultures were transferred to fresh TGY liquid medium and incubated on a rotary shaker (180 r.p.m.) overnight at 25 °C. These starter cultures were then used to inoculate (1%, v/v) 50 ml of the growth/bioconversion medium in 125-ml Erlenmeyer flasks. After 12–18 h aerobic incubation at 30 °C, 0.3 ml oleic acid was added to initiate the 3-day bioconversion. For substrates (50 mg or 0.05 ml) other than oleic acid, 25 ml medium per flask was used to determine growth/bioconversion in duplicate aerobic cultures at 30 °C.

Resting cells were prepared by centrifuging 24-h batch cultures grown in the appropriate medium (800 ml per Fernbach flask) and by suspending the cells in 50 mM sodium phosphate buffer (pH 6.8) containing 10 mM dithiothreitol (Cleland's sulfhydryl reagent) and 0.2 mM EDTA. Resting cell suspensions were stored at −70 °C and thawed before use. Aliquots of resting cells were also centrifuged, and the particulate, cellular fractions were lyophilized to obtain dry

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weights. The B-14797 suspension contained 13. 1 mg dry wt per ml, and the *Nocardia* 5767 suspension contained 9.9 mg ml^{-1}. Cell-free extracts were prepared from suspensions by cell breakage with 0.15-mm diameter glass beads in a Bead-beater™ (Biospec Products, Bartlesville, OK, USA), followed by centrifugation (15 000 × *g*) at 4 °C for 20 min.

Both isolate B-2994 [12] and isolate B-14797 elicited enhanced bioconversions in a modified Wallen fermentation (WF) medium containing (g L^{-1}): yeast extract, 5; glucose, 4; K_2HPO_4, 4; $MgSO_4 \cdot 7H_2O$, 0.5; and $FeSO_4 \cdot 7H_2O$, 0.01; and adjusted to pH 7.3 before autoclaving. For determination of glucose requirement, the WF medium contained 5 g L^{-1} amounts of either potassium gluconate, mannitol or glycerol as glucose replacements. Growth/bioconversion medium appropriate for noninduced *Nocardia* 5767 contained (g L^{-1}); yeast extract, 4; malt extract, 10; glucose, 4; and adjusted to pH 6.1 [5].

Culture pH was recorded following growth/bioconversion. Lipid product was recovered from cultures by acidification below pH 3 with 5 N H_2SO_4 and subsequent solvent extraction. The product was extracted twice with one volume of 10% (v/v) methanol in ethyl acetate [2] and concentrated in a rotary evaporator. The concentrate was solubilized in a small volume of methanolic ethyl acetate, dried over anhydrous Na_2SO_4, transferred to a small vial, reconcentrated to dryness over a stream of nitrogen, and then weighed after vacuum desiccation.

Product analyses

Lipid extracts of selected cultures grown in WF medium were analyzed by TLC on Silica Gel 60 plates (EM Sciences, Cherry Hill, NJ, USA) developed in a solvent system of ethyl acetate:hexane:glacial acetic acid (50:50:1, v/v). Unsaturated compounds were made visible by exposure to iodine vapor; all other spots were made visible by a color-enhancing combination of sulfuric acid–vanillin sprayings and heating at 130 °C [4,11].

Dried samples (1–3 mg) were esterified with diazomethane and analyzed by capillary-column GC. Operating conditions for the GC included He carrier gas flow of 1 ml min^{-1}, 230 °C injector port temperature, 250 °C detector temperature, and flame ionization detection. Purities or extents of substrate conversions (method A) were estimated from the percentages of total peak areas. For quantitative hydroxylation determinations (method B), 25 mg of palmitic acid was used as an internal standard to each 25 ml of medium, which contained 140 mg oleic acid substrate [5]. Accordingly, ether extraction was employed in order to obtain a HSA/palmitic acid peak area ratio. The amount of HSA product was determined from a standard curve of peak area ratios generated when 0.5 mg of palmitic and 0.1–3.0 mg HSA served as standard acids. All GC samples were chromatographed (185 °C) on a SPB-1 capillary column (15 m × 0.32 mm i.d.), and emergent peaks were identified according to retention times relative to standard compounds.

GC/MS was used to confirm the structure of hydroxylated fatty compounds [2,5], which were separated on a 25-m fused silica capillary column in tandem with a Hewlett-Packard 5970 Mass Selective Detector (70 eV; Palo Alto, CA, USA). Separations were conducted by temperature programming from 160–220 °C while ramping at 5 °C per min, and holding at 220 °C for 15 min. Molecular ion and mass ion fragments (5,6,9] were used to identify the oxygenated fatty esters.

The ^{1}H-NMR spectra of methyl-10-hydroxystearate derivatives in $CDCl_3$ solutions were obtained with a Bruker ARX 400 MHz Spectrometer (Bruhe, Billerich, NH, USA). The diastereoisomeric (*S*)-(+)-*O*-acetylmandelate esters of methyl-10-hydroxystearate samples were prepared according to the procedure of El-Sharkawy et al. [2,13] in order to determine the C-10 hydroxyl configuration. Two signals (3.6693 and 3.6639 p.p.m.) were analyzed for the methyl ester protons (CO_2CH_3) of the resulting diastereoisomers. Thus, a racemic HSA was synthesized by $NaBH_4$ reduction of 10-ketostearic acid [2,13] for comparison with HSA produced by isolate B-14797.

Biochemicals and substrates

Oleic [9(*Z*)-octadecenoic] acid and all other reagent grade chemicals were purhcased from Sigma Chemical Co. (St Louis, MO, USA) and used without further purification. Fatty acid substrates screened for bioconversions included: linoleic [9(*Z*)-12(*Z*)-octadecadienoic], ricinoleic [12(*R*)-hydroxy-9(*Z*)-octadecenoic], elaidic [9(*E*)-octadecenoic], petroselinic [6(*Z*)-octadecenoic], and *cis*-vaccenic [11(*Z*)-octadecenoic] acids and oleyl alcohol. 10-Ketostearic acid was purified (> 99%) from bioconversion products in our laboratory.

Crude soybean soapstock from ADM (Decatur, IL, USA) [3] was adjusted to pH 7.4, diluted to 10% (w/v) and autoclaved before use. A dried, soapstock extract was also prepared by acidification and methanolic ethyl acetate extraction of the crude soapstock, and by subsequent rotary evaporation of the solvent. Both types of soapstock were added in 180 mg amounts to 25-ml cultures.

RESULTS AND DISCUSSION

Screening of soil-compost microorganisms yielded a novel eubacterial isolate, B-14797, that transformed approximately 70% of oleic acid in a yeast extract–glucose (WF) growth medium. For example, when 0.35 ml (approximately 0.32 g) of oleic acid was added to 35-ml aerobic cultures, a mean value of 0.23 g polar lipid product was recovered by solvent extractions. The isolate B-14797, grown initially in a WF medium for approximately 15 h at 30 °C, transformed subsequent additions of 1% (v/v) oleic acid completely in 3–4 days (Fig. 1(A)).

Biotransformation to a polar, long chain fatty acid was detected as a spot comigrating with HSA (R_F: 0.24) on solvent-developed TLC plates. As expected, the product was not detected with iodine vapor, and gave a purple spot with sulfuric acid–vanillin indicator sprays. Upon GC of the product (Fig. 1), the methylated fatty ester peak corresponding to oleate substrate decreased to 1% of total peak areas,

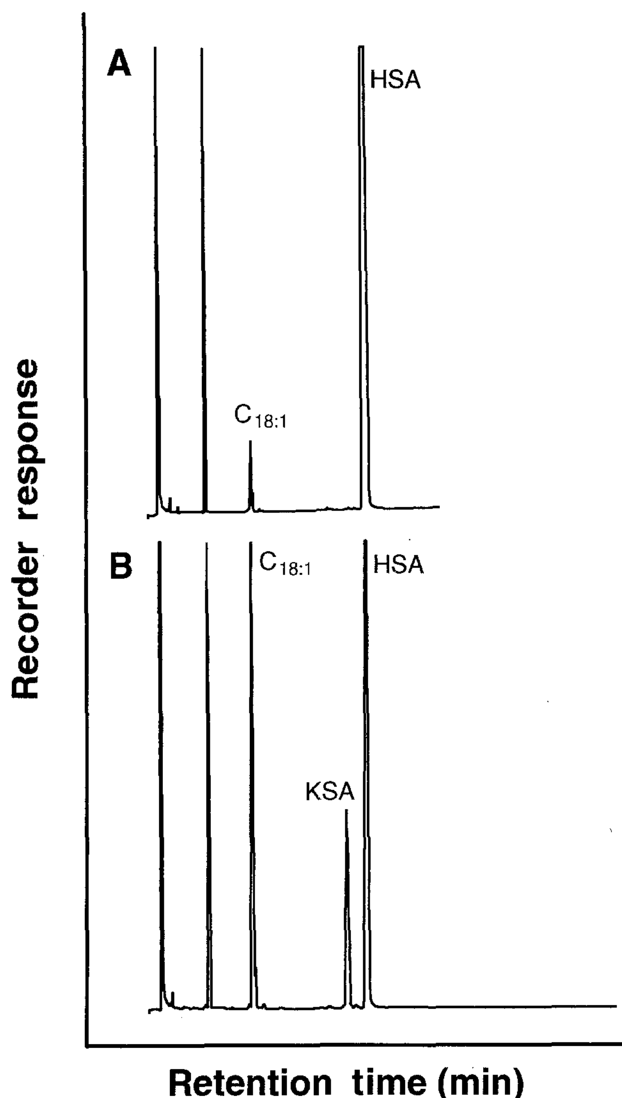

Fig. 1. Gas chromatograms of methyl esters recovered after bioconversions of oleic acid by isolate B-14797 (A) and *Nocardia* 5767 (B). Retention times relative to standards are labeled: $C_{18:1}$, oleate; HSA, 10-hydroxystearate; KSA, 10-ketostearate.

but only one major peak (75%) corresponding to methyl 10-hydroxystearate was detected.

GC/MS confirmed the identity of the product as methyl 10-hydroxystearate of molecular weight 314 [2,5,9]; i.e. prominent mass ion (m/z) fragments were 201 (M-113), 169 (201-31-1) and 143 (M-171). Similarly, a previously identified methyl 10-ketostearate of *Nocardia* 5767 [5 and Fig. 1(B)] yielded a molecular ion (312) and mass fragments: 281 (M-31), 214 (M-101+1), 199 (M-113), 171 (M-141), 156 (M-157+1) [6,9]. Thus, the isolate B-14797 produced HSA exclusively in aerobic batch cultures. Although 10-hydroxy- and 10-ketostearates have been found as mixtures (Fig. 1(B)) resulting from aerobic biotransformations of oleate by several microorganisms [1,5,6], an exclusive aerobic conversion to HSA has not been observed with eubacterial strains other than B-14797.

Similar to the HSA products of *Pseudomonas* strains B-2994 and B-3266 [10,13], HSA produced by B-14797 bioconversion was characterized as the 10(*R*)-hydroxystearic acid enantiomer by ^{1}H-NMR. The (*S*)-(+)-*O*-acetylmandelate derivative of methylated HSA in $CDCl_3$ gave the following 400-MHz ^{1}H-NMR signals: 0.88 p.p.m. (3H, t, J = 7, $18CH_3$), 1.0–1.3 (18H, m, $[CH_2]_9$), 1.35–1.39 (2H, m, H-9 or H-11), 1.5–1.7 (2H, m, H-3), 2.19 (3H, s, CH_3CO), 2.30 (2H, t, J = 7.5, H-2), 3.665 (3H, s, CO_2CH_3), 4.88 (1H, m, H-10), 5.87 (1H, s, H-2′), 7.37 (3H, m, H-3″, H-4″, H-5″), 7.47 (2H, m, H-2″, H-6″). Unlike racemic R,S enantiomers where two peaks are observed [2,13], only one signal appeared for the methyl ester protons with HSA derived from B-14797. This is consistent with a stereospecific 10(*R*)-hydroxy enantiomer.

Growth/bioconversion by strain B-14797

When one-step bioconversions by isolate B-14797 were compared with those by *Pseudomonas* B-2994 (Table 1), strain B-14797 gave only one product, HSA, with consistent high yields. Carbohydrates such as glucose, gluconate, mannitol or glycerol were added to a suboptimal growth medium [12] in order to identify repressed cultures [7]. Supplemented carbohydrates did not affect bioconversions by strain B-14797, but they resulted in erratic yields with B-2994. Erratic bioconversions with cultures of *Pseudomonas* B-2994 are unexplained although not unusual [1,12].

As in *Pseudomonas* B-2994 cultures [12], HSA production by isolate B-14797 (47 mg) was enhanced in a pH 7.0–7.3 WF medium, which decreased to pH 5.5–6.8 during growth/bioconversion. However, the HSA production (3 mg) was inhibited in a low pH 6.1 yeast extract/malt extract/glucose

TABLE 1

Growth/bioconversions of oleic acid[a] by *Pseudomonas* B-2994 and isolate B-14797 during growth on various carbon supplements

Carbon supplement[b]	Product of bioconversions by strains:				
	Pseudomonas B-2994			Strain B-14797	
	Recovered[c]	KSA[d]	HSA	Recovered[c]	HSA[d]
	(mg)	(Area %)		(mg)	(Area %)
Glucose	32–62[c]	0.5	0.4–4	80	97
Gluconate	15–63	0.6	0.8–8	89	88
Mannitol	43	0	2	ND	ND
Glycerol	50	0	1	84	97
None	8–27	0–2	1–40	76	89[a]

[a]One-step growth/bioconversion of 0.3 ml oleic acid in WF medium (see Methods) for 3–4 days at 30 °C. Without glucose, bioconversion by strain B-14797 after 2 days was 30% HSA.
[b]Carbon supplements (5 g L^{-1}) in place of glucose.
[c]Lipid product (mg) recovered by solvent extraction. Normalized to 100 mg oleic acid additions (method A). Ranges given for 3–6 replicate cultures.
[d]Area % by GC analysis. Abbreviations: 0, not detected; ND, not determined; HSA, 10-hydroxystearate; KSA, 10-ketostearate.

medium, when compared to that (21 mg HSA, 8 mg KSA) by *Nocardia* 5767. Even so, only HSA was formed by strain B-14797.

A time-course study of quantitative hydroxylations, normalized to 100 mg substrate, indicated that strain B-14797 produced 23 mg HSA after 1 day, 32–35 mg after 2–4 days, and 26 mg after 5 days. In contrast to a gravimetric estimate of 78% conversion (0.97 × 80; method A of Table 1), the palmitic acid internal standard method B indicated a 47% conversion of oleic acid to HSA. The samples of Table 1 apparently contained appreciable nonvolatile lipid or nonlipid materials.

Bioconversions comparing isolate B-14797 and Nocardia

Unlike *Nocardia* 5767 cultures, isolate B-14797 converted the 0.6% oleic acid substrate (v/v) efficiently in a one-step batch process. Two-day bioconversions, when 15-h cultures (100 ml) were supplemented before growth with 25 mg oleic acid, gave no detectable enhancement of oleic bioconversions (60% area HSA by GC analyses). However, all cultures required glucose as a stimulatory ingredient in WF medium (95% area bioconversion to HSA). Cultures grown without glucose exhibited only 30% area bioconversions.

Freeze-thawed resting cells of strains B-14797 and *Nocardia* 5767 gave maximum bioconversions when cell suspensions equivalent to 5 mg dry cells were incubated at 35 °C aerobically with 20 μl oleic acid for 8 h. For both strains, the maximum yield of HSA was 8–10 mg per 18 mg (20 μl) oleic acid. Greater concentrations (8–16 mg) of cell suspensions elicited a different pattern of bioconversion for the two strains. At 8 h and 8–16 mg, strain B-14797 suspensions still contained approximately 50% area oleic acid, whereas the *Nocardia* suspensions showed less than 1% area residual oleic acid. When incubation periods were extended to 36 h, however, the amount of bioconversion products by more concentrated suspensions (14–30 mg) were similar for both strains. These different patterns suggested a regulatory difference in cellular production of HSA by strain B-14797 and of HSA-KSA by *Nocardia* 5767.

Resting cells and cell-free extracts of both strains yielded hydroxylated products that were indicative of hydratase catalysis [1,2,5]. For example, aerobic cultures of *Nocardia* 5767 possessed both hydratase and enhanced secondary alcohol drhydrogenase activities [5]. Cell-free extracts of aerobic cultures of strain B-14797 gave consistent results, indicating absence of a secondary alcohol dehydrogenase and presence of a hydratase that is sensitive to air. Thus, an anaerobic mixture flushed repeatedly with nitrogen enhanced oleic acid bioconversion twofold (1.6 mg HSA produced from 9 mg oleic acid at 35 °C in 10 h). Under different conditions (30 °C for 2.6 days), 1 mg protein (BioRad protein assay) of the strain B-14797 cell-free extract converted 18 mg oleic acid aerobically to 0.5 mg HSA.

As was found for *Nocardia* [5], the strain B-14797 cultures converted compounds possessing both 9(*Z*)-unsaturation and carboxylic entities specifically (Table 2). Cultures of strain B-14797 converted oleic acid most readily (62–93%) and 9(*E*)-octadecenoic and 11(*Z*)-octadecenoic acids the least.

TABLE 2

Bioconversion of fatty substrates[a] by cultures of strain B-14797

Substrate	Recovered compound:	
	Substrate[b]	Polar C_{18} products
	Area %	
9(*Z*)-Unsaturated compounds		
Oleic acid	5–30	62–93
Linoleic acid	77–79	12–16
Ricinoleic acid	88–89	7–9[d]
Oleyl alcohol	98	2
Other unsaturates		
Elaidic acid (9-*E*)	99–100	0
cis-Vaccenic acid (11-*Z*)	100	0
Petroselinic acid (6-*Z*)	98	2
Soapstock (0-time control)[c]	67	0.5
Crude soapstock	11	1–5
Soapstock extract	34	26

[a]One-step growth/bioconversions in 25 ml WF medium (see Methods) obtained with oleic acid and other fatty substrates (50 mg or 0.05 ml).
[b]Substrate recovered after solvent extraction. Determined as % area of total GC peaks (Method A).
[c]Soapstocks added in 180 mg amounts per 25 ml cultures.
[d]Dihydroxy fatty ester.

In contrast to conversions by *Nocardia* 5767, however, bioconversions by strain B-15797 exhibited a 9(*Z*)-fatty acid preference in a descending order: oleic>linoleic>ricinoleic. Also, 6(*Z*)-octadecenoic acids and 9(*Z*)-octadecenol were not substrates.

With 180 mg of soapstock extract (67% oleic, linoleic and linolenic acids as substrate), B-14797 cultures grown in WF medium (25 ml) converted approximately 26% of the recoverable acids to hydroxy fatty acids (Table 2). Unprocessed, commercial (crude) soapstock was a poor substrate for this bioconversion. The GC analyses indicated that an initial 67% unsaturated fatty acid content of the crude soapstock decreased to 11%, apparently through metabolism of shorter-chain fatty acids. In contrast, the extracted fatty acids were poor metabolites, but they were utilized better for the hydroxy bioconversions than either crude soapstock or pure linoleic acid (12–16% conversion).

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