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Hydroxy Fatty Acids in *Bacteroides* Species: D-(−)-3-Hydroxy-15-Methylhexadecanoate and Its Homologs

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Acid hydrolysates of 140 strains, representing 11 species of the genus *Bacteroides*, were analyzed by capillary gas-liquid chromatography for total cellular fatty acid. All samples contained components which appeared to be hydroxy fatty acid. The relative amount and chain length distribution of the hydroxy fatty acids, as well as the nonhydroxy fatty acids, varied according to species. To characterize the presumed hydroxy acids, a composite of some 40 of these samples was analyzed by thin-layer and capillary gas-liquid chromatography, mass spectrometry, infrared spectrophotometry, and polarimetry. The hydroxy acids were shown to be of the D-(−)-3-hydroxy acid family. The predominant component was the iso-branched D-(−)-3-hydroxy-15-methylhexadecanoic acid. Lesser amounts of the iso-branched 15-carbon, straight-chain 16-carbon, and anteiso-branched 17-carbon acids were also found.

A number of workers (3, 6–8, 20, 21, 23) have reported that the genus *Bacteroides* contains iso- and anteiso-branched cellular fatty acids, predominantly of 15-carbon chain length, as well as branched long-chain bases of the sphinganine type (6–8, 21, 23, 24). In most such reports, no mention is made of the hydroxylated fatty acids, except, perhaps, as a comment that such compounds were not detected (3, 6, 7, 13, 14, 21), a generally unexpected result. The lone exception is the work of Stoffel et al. (23), who reported "3-hydroxyhexadecanoic" acid as a significant component of the cellular fatty acids of *Bacteroides thetaiotaomicron*.

In a survey of the fatty acid profiles of 140 strains representing 11 *Bacteroides* species (W. R. Mayberry, D. W. Lambe, Jr., and K. P. Ferguson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C39, p. 281), each sample, without exception, was found to contain several components whose gas chromatographic behavior suggested the presence of relatively high concentrations of branched hydroxylated fatty acids.

In an effort to define the spectrum of fatty acids, particularly the hydroxy acids, which can be found in the genus *Bacteroides*, 40 samples from the survey study were pooled to form a composite cellular fatty acid sample. This approach permits the identification of all the hydroxy fatty acids detectable, without regard to the presence, absence, or relative concentration of any individual compound in any given species.

This report, then, identifies the structure, configuration, and chain length distribution of the hydroxy fatty acids of *Bacteroides* spp., as derived from pooled samples from the survey study.

MATERIALS AND METHODS

Organisms. Strains representing the species *B. asaccharolyticus, B. disiens, B. distasonis, B. bivius, B. oralis, B. melaninogenicus* subsp. *melaninogenicus, B. melaninogenicus* subsp. *intermedius, B. melaninogenicus* subsp. *levii, B. fragilis, B. vulgatus*, and *B. thetaiotaomicron* were provided by D. W. Lambe, Jr., Department of Microbiology, East Tennessee State University College of Medicine.

Cells were grown in an anaerobic chamber, with an atmosphere of 80% N_2, 10% H_2, and 10% CO_2, in 10 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented (9) with yeast extract (5 g/liter), hemin (5 mg/liter), and menadione (0.5 mg/liter). After 48 h at 35°C, cells were harvested and washed free of medium by repeated centrifugation from distilled water at 4°C.

Hydrolysis and preparation of composite sample. Pellets were suspended in 2.5 ml of 2 N HCl and hydrolyzed for 12 h at 100°C in tubes with Teflon-lined screw caps. Hydrolysates were extracted once with 5 ml of $CHCl_3$, and the aqueous layers were discarded. The organic layers were treated with 2.5 ml of 1 N HCl in methanol and esterified for 1 h at 100°C. The cooled reaction mixtures were partitioned and washed several times with 5-ml volumes of distilled water. The esterified samples were evaporated to dryness under N_2. Gas-liquid chromatography (GLC) analysis of the individual samples was performed, both before and after trifluoroacetylation. The unused portions of 40 of these samples, selected to represent all of the species studied, were pooled and stored at 4°C to provide the composite sample described in this report.

The composite sample was subjected to acid meth-

anolysis to remove trifluoroacetate (TFA) groups, partitioned, and washed as described above. Equal portions were taken for chromatographic analysis of the total profile under various conditions of derivatization. The balance was separated by preparative thin-layer chromatography (TLC) into the nonhydroxy and hydroxy fatty acid ester fractions.

Chromatography. TLC was carried out on glass plates (20 by 20 cm) coated with 0.5-mm layers of Silica Gel H (EM Laboratories, Inc., Elmsford, N.Y.), activated at 60°C in a vacuum oven at 60 torr. Plates were developed to 15 cm in hexane-diethyl ether (1:1, vol/vol). Zones of interest were located by spraying the plates with water to transparency. Zones containing nonhydroxy esters (R_f 0.7 to 0.9) and hydroxy esters (R_f 0.3 to 0.5) were scraped from the plates, eluted under vacuum filtration with 5 ml of chloroform-methanol (1:1, vol/vol), and freed of residual silicate fines by repeated partition against water.

GLC was carried out with a Hewlett-Packard 5840A gas chromatograph equipped with flame ionization detectors. Samples were analyzed on a 10-m capillary column coated with the dimethylsiloxane liquid phase SP-2100 (J & W Scientific Co., purchased through Supelco, Inc., Bellafonte, Pa.), operated in the splitless mode, with a carrier gas (He) velocity of approximately 20 cm s^{-1}. Other operating conditions were: initial temperature, 90°C, held 30 s, followed by a 25-degree/min rise to 127.5°C, at which point was initiated the analytical temperature program of 4.5 degrees/min to a final oven temperature of 230°C. The splitless injection interval was 30 s, coincident with the initial temperature hold period.

Other analytical methods. Mass spectrometry was carried out with a Finnigan 4000 GC/MS spectrometer, operated in the electron impact ionization mode, at an ionizing energy of 70 eV. Samples were introduced via a probe heated from 50 to 250°C at 10 degrees/min.

Infrared spectra were acquired by using a Perkin-Elmer 710B infrared spectrophotometer with samples analyzed as dried films between salt plates.

Optical rotation was determined by using a Reichert model 3477 polarimeter. Samples were dissolved in chloroform, and the optical rotation was determined at the sodium D line.

Chemicals. Chloroform, methanol, and hexane were redistilled before use. Pyridine and diethyl ether (Fisher Scientific Co., Fairlawn, N.J.), acetic anhydride and bromine (J. T. Baker Chemical Co., Phillipsburg, N.J.), and trifluoroacetic anhydride (Eastman Kodak Co., Rochester, N.Y.) were used as received, as were the trimethylsilylating reagents hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS), and *N,O*-bistrimethylsilyltrifluoroacetamide (BSTFA), which were purchased from Sigma Chemical Co., St. Louis, Mo.

Standards. Straight-chain saturated nonhydroxylated fatty acids of both even and odd carbon number were purchased individually (Sigma Chemical Co.) and weighed to provide a standard mixture. Unsaturated fatty acids were obtained as components of standard mixtures ANHI-D and GLC-10 (Supelco, Inc.). Branched-chain fatty acids were obtained in standard mixtures BC-Mix L and BC-Mix 1 (Applied Science Laboratories, Inc., State College, Pa.). Straight-chain 3-hydroxy fatty acids of 12 and 14 carbons were prepared from acid hydrolysates of *Escherichia coli*, and fatty acids of 14 to 20 carbons were prepared from hydrolysates of *Acholeplasma axanthum* S743.

These groups of standards provided the basis for derivation of a least-squares regression equation for determination of "equivalent chain length" (ECL) as a function of relative retention time, nonadecanoic acid (C_{19} fatty acid; retention time, 21.25 min) being used as the reference compound. Under the temperature programming conditions used, retention time and length of carbon chain were linearly related for the methyl esters. These standards were also used to determine relative molar responses of the various fatty acid esters and their derivatives.

Derivative formation. Acetylated derivatives of the hydroxy esters were formed by heating the samples at 100°C for 30 min in 0.5 ml of pyridine-acetic anhydride (1:1, vol/vol). Trimethylsilyl (TMS) ethers were formed by incubating the samples at room temperature 30 min in 0.5 ml of a mixture of pyridine-HMDS-TMCS-BSTFA (2:2:1:1, vol/vol) (15). TFA derivatives were prepared by incubating the samples for 30 min at room temperature in 0.5 ml of trifluoroacetic anhydride-dichloromethane (1:2, vol/vol). Bromination was carried out by adding a few drops of Br_2 to the samples in 0.5 ml of chloroform and incubating for 30 min at room temperature. Reagents were removed under a stream of dry N_2, and the derivatized sample was dissolved in chloroform for analysis.

RESULTS

The spectrum of cellular fatty acids found in *Bacteroides* spp. is demonstrated in Fig. 1. This composite sample represents some 40 strains, encompassing the several species mentioned above. Therefore, the profile shown is not typical of any particular species, but indicates the types of fatty acids which might be found in members of the genus.

This figure also demonstrates the concentration sensitivity exhibited by underivatized hydroxy fatty acid esters during GLC, particularly on nonpolar columns. In the chromatogram of underivatized methyl esters, the small, poorly shaped group of peaks eluting between 18 and 20 min, which at first glance appear to be minor components of approximately 18-carbon chain length, are, in fact, the chromatographic "survivors" of the hydroxy esters. These esters, when appropriately derivatized, are seen to be major components of the mixture, i.e., approximately one-third of the total fatty acid ester in this particular composite sample.

Finally, Fig. 1 provides some insight as to the chromatographic behavior, on a nonpolar column, of the various derivatives of the hydroxy

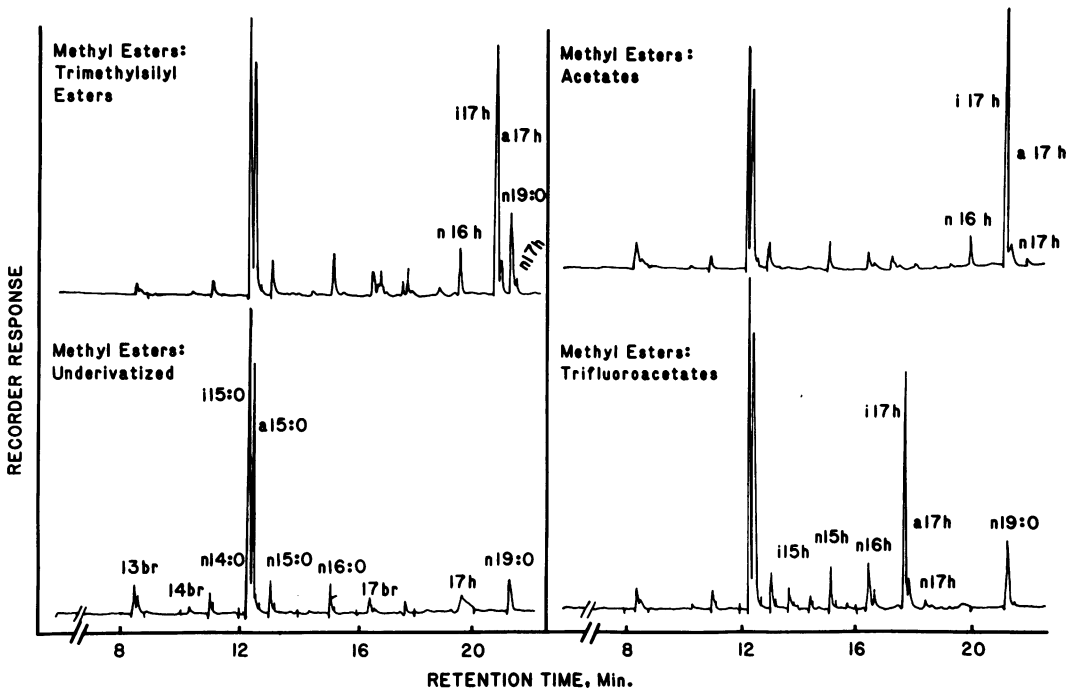

FIG. 1. *Capillary gas chromatographic patterns of methyl esters, cellular fatty acids of composite sample as various derivatives. Chromatography on a 10-m capillary of SP-2100. See text for conditions. Abbreviations: i, iso-branched; a, anteiso-branched; n, normal (straight chain); h, hydroxy; br, branched (position unspecified).*

acid methyl esters relative to the nonhydroxylated components of the mixture. These chromatograms indicate areas of potential interference and suggest that some derivatives might be more useful than others, depending on the information sought.

The tendency toward mutual interference is demonstrated numerically in Table 1, in which the hydroxy and nonhydroxy esters, after class separation by TLC, are arranged according to their chain length and branching, with their relative retention times indicated as ECL values. Note that, as the TFAs, the straight-chain hydroxy chain esters cochromatographed with the next-higher branched-chain nonhydroxy esters (e.g., n16h and i17:0 coincided at ECL = 16.6). As the acetates, the branched-chain hydroxy esters cochromatographed with the normal esters of two more carbons (e.g., i17h and n19:0 coincided at ECL = 19.0).

The major nonhydroxy acids in the genus *Bacteroides* are branched 15-carbon acids, a fact previously shown by a number of other workers. The branched 17-carbon hydroxy acids, in particular the iso-branched member, are the major hydroxy fatty acids (Table 1).

The relative constancy of the profiles (expressed as moles percent) of the hydroxy acids, when analyzed as any of the three derivatives (TFA, TMS, or acetate), indicates that unlike the underivatized compounds, the derivatized hydroxy acid methyl esters do not exhibit the concentration sensitivity toward GLC. As shown in the last column of Table 1 (for which a fivefold more concentrated sample was required to determine the ECL values), the lesser components of the underivatized sample were readily lost.

Finally, the fact that each of the hydroxy esters varied in ECL over a range of less than three carbon units, from the rapidly moving TFA to the much slower acetate, suggests that the compounds are monohydroxylated. Addition of another hydroxyl group generated an increase of only approximately 0.3 ECL units in the TFA derivative, but an additional 2 to 3 ECL units in the acetate and TMS derivatives. This correlates well with the TLC behavior of the hydroxy esters, which display an R_f of approximately 0.4 in the system used (16), compared with an R_f of approximately 0.1 for methyl 9,10-dihydroxyoctadecanoate.

None of the fatty esters, hydroxy or nonhydroxy, responded to bromination by a change of retention time or peak area, suggesting that all are saturated. Under the conditions used, all of the unsaturated esters and 30 to 50% of the cyclopropyl esters were removed from a sample of cellular fatty acid esters from *E. coli*.

TABLE 1. *Gas chromatographic behavior of fatty acid methyl esters of composite sample*

Chain identification[a]	Methyl esters									
	Nonhydroxy fatty acids (67%)[b]		Hydroxy[c] fatty acids (33%)[b]							
			TFA		TMS ethers		Acetates		Underivatized	
	ECL	Mol%	ECL	Mol%	ECL	Mol%	ECL	Mol%	ECL	Mol%
n12:0	12.0	tr[d]	12.6		14.2		14.4		13.5	
i13:0	12.6	2.8								
a13:0	12.7	1.3								
n13:0	13.0	tr	13.6		15.2		15.4		14.5	
i14:0	13.6	0.8								
a14:0	13.7	tr								
n14:0	14.0	2.8	14.6		16.2		16.4		15.5	
i15:0	14.6	41.9	15.3	5.2	16.8	4.8	17.0	4.7	16.1	0.8
a15:0	14.7	35.2	15.4	tr	16.9	tr	17.1	tr		
n15:0	15.0	5.4	15.6	1.8	17.2	1.9	17.4	0.6		
i16:0	15.6	0.2	16.3	1.3	17.8	1.6	18.0	0.6		
a16:0	15.7	tr	16.4		17.9		18.1			
n16:0	16.0	5.6	16.6	12.0	18.1	12.0	18.4	11.8	17.5	6.0
i17:0	16.6	2.6	17.3	69.4	18.8	69.5	19.0	71.9	18.1	93.2
a17:0	16.7	1.2	17.4	8.7	18.9	8.3	19.1	8.8		
n17:0	17.0	0.2	17.6	1.5	19.1	1.9	19.4	1.6		
i18:0	17.6	tr								
a18:0	17.7	tr								
n18:0	18.0	tr	18.6		20.1		20.4		19.5	

[a] Number of carbons/number of double bonds. i, Iso-branched; a, anteiso-branched; n, normal (straight chain).
[b] Percentage of total micromoles of fatty acid ester in composite sample.
[c] Hydroxy acids are D-(−)-3-hydroxy fatty acids.
[d] tr, Trace.

Infrared examination of the hydroxy ester fraction showed broad hydroxyl absorption centered at 3,500 cm^{-1}, the hydrocarbon multiplet between 3,000 cm^{-1} and 2,800 cm^{-1}, ester absorption at 1,740 cm^{-1}, and a doublet at 1,380 to 1,360 cm^{-1} attributable to a geminal dimethyl group (1, 19), suggesting iso-branching. The acetylated derivative yielded a spectrum devoid of hydroxyl absorption, with enhanced ester absorption, but no amide absorption at 1,650 to 1,690 cm^{-1}, eliminating the possibility of an amine group.

Mass spectral analysis of the hydroxy ester fraction showed a base peak at *m/e* 103, characteristic of 3-hydroxy methyl esters (16–19, 22) and a relatively strong peak at *m/e* 282, which agrees with the calculated (m^+-18) value for a hydroxyheptadecanoic methyl ester. The TMS derivative exhibited a base peak at *m/e* 175, also attributable to 3,4-cleavage of 3-hydroxy methyl ester (17, 18, 22), as well as a strong (M^+-15) peak at *m/e* 357, consistent with a molecular weight of 372 for the TMS ether of a hydroxyheptadecanoate methyl ester. The TFA derivative was sufficiently stable to show a significant molecular ion at *m/e* 396. An abundant ion at 353, ($M^+ - 43$), attributable to cleavage at the geminal dimethyl terminus of the compound (17, 18, 22), was also present. The abundance ratio (M^+-43/M^+) was somewhat greater than 2:1, as compared with (M^+-43/M^+) less than 1:2 in a spectrum of the straight-chain homolog, confirming the iso-branched structure.

The relative concentrations of the minor compounds of the hydroxy ester fraction were so low that the mass spectra were reflective almost exclusively of the major component. No ions attributable to 2-hydroxy esters were detected, indicating that such compounds were present in extremely low concentrations, if not absent.

Polarimetry indicated that the hydroxy ester fraction was levorotatory, $[\alpha]_D^{20}$ approximately −15 degrees $cm^3\ g^{-1}\ dm^{-1}$ (c.1) in chloroform, comparable to the values reported for the D-(−)-3-hydroxy straight-chain methyl esters of *A. axanthum* S743 (16), indicating that the hydroxy acids of *Bacteroides* spp. are of the D family in absolute configuration.

Table 2 shows the fatty acid profiles of typical strains of representative *Bacteroides* spp., the data taken from individual samples from the survey study mentioned above. These individual samples are among those pooled in the composite sample. The acids are identified on the basis of the conclusions drawn from the composite sample and presented in Fig. 1 and Table 1. The

TABLE 2. *Typical fatty acid profile of representative strains of Bacteroides spp.*

Fatty acid[a]	Fatty acid composition (mol%)					
	B. asaccharolyticus	*B. melaninogenicus* subsp. *levii*	*B. melaninogenicus* subsp. *melaninogenicus*	*B. oralis*	*B. fragilis*	*B. thetaiotaomicron*
n12:0	0.3					
i13:0	5.9		2.1	3.6		
a13:0				1.6		
i14:0			4.1	7.7		0.8
n14:0	3.4		0.3	0.7		1.1
i15:0	73.2	23.6	23.8	21.5	15.8	9.1
a15:0	2.2	11.6	44.3	42.9	32.6	32.2
n15:0	0.2				7.3	7.9
i16:0			0.4			
a16:0						
n16:0	1.6	4.2	3.0		3.6	4.0
i17:0	0.2	9.7				
a17:0		5.2				
n17:0						1.7
i15h	4.8	18.6		2.7		
a15h		1.1				
n15h					0.7	2.3
i16h	0.2			3.4		1.0
n16h		7.9	7.5	1.7	5.3	6.3
i17h	8.2	12.0	14.4	6.8	23.8	26.3
a17h		1.9		3.7	2.1	5.2
n17h					1.2	2.0

[a] See Table 1, footnote *a*, for abbreviations. h, Hydroxy.

quantitative values are presented as moles percent.

B. melaninogenicus subsp. *intermedius* displayed a profile similar to that presented for a typical strain of *B. melaninogenicus* subsp. *melaninogenicus*. *B. bivius* and *B. disiens* had profiles similar to that shown for *B. oralis*. *B. vulgatus* and *B. distasonis* had profiles similar to those shown for *B. fragilis* and *B. thetaiotaomicron*.

These data show that: (i) *Bacteroides* spp. contain hydroxy fatty acids as a significant component of their cellular fatty acids; and (ii) the predominant hydroxylated fatty acid is D-(−)-3-hydroxy-15-methylhexadecanoic acid.

DISCUSSION

It is tempting to suggest that the hydroxy acids of *Bacteroides* spp. arise by selective chain elongation of the nonhydroxylated fatty acids of two fewer carbons. This is supported by the fact that the predominant fatty acids are branched C_{17}, whereas the predominant nonhydroxy acids are branched C_{15}. Further support derives from the fact that the hydroxy acids are of the D-(−)-3-hydroxy family, which are intermediates of fatty acid biosynthesis, whereas the L-(+)-3-hydroxy acids are intermediates of β-oxidation (11, 12, 16). Stoffel et al (23) were unable to demonstrate incorporation of radioactivity into "3-hydroxy palmitate" from [1-^{14}C]palmitate, although label was incorporated into long-chain base fractions. This is consistent with the idea that the hydroxy fatty acids arise by chain elongation.

Fritzche and Thelen (3) describe a number of "unidentified fatty acids" in *Bacteroides* spp. These are of relatively high concentration, and most likely represent hydroxy acids.

Several other gram-negative genera have been reported to contain predominantly iso- and anteiso-branched nonhdroxy acids. Included are the aerobic gliding bacteria in the genera *Myxococcus*, *Stigmatella*, *Cytophaga*, and *Flexibacter* (2), as well as *Sporocytophaga* and the anaerobic to microaerophilic *Capnocytophaga* (5, 10).

Fautz et al. (2) have demonstrated 2- and 3-hydroxy, predominantly branched-chain C_{17} acids in their studies and have speculated that the apparent delay in finding these significant components of the fatty acid profile may be due to technical factors. Holt et al. (5) do not report such compounds in *Sporocytophaga* and *Capnocytophaga*, but these organisms may also contain hydroxy acids. Since some members of the genus *Capnocytophaga* have until recently been included in the genus *Bacteroides* (10), it is possible that some of the unidentified phospholipids reported by Holt et al. (4) are phospho-

phingolipids as reported in *Bacteroides* species by several groups (3, 6–8, 23, 24).

It can be seen in Table 2 that, in the *Bacteroides* species considered in this report, the hydroxy acid concentration varies with species, from approximately 10 to 50% of the total fatty acids, a concentration too high to be accounted for entirely as nonextractable (i.e., cell-bound, lipopolysaccharide-linked, etc.) fatty acid. In fact, 50 to 80% of the total hydroxy fatty acid, depending on species, can be found in the extractable lipid fraction (W. R. Mayberry, unpublished data). The chain length distribution also varies with species, in both the hydroxylated and nonhydroxylated acids, as does the branching pattern. The high resolution afforded by capillary column gas chromatography permits ready evaluation of these variations. The cellular distribution of hydroxy acids and the species-related variation of fatty acid profiles will be presented in subsequent reports.

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