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Cellular Distribution and Linkage of D-(−)-3-Hydroxy Fatty Acids in *Bacteroides* Species

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Two strains of *Bacteroides asaccharolyticus* and two strains of *Bacteroides fragilis* were analyzed for total fatty acid, total lipid fatty acid, and total bound fatty acid profiles. Extracted lipids and defatted cell residues were subjected to sequential alkaline and acid methanolyses to distinguish ester- and amide-linked fatty acids in each fraction. In the lipid fractions, all the ester-linked fatty acids were nonhydroxylated, whereas all of the amide-linked fatty acids were hydroxylated. In the nonextractable fractions, both hydroxy and nonhydroxy fatty acids were found in both ester and amide linkage, although hydroxy acids predominated. The fatty acid profiles of the bound fractions differed widely from those of the lipid fractions. Bound fatty acid represented approximately 10% of the total cellular fatty acids.

Members of the genus *Bacteroides* have been shown to contain hydroxy fatty acids as major constituents of their total cellular fatty acids (20). These acids, which are predominantly iso-branched and of the D-(−)-3-hydroxy structural family, comprise an apparently species-related proportion of the total profile, ranging from approximately 10% in some species to 50% or more in others (unpublished data). This relatively high level of hydroxy acid is too great to be accounted for as only cell envelope-bound (e.g., lipopolysaccharide) fatty acid.

Members of the genus *Bacteroides* have been shown to contain sphingolipids (9, 10, 11, 24, 25, 27), which suggests that the hydroxy acids may be associated with this fraction of the cell lipids as well. Therefore, studies were undertaken to determine the cellular location and linkage of the fatty acids of representative strains. This report describes the analysis of strains representing organisms with relatively low hydroxy acid levels and those with relatively high hydroxy acid levels.

MATERIALS AND METHODS

Organisms and culture methods. *Bacteroides asaccharolyticus* strains 207-72 and 783-75, which are representative of species with relatively low hydroxy fatty acid concentration, and *Bacteroides fragilis* strains 289-71 and 814-72A, which are representative of species having relatively high hydroxy fatty acid concentration, were provided by D. W. Lambe, Jr., Department of Microbiology, East Tennessee State University College of Medicine. These strains have been documented previously (12-15).

Cultures were grown in an anaerobic chamber with an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. Cells were grown in 5-ml lots of brain heart infusion broth (Difco) supplemented with yeast extract, hemin, and menadione (15). After 48 h of incubation at 35°C, 50 ml of each culture was harvested and washed free of medium by centrifugation at 4°C from distilled water.

Sampling and extraction. Cell pellets were suspended in 5 ml of distilled water, and 0.5-ml portions were taken for acid hydrolysis to determine total cellular fatty acid profiles. The balance of each suspension was extracted at room temperature by the addition of 100 ml of methanol and two 100-ml portions of chloroform, with stirring for 30 min between additions. After the final chloroform addition, resulting in a mixture of chloroform, methanol, and water (200:100:4.5 [vol/vol]), the samples were stirred an additional hour and allowed to stand overnight to insure complete extraction.

The extracts were filtered through coarse sintered glass funnels containing 0.5 g of Chromosorb W and 0.2 g of silica gel H to retain cell debris. Flasks and filters were washed twice with 10-ml portions of chloroform-methanol (1:1 [vol/vol]). Extracts were evaporated to dryness in a rotary evaporator, reconstituted in 4.5 ml of chloroform, and divided into portions for acid methanolysis, to determine total lipid fatty acids, and for sequential alkaline and acid methanolyses, to distinguish alkali-labile and alkali-stable fatty acids. The filter cakes were treated with sequential alkaline methanolysis and acid hydrolysis to determine alkali-labile and alkali-stable bound fatty acids.

Hydrolytic procedures. Samples for total cellular fatty acid analysis were treated with 0.5 μmol of nonadecanoic acid and hydrolyzed overnight at 100°C in 2.5 ml of 2 M HCl. Hydrolysates were extracted once with 5 ml of chloroform, and the organic phases were esterified with 2.5 ml of 1 M HCl in methanol for 1 h at 100°C. Samples were partitioned against an equal volume of water, washed once with water, and dried under a stream of N_2 with gentle heating.

Samples for total lipid fatty acid analysis were

treated with 0.5 μmol of nonadecanoic acid and subjected to acid methanolysis in 2 ml of 1 M HCl in methanol overnight at 100°C. Samples were treated with 5 ml of chloroform, washed with water, and dried under N_2.

To determine alkali-labile fatty acids, samples of the lipid extract, in 3 ml of chloroform, were treated with 0.5 μmol of nonadecanoic acid and 2 ml of 1 M KOH in methanol. The mixtures were incubated at room temperature overnight to insure complete interesterification of all ester-linked fatty acids, including those which might be protected by an alkali-stable bond at an adjacent carbon (1, 23). The reaction mixtures were treated with 2 ml of an aqueous suspension of Dowex 50x8 (H^+ form, 1.8 meq/ml) ion-exchange resin, and filtered. Filters and resins were washed with 7.5 ml of chloroform-methanol (2:1 [vol/vol]), followed by 5 ml of chloroform. After phase separation, the slightly acidic aqueous layers were discarded, and the organic layers were washed twice by partition against 2-ml portions of water. The organic phases were evaporated to dryness in vacuo, dissolved in 1 ml of hexane, and separated into fatty ester-free fatty acid and partially deacylated lipid fractions by column chromatography. The fatty ester-free fatty acid fractions were heated with acid methanol as described above.

The partially deacylated lipid fractions were treated with 0.5 μmol of DL-12-hydroxyoctadecanoic acid and subjected to acid methanolysis in 1 ml of 1 M HCl in methanol at 100°C overnight. Samples were evaporated to dryness under N_2, dissolved in 1 ml of hexane, and separated into fatty ester and fully deacylated lipid fractions by column chromatography.

The filter cakes containing defatted cell debris were examined for alkali-labile bound fatty acids by treatment with 5 ml of 1 M KOH in methanol. The mixtures, containing 0.5 μmol of nonadecanoic acid as the internal standard, were incubated overnight at room temperature. The reaction mixtures were acidified with 0.5 ml of concentrated HCl, diluted with 2 ml of water, and extracted twice with 5-ml portions of hexane-diethyl ether (3:2 [vol/vol]). The organic phases, which contained alkali-labile fatty acids and esters, were evaporated to dryness and esterified with acid methanol.

To determine alkali-stable and acid-labile bound fatty acids, the aqueous phases were treated with 0.5 μmol of DL-12-hydroxyoctadecanoic acid and an additional 1 ml of concentrated HCl (final concentration, approximately 2.2 M) and hydrolyzed for 2 h at 121°C in an autoclave. The hydrolysates were extracted twice with 5-ml portions of hexane-diethyl ether (3:2 [vol/vol]). The organic phases were evaporated to dryness and esterified with acid methanol.

Analytical methods. Column chromatography of deacylation mixtures was performed on 1-g columns of silicic acid (Unisil; 100/120 mesh, Clarkson Chemical Co., Williamsport, Pa.) packed in hexane. Samples were loaded onto the columns in 1 ml of hexane and eluted with 4 ml of hexane-diethyl ether (1:1 [vol/vol]) followed by 2 ml of chloroform, yielding free fatty acids and esters. Deacylated products were eluted from the columns with 5 ml of chloroform-methanol (1:1 [vol/vol]). Fractions were evaporated to dryness in vacuo.

Gas-liquid chromatography was carried out on a Hewlett-Packard 5840A gas chromatograph equipped with flame ionization detectors. Samples were analyzed on a capillary column (10 m) coated with the dimethylsiloxane liquid phase SP-2100 (J & W Scientific Co., purchased through Supelco, Inc., Bellafonte, Pa.) operated in the splitless mode. Operating conditions for the temperature-programmed analysis were described previously (20). Fatty acid esters and their derivatives were identified by their equivalent chain length values.

Infrared spectra of the intact, partially deacylated, and fully deacylated lipids were acquired by using a Perkin-Elmer 710B infrared spectrophotometer. Samples were analyzed as dried films between salt plates.

Chemicals. Chloroform, methanol, and hexane were redistilled before use. Pyridine and diethyl ether (Fisher Scientific Co., Fairlawn, N.J.) were used as received, as were the following trimethylsilylating reagents: hexamethyldisilazane, trimethylchlorosilane, and *N,O*-bis-trimethylsilyltrifluoroacetamide, which were purchased from Sigma Chemical Co., St. Louis, Mo.

Standards. Straight-chain saturated nonhydroxylated fatty acids were purchased individually (Sigma Chemical Co.) and carefully weighed to provide a standard mixture. 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 acids of C_{12} to C_{20} were prepared from acid hydrolysates of *Escherichia coli* and *Acholeplasma axanthum* S743. Nonadecanoic acid (Sigma Chemical Co.) and DL-12-hydroxyoctadecanoic acid (Applied Science Laboratories, Inc.) were used as internal standards.

Derivative formation. Samples were treated at room temperature for 1 h with 0.5 ml of pyridine-hexamethyldisilazane-trimethylchlorosilane-*N,O*-bis-trimethylsilyltrifluoroacetamide (2:2:1:1 [vol/vol]) (21) to convert any hydroxy acid esters to the trimethylsilyl ether derivatives. Reagents were removed under a stream of dry N_2, and the derivatized samples were dissolved in chloroform for analysis by gas-liquid chromatography.

All glassware, filter aids, and ion-exchange resins were rinsed with chloroform-methanol (1:1 [vol/vol]) and chloroform before use.

RESULTS

Each 50-ml culture yielded approximately 800 mg (wet weight; an estimated 75 mg dry weight) of cells, of which fatty acid comprised approximately 7%. The extractable (lipid) fractions contained approximately 90% of the total fatty acid, with 10% remaining as nonextractable (bound) fatty acids.

Infrared spectra were recorded on the intact lipids and on the deacylated products from alkaline and acid methanolyses, after chromatographic removal of the released fatty acids. Ab-

sorption at the ester carbonyl (1,740 cm^{-1}) and the amide I and II (1,640 cm^{-1} and 1,530 cm^{-1}) bands (3) was monitored to determine if the deacylation reactions had gone to completion. The intact lipids showed both ester and amide absorption. The *B. asaccharolyticus* strains showed relative intensities of ester to amide I of approximately 2:1, whereas the *B. fragilis* strains showed the reverse pattern. After alkaline methanolysis, ester absorption was eliminated in all samples. After acid methanolysis, the amide bands were absent as well.

There was no essential difference between the two strains of *B. asaccharolyticus* nor between the two strains of *B. fragilis*, as regards the profiles of fatty acids released by any of the treatments. Therefore, the results are reported as average values for each species. The fatty acid profiles resulting from the various procedures are shown for *B. asaccharolyticus* in Table 1 and for *B. fragilis* in Table 2.

DISCUSSION

In both species, all of the extractable (lipid) nonhydroxy fatty acids were linked in alkali-labile (presumably ester) bonds (Tables 1 and 2). By contrast, all the lipid hydroxy acids were involved in alkali-stable, acid-labile (presumably amide) bonds. The infrared spectra support these assignments, since ester absorption was lost after mild alkaline methanolysis, which yielded only nonhydroxylated fatty esters. Amide absorption was lost after subsequent acid methanolysis, which liberated only hydroxylated fatty esters. The nitrogen donors for the amide bonds in the lipid fractions are assumed to be long-chain bases of the sphinganine type, which have been shown to exist in members of the genus *Bacteroides* (9, 10, 11, 24, 25, 27). Stoffel et al. (25) reported that 3-hydroxy palmitate was the predominant amide-linked fatty acid in the sphingolipids of *Bacteroides thetaiotaomicron* and that the ester-linked fatty acids were predominantly nonhydroxylated. An analogous situation has been demonstrated in *A. axanthum*; most, if not all, of the amide-linked fatty acids of the sphingolipids are D-(−)-3-hydroxy acids (22, 23).

An attempt to determine sphinganines in the *Bacteroides* species studied here did not yield 1:

TABLE 1. *Fatty acid profiles of various fractions of B. asaccharolyticus strains*

Fatty acid[a]	Fatty acid fraction (mol%)						
	Total	Total lipid	Lipid, ester-linked	Lipid, amide-linked	Total bound[b]	Bound, ester-linked	Bound, amide-linked
Nonhydroxy[c]							
n14	2.0	2.2	1.4	—[d]	—	—	—
i15	72.2	71.9	88.8	—	6.0	9.8	5.1
a15	1.9	2.1	2.9	—	—	—	—
n15	—	—	—	—	—	—	—
n16	3.9	5.9	4.0	—	4.0	—	5.0
i17	tr	2.5	2.1	—	—	—	—
a17	tr	1.0	1.0	—	—	—	—
Hydroxy[e]							
i15	7.7	4.4	—	10.6	53.4	90.2	44.3
n15							
n16	1.0	1.8	—	1.0	7.2	—	9.0
i17	10.7	7.8	—	88.7	29.3	—	36.6
a17							
n17							
% Nonhydroxy	80.5	86.1	100.0		10.0	9.8	10.1
% Hydroxy	19.5	13.9		100.0	90.0	90.2	89.9
% Total[f]	100	90	77	13	10	2	8

[a] Number of carbon atoms is preceded by description of the chain type as follows: n, normal (straight chain); i, iso branched; a, anteiso branched.
[b] Calculated from ester- and amide-linked bound fatty acid.
[c] No double bonds.
[d] —, None found.
[e] Hydroxy acids on D-(−)-3-hydroxy fatty acids.
[f] Approximate values.

TABLE 2. *Fatty acid profiles of various fractions of B. fragilis strains*

Fatty acid[a]	Fatty acid fraction (mol%)						
	Total	Total lipid	Lipid, ester-linked	Lipid, amide-linked	Total bound[b]	Bound, ester-linked	Bound, amide-linked
Nonhydroxy[c]							
n14	1.0	1.8	1.3	—[d]	—	—	—
i15	14.2	13.5	20.0	—	20.4	34.0	—
a15	31.2	37.4	58.7	—	8.2	13.6	—
n15	6.0	7.8	10.4	—	1.7	2.8	—
n16	5.1	5.7	9.5	—	—	—	—
i17	—	—	—	—	—	—	—
a17	—	—	—	—	—	—	—
Hydroxy[e]							
i15	—	—	—	—	—	—	—
n15	2.6	2.0	—	1.0	7.8	9.4	5.4
n16	6.8	3.6	—	8.0	28.4	30.7	25.0
i17	27.4	24.3	—	80.7	24.8	3.2	57.3
a17	3.4	2.7	—	7.9	0.6	—	1.0
n17	1.8	1.0	—	1.2	8.3	6.1	11.4
% Nonhydroxy	56.8	66.3	100.0		30.2	50.5	
% Hydroxy	43.2	33.7		100.0	69.8	49.5	100.0
% Total[f]	100	90	59	31	10	6	4

[a,b,c,d,e,f] See Table 1 footnotes.

1 stoichiometry between long-chain base and lipid hydroxy acid, but did indicate a proportionality between the two moieties (i.e., strains with higher lipid hydroxy acid concentration also contained more sphinganine).

The profiles of the nonextractable (bound) fatty acids differed considerably from those of the lipid fatty acids. In both species, the proportions of nonhydroxy and hydroxy acids were reversed in extractable and nonextractable fatty acid fractions. The relative amounts of iso- and anteiso-branched pentadecanoate were reversed in *B. fragilis*, confirming that the nonhydroxy fatty acid contribution to the bound fatty acid profile was not a result of incomplete extraction of the lipids.

Note that, whereas the iso-branched hydroxyheptadecanoate (Tables 1 and 2) was the predominant hydroxy acid in the total fatty acid and lipid fractions of both species, the profiles of the bound fatty acids were high in components which are relatively minor in the total fatty acid profile: the iso-branched hydroxypentadecanoate (Table 1) in *B. asaccharolyticus* and the straight-chained hydroxyhexadecanoate (Table 2) in *B. fragilis*. Further disproportionation can be seen by comparing the profiles of the ester- and amide-linked bound fatty acids. Note that iso-branched hydroxypentadecanoate is the sole ester-linked hydroxy acid in *B. asaccharolyticus* and that straight-chained hydroxyhexadecanoate is the major ester-linked hydroxy acid in *B. fragilis*. These compounds are also major components of the amide-linked bound fatty acids in both species, although iso-branched hydroxyheptadecanoate is also found in high concentration. Selectivity of this sort implies considerable specificity among the acylating enzymes involved in cell envelope biosynthesis.

Rizza et al. (24), studying a strain of *Bacteroides melaninogenicus*, remarked on the apparent low yield of nonextractable fatty acid (approximately 3%). They also commented on the difference in fatty acid profile between the lipid and bound fractions, as well as on the apparent absence of β-hydroxy fatty acids in this gram-negative organism. Low levels of wall-associated fatty acid and apparent lack of hydroxy acid in *Bacteroides* species have been noted by other workers as well (2, 4, 5) and have been suggested as part of the reason for the relative biological inactivity of lipopolysaccharides from *Bacteroides* species (7, 18, 19). The data presented here may aid in resolving the apparent anomaly regarding the fatty acid composition of the cell walls of *Bacteroides* species. This information also suggests that lack of β-hydroxy acid is a less likely reason for the biological impotency of *Bacteroides* lipopolysaccharides.

The substrates for ester linkage of these bound fatty acids may be the hydroxyls of carbohydrates in cell wall polymers as well as the hydroxyls of the hydroxy acids themselves, as has been shown in other gram-negative organisms (16). The substrates for amide linkage are assumed to be the nitrogens of amino sugars. However, the possibility that bound sphinganine, perhaps as a constituent of a lipoteichoic acid-like moiety, serves as a substrate cannot be discounted without examination.

Another possible nitrogen donor, for both lipid and bound amide-linked fatty acids, is ornithine or a similar dibasic amino acid. Ornithine-containing lipids have been demonstrated in several bacterial species (6, 8, 17, 26, 28). In these compounds, the amide-linked fatty acid is hydroxylated, with another fatty acid, either hydroxy or nonhydroxy, in ester linkage via the 3-OH group.

In summary, the hydroxy acids of (at least some) members of the genus *Bacteroides* are present in both extractable and nonextractable moieties of the cell. The chain length distribution varies between the lipid and bound fractions. With this demonstration of significant concentrations of hydroxy acid in what is likely to be a cell wall-associated state, the genus *Bacteroides* need no longer be considered an unusual gram-negative organism in regard to the fatty acid composition of the cell envelope.

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