CELLULAR FATTY ACID COMPOSITION IN GRAM-NEGATIVE OBLIGATELY ANAEROBIC RODS

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The cellular fatty acid composition of 27 strains of gram-negative obligately anaerobic rods was determined by gas-liquid chromatography. Results showed that a variety of acids was detected in these organisms, and comparison of the relative amount of each of the acids was useful in distinguishing several species. A dendrogram based on their fatty acid composition was made, and this analysis appeared to be an aid in the grouping of the bacteria.

The application of gas-liquid chromatography (GLC) of fatty acids for the classification of bacteria was first attempted by ABEL and PETERSON (1) in 1963 and many laboratories are currently using GLC to study the cellular fatty acid composition of microorganisms because of its convenience and usefulness. These studies have rapidly expanded our knowledge of chemistry and taxonomy of closely related bacteria. Members of the genera *Bacteroides* and *Fusobacterium* in the family Bacteroidaceae that are Gram-negative, obligately anaerobic rods are commonly isolated from oral cavity, upper respiratory tract, and intestinal tracts of man and animals (2). They are also significant anaerobic pathogens in many clinical conditions (3). In recent years, they have been isolated at a high rate from the abscess of cattle and pigs. At present, 22 species are registered in the genus *Bacteroides* and 15 species in the genus *Fusobacterium* (2). The features available for the differentiation of these species are morphology, carbohydrate fermentation, final products of the metabolism, nutritional requirements, and so on. It is not easy, however, to apply all these techniques, and much time is needed to obtain final judgement according to results of these tests.

There have been some reports for arrangement of Bacteroidaceae by means of the bacterial cell components. HOFSTAD (4) analysed lipopolysaccharide (LPS), while FRITSCH (5) and the authors (6) investigated lipids, in these organisms. These studies supplied an interesting information which might serve as a useful taxonomic criterion, but few reports have appeared on fatty acid composition of

Bacteroidaceae (5, 7).

This paper describes the results obtained in the preliminary experiments on analysis of cellular fatty acids, centering around reference strains to clarify the taxonomical position of gram-negative, obligate anaerobes of cattle and pig origin.

MATERIALS AND METHODS

Organisms. Twenty-seven strains were used. The strains and their sources are listed in Table 1. All of them were preserved in a freeze-dried state until just before the experiment.

Table 1. Bacterial strains used.

Strains	Source of strain
1. <i>Bacteroides fragilis</i>	Dr. R. Tanaka←NCTC ^a 9343
2. <i>B. fragilis</i> var. <i>fragilis</i>	ATCC 23745
3. <i>B. fragilis</i> var. <i>vulgatus</i>	Dr. R. Tanaka←ATCC ^b 8482
4. <i>B. fragilis</i> var. <i>ovatus</i>	Dr. R. Tanaka←ATCC 8483
5. <i>B. fragilis</i> var. <i>thetaiotaomicron</i>	Dr. R. Tanaka←NCTC 10582
6. <i>B. fragilis</i> var. <i>thetaiotaomicron</i>	Dr. R. Tanaka←ATCC 12290
7. <i>B. ruminicola</i> var. <i>brevis</i>	ATCC 19188
8. <i>B. ruminicola</i> var. <i>ruminicola</i>	ATCC 19189
9. <i>B. oralis</i>	Dr. R. Tanaka←ATCC 15930
10. <i>B. oralis</i> (2467 B)	Dr. K. Ueno
11. <i>B. melaninogenicus</i> var. <i>melaninogenicus</i>	ATCC 25261
12. <i>B. melaninogenicus</i> var. <i>melaninogenicus</i>	ATCC 25285
13. <i>B. melaninogenicus</i> var. <i>melaninogenicus</i> (Gui-1034)	Dr. K. Ueno
14. <i>B. melaninogenicus</i> var. <i>intermedius</i> (O-33)	Dr. K. Ueno
15. <i>B. melaninogenicus</i> var. <i>asaccharolyticus</i> (28-3)	Dr. K. Ueno
16. <i>B. splachnicus</i>	Dr. H. Werner (NCTC 10825)
17. <i>B. succinogenes</i>	ATCC 19169
18. <i>B. hypermegas</i>	ATCC 25560
19. <i>B. furcosus</i>	ATCC 25662
20. <i>B. multiacidus</i>	Dr. T. Mitsuoka (ATCC 27723)
21. <i>B. amylophilus</i>	Bovine rumen
22. <i>Fusobacterium varium</i>	Dr. T. Tanaka←ATCC 8501
23. <i>F. mortiferum</i>	Dr. T. Tanaka←ATCC 9817
24. <i>F. nucleatum</i>	Dr. T. Tanaka←ATCC 25586
25. <i>F. nucleatum</i> (polymorphum)	Dr. T. Tanaka←ATCC 10953
26. <i>F. fusiforme</i>	ATCC 23726
27. <i>F. necrophorum</i>	ATCC 25286

^a NCTC: National Collection of Type Culture.

^b ATCC: American Type Culture Collection.

Condition and harvest. These organisms, except *Bacteroides amylophilus*, *Bacteroides ruminicola* var. *ruminicola*, and *Bacteroides succinogenes*, were cultivated in meat extract-yeast extract broth (VL medium) (8) containing 1% glucose in deoxygenated CO₂ atmosphere for 72 hr. Of the three species mentioned above,

B. amylophilus was cultivated in VL broth containing 1% maltose and the other two in rumen fluid broth (9) containing 0.5% glucose. The cells were harvested by centrifugation, washed three times with 0.85% saline, and freeze-dried for the following methanolysis.

Methanolysis and extraction of methyl esters. Freeze-dried cells (about 20 mg) were suspended in 2 ml of methanol containing 5% HCl in a screw-capped glass tube and heated at 105° for 3 hr. After cooling, 1 ml of water and 3 ml of hexane were added, the mixture was shaken well and centrifuged at 2000×*g* for 5 min. The upper phase was transferred to another tube. After this procedure was repeated three times, the hexane fraction was washed with the same volume of water, dried over anhydrous Na₂SO₄, and evaporated to dryness *in vacuo*. The residue which contains fatty acid methyl esters was dissolved in 60 μl of acetonitrile and subjected to GLC. The samples obtained were preserved at -20° until analysis by GLC.

Thin-layer chromatography. In case of need to confirm hydroxy-fatty acid and dimethylacetal, part of the methyl esters was developed on a silica gel G thin-layer plate with hexane: ether (8: 2, v/v), or toluene: ether (97: 3, v/v) as a solvent.

Gas-liquid chromatography. GLC of fatty acid methyl esters was carried out on a gas chromatograph (Nihon Chromato Co., Tokyo) equipped with FID. The coiled glass column used was 0.3×200 cm, packed with 10% diethylene glycol succinate polyester (DEGS) on Chromosorb W, silanized, 80–100 mesh. A nonpolar liquid-phase column (5% SE-30, 0.3×200 cm) was also used for further characterization of fatty acids. The column temperature was 180° for DEGS and 200° for SE-30. Nitrogen was used as a carrier gas at a flow rate of 30 ml/min.

Identification of fatty acids. Fatty acids were primarily identified by comparison of the relative retention time of their methyl esters with that of standard fatty acid methyl esters (C_{15:0}, C_{16:0}, C_{17:0}, C_{18:0}, C_{18:1}, C_{19:0}, C_{20:0}, and C_{22:0}, Applied Science Laboratories, Inc., Chicago) using both the polar and nonpolar columns. Hydrogenation and bromination of the esters were performed by the method of BRIAN and GARDNER (10). The equivalent chain length (ECL), percentage of each acid, retention time, and the amount of each peak were calculated by the programmed computer (YHP 2108 A, Yokokawa Hewlett-Packard Co., Tokyo) and these were printed out on a teletype (YHP 2752A, Yokokawa Hewlett-Packard Co.). Details of this computation method will be reported elsewhere.

Numerical analysis. There are several ways of calculating the similarity. The following formula of BERGAN (11) was adopted:

$$\gamma = \frac{\sum a_i b_i - n \frac{\sum a_i}{n} \frac{\sum b_i}{n}}{\sqrt{\left\{ \sum a_i^2 - n \left(\frac{\sum a_i}{n} \right)^2 \right\} \left\{ \sum b_i^2 - n \left(\frac{\sum b_i}{n} \right)^2 \right\}}}$$

where γ is the similarity, a_i is the peak area of a fatty acid, b_i is the peak area of the fatty acid which corresponds to that of a_i and n is the number of peaks which

correspond with each other. Calculation of the similarity was carried out by using the programmed computer mentioned above.

Mass spectrometry. Gas chromatographic and mass spectrometric analyses were performed on Shimadzu-LKB 9000. The mass spectrum of fatty acid methyl esters was recorded at an electron energy of 20 eV, a trap current of 60 μ A, an accelerating voltage of 3.5 kV, and ion source temperature of 250°. A coiled stainless steel column used was 0.3 $\times$ 200 cm, packed with 10% polyethylene glycol succinate.

RESULTS

Fatty acid composition of Gram-negative, obligately anaerobic rods

A variety of gas-liquid chromatographic profiles that depended on bacterial species used were found and 53 peaks were demonstrated in them within 60 min. The fatty acid compositions of the representative bacteria are shown in Fig. 1. Label, ECL, name, and content of these peaks are presented in Table 2. When the materials that correspond to label 19.2, 20.2, 22.0, and 22.5 were developed on the silica gel G thin-layer plate with hexane: ether (8: 2, v/v) as a solvent, their *R_f* value (0.13) agreed well with that of authentic 3-hydroxy-fatty acid methyl esters. The mass spectra of these peaks (Fig. 2) showed very intense peaks at *m/e* 103 and *m/e* 74 due to C₃-C₄ and C₂-C₃ cleavage, respectively. The molecular ion peak was weak, but the peaks at *m/e* M-18 (226, 240, 268, and 282, respectively) due to loss of H₂O and M-50 (194, 208, 236, and 250, respectively) due to loss of H₂O + CH₃OH appeared, this being a typical mass spectrum for 3-hydroxy-fatty acid methyl esters (12). From these results, peaks of label 19.2, 20.2, 22.0, and 22.5

Fig. 1. Fatty acid compositions in the representative bacteria.

Fig. 2. Mass spectra of 3-hydroxy-fatty acid methyl esters.

were identified as 3-OH-13:0, 3-OH-14:0, 3-OH-16:0, and 3-OH-*iso*-17:0, respectively. Label 18.5 or 18.6 are regarded as $C_{18:1}$ fatty acid and, by comparison with authentic standards, the former is oleic acid and the latter is *cis*-vaccenic acid. Both *Fusobacterium* species and *B. amylophilus* contained *cis*-vaccenic acid but the others contained oleic acid. The branched and odd-numbered acids were present in a large amount in all the strains belonging to *Bacteroides oralis*, *B. ruminicola*, *Bacteroides fragilis*, and *Bacteroides melaninogenicus*, but were hardly seen in all the strains belonging to *Bacteroides hypermegas*, *Bacteroides furcosus*, *Bacteroides multiacidus*, *B. amylophilus*, and *Fusobacterium* species. In the percentage composition of fatty acids of *Bacteroides oralis* (2467 B), *Bacteroides splanchnicus*, and *B. melaninogenicus* var. *asaccharolyticus* (28-3), a few differences were found, while they commonly contained *iso*-15:0 acid in a large quantity. Cellular fatty acids of all the strains belonging to *B. fragilis*, *B. ruminicola*, *B. melaninogenicus*, and *B. oralis*, excepting the strains, the main fatty acid of which was *iso*-15:0, mainly consisted of *anteiso*-15:0, 3-OH-*iso*-17:0, 16:0, and 3-OH-16:0 acids, although a

Table 2. Cellular fatty acid composition in

Label	ECL ^a	Fatty acids ^b	1 ^c	2	3	4	5	6	7	8	9	10
10.7	10.74	?										
11.4	11.37	?										
12.2	12.21	?								tr ^d		tr
12.3	12.25	?										
12.5	12.49	?										
12.7	12.66	<i>i</i> 13:0					tr	tr	1.7	1.9	tr	1.2
12.8	12.83	<i>ai</i> 13:0			1.0			tr		1.2		
13.1	13.09	13:0								0.7		
13.4	13.42	?										
13.5	13.53	?										
13.6	13.58	<i>i</i> 14:0			1.5	tr	2.1	0.6	0.8	1.9	2.1	tr
14.0	14.03	14:0		tr	4.0	1.3	0.7	3.3	1.3	2.0	7.0	1.1
14.5	14.52	?										
14.6	14.56	<i>i</i> 15:0	10.5	11.0	11.1	8.5	6.3	7.1	17.1	4.8	16.7	56.6
14.7	14.73	<i>ai</i> 15:0	41.5	41.3	42.5	40.8	48.5	45.0	22.0	28.0	20.8	
15.0	14.98	15:0	2.0		4.5	7.5		5.6	22.1	5.6	6.1	2.3
15.3	15.32	?										
15.5	15.52	<i>i</i> 16:0	0.7	0.7	1.1	1.7	3.2	0.8	2.2	1.1	0.7	tr
15.6	15.58	15:1										
16.0	15.99	16:0	14.6	11.9	11.3	15.1	10.0	6.4	3.2	12.9	30.4	9.6
16.3	16.30	?										
16.4	16.54	16:1(ω -9)										
16.5	16.52	<i>i</i> 17:0		1.4		0.6	2.9	0.6	3.6	1.4	0.9	3.9
16.6	16.59	16:1(ω -7)										
16.7	16.70	<i>ai</i> 17:0	3.5	2.3	0.7	3.1	2.8	1.8	4.8	2.1		
17.0	16.97	17:0								0.9		
17.2	17.22	?										
17.3	17.28	?		0.7			0.6		tr	tr		tr
17.5	17.53	17:1										
17.9	17.86	?										
18.0	17.97	18:0								12.9		tr
18.2	18.15	?					0.6					
18.3	18.28	?										
18.4	18.51	<i>i</i> 19:0										
18.5	18.48	18:1(ω -9)								5.8	0.8	0.6
18.6	18.55	18:1(ω -7)										
19.1	19.07	?	1.1	1.1	tr		1.0		1.2			
19.2	19.21	3-OH13:0										
19.3	19.26	?								1.3		tr
19.5	19.48	19:1										
20.1	20.10	?										
20.2	20.17	3-OH14:0							tr	0.8		
20.4	20.43	20:1										
20.5	20.49	?										
20.7	20.74	?							1.2			0.6
21.1	21.11	?						1.0		2.3		
21.3	21.25	?										0.7
21.5	21.49	?					1.6			0.6		
22.0	22.02	3-OH16:0	8.0	6.1	9.7	7.7	2.1	10.2	0.7	5.2	10.1	9.2
22.5	22.49	3-OH17:0	18.1	23.2	11.9	13.5	17.5	10.7	13.6	4.3	4.3	13.0
22.7	22.68	3-OH <i>ai</i> 17:0						6.7	3.8			
23.0	22.99	3-OH17:0								2.0		
23.4	23.37	?										

^a Equivalent chain length: Values are an average of its peaks.^b *i*, *iso*; *ai*, *anteiso*; 3-OH, 3-hydroxy.

Gram-negative obligately anaerobic rods.

[illegible]

^c Corresponds to number in Table 1.

^d Number refers to percentage of total acids: tr, less than 0.5%; blank spaces, not detected.

few differences that depended on species and/or strains used were found. *B. succinogenes* had mainly 16:0, 15:0, and 18:0 acids. An acidic dinitrophenylhydrazine-positive spot, assumed as a dimethylacetal, was detected, corresponding to authentic standard by thin-layer chromatography with toluene: ether (97:3, v/v) as a solvent, but these peaks on gas chromatogram agreeing with this substance was not fully analyzed. The major fatty acids were 15:0, 17:1, and 3-OH-13:0 in *B. hypermegas*, 18:1, 18:0, and 16:0 in *B. furcosus*, 16:1, 3-OH-14:0, and 16:0 in *B. multiacidus*, and 16:0, 18:1, and 14:0 in *B. amylophilus*. The most abundant fatty acids in *Fusobacterium* species were 16:0, 16:1, 14:0, and 3-OH-14:0.

Numerical analysis

After calculating the correlation coefficient among all the strains used by BERGAN's formula (11), a dendrogram based on their fatty acid composition was made (Table 3). High similarity indices ($S \geq 0.95$) were found among inter-subspecies of *B. fragilis*. High value ($S \geq 0.94$) was found between *B. fragilis* and *B. melaninogenicus*, excepting *B. melaninogenicus* var. *asaccharolyticus*. Therefore, it seems difficult to distinguish *B. fragilis* from *B. melaninogenicus* simply on the basis of fatty acid composition using a packed column GLC, but a lower similarity was observed between *B. ruminicola* var. *ruminicola* and *B. fragilis* ($S=0.842$). Also, similarity between *B. oralis* ATCC 15930 and *B. fragilis*, and that between *B. rumini-*

Fig. 3. Dendrogram of Gram-negative obligately anaerobic rods based on their fatty acid composition.

cola var. *ruminicola* and *B. fragilis* is 0.660 and 0.604, respectively. High value ($S \geq 0.95$) was noted among three strains including *B. splachnicus*, *B. oralis* (2467 B), and *B. melaninogenicus* var. *asaccharolyticus*, but these strains exhibited the value ($S \geq 0.379$) away from that of the strains including *B. fragilis*, *B. oralis* ATCC 15930, *B. melaninogenicus*, and *B. ruminicola*. *Bacteroides amylophilus* showed a higher degree of similarity to *Fusobacterium* species rather than to *Bacteroides* species, that is, high similarity indices ($S \geq 0.96$) were obtained between *B. amylophilus* and two *Fusobacterium* species (*F. fusiforme* and *F. mortiferum*). In respect to the inter-species similarity of *Fusobacterium*, similarity values more than 0.69 were obtained. A low similarity ($S=0.49$) was seen between *B. succinogenes* and *Fusobacterium* spp. *B. hypermegas*, *B. furcosus*, and *B. multiacidus* showed a very low degree of similarity to the bacteria mentioned above, maximal value was 0.20.

DISCUSSION

Previously, FRITSCH (5) reported that 3-OH-16:0 and/or 3-OH-iso-17:0 was present in lipids from *B. fragilis* and *B. melaninogenicus*. Our results showed these fatty acids in large quantities were also contained in *B. oralis* and *B. ruminicola*. STOFFEL *et al.* (13) analysed sphingolipids from *B. fragilis* var. *vulgatus* and reported that their main fatty acid was 3-OH-16:0. In sphingolipids from *B. fragilis* NCTC 9343, 3-OH-iso-17:0 (14) was their major fatty acid. Hence it is considered that these 3-hydroxy-fatty acids are derived from sphingolipids that are present in these bacteria. Hydroxy-fatty acids (15) are generally found in lipopolysaccharide (LPS) of some Gram-negative bacteria. HASE *et al.* (16) analyzed the fatty acid compositions of LPS from several *Fusobacterium* species, and it was found that a large amount of 3-hydroxy-fatty acids was present in LPS of these organisms. The results of our study demonstrated that *Fusobacterium* species were rich in 3-OH-14:0. This acid, therefore, was presumed to be of LPS origin.

UCHIDA and MOGI (17) obtained useful information about the relationships between bacteria and the other from the point of fatty acid biosynthesis. Octadecenoic acid in *B. amylophilus* and *Fusobacterium* species was composed of *cis*-vaccenic acid, whereas that in other *Bacteroides* was composed of oleic acid. This difference in fatty acids serves as a useful taxonomic criterion.

The genus *Bacteroides* (2) is characterised by the formation of succinic acid and acetic acid as a final product of the metabolism. *Fusobacterium* species produce a large amount of butyric acid from glucose while most *Bacteroides* species do not. The major fatty acids of *B. fragilis*, *B. oralis* ATCC 15930, and *B. melaninogenicus*, except *B. melaninogenicus* var. *asaccharolyticus*, that produced a large amount of succinic acid, were *anteiso*-15:0, 3-OH-iso-17:0, *etc.*, but in spite of producing succinic acid, the fatty acid compositions of *B. amylophilus* were similar to that of *Fusobacterium* rather than to *Bacteroides* mentioned above. *B. succinogenes* that produces succinic acid also showed fatty acid compositions not to those

of these *Bacteroides*. The fatty acid compositions of *B. multiacidus*, *B. hypermegas*, and *B. furcosus* which produce no or little succinic acid, were different from those of other *Bacteroides* and *Fusobacterium*. FRITSCH (5) claimed that the presence of phosphosphingolipids should be an important feature to define the genus *Bacteroides*. At present, we are of the same opinion. Accordingly, it is considered that *B. amylophilus*, *B. succinogenes*, *B. hypermegas*, *B. furcosus*, and *B. multiacidus* may be excluded from genus *Bacteroides* because these bacteria do not contain sphingolipids (6) and their fatty acid compositions are different from those of *Bacteroides* that contain sphingolipids. MITSUOKA *et al.* (18) studied a variety of characters of *B. multiacidus* and mentioned that it seemed appropriate that this organism was retained within the genus *Bacteroides*, because at present there is insufficient evidence to prepare a new genus. Further investigations are needed in order to place these bacteria that do not contain sphingolipids in a suitable position on taxonomy. FRITSCH (5) placed a few bacteria in genus *Bacteroides* because they contained sphingolipids, although they produced butyric acid. There are three strains that come under this category in our experiment, that is, *B. splachnicus* that FRITSCH studied after Dr. Welner offered it to him, *B. oralis* (2467 B), and *B. melaninogenicus* var. *asaccharolyticus* obtained from Dr. Ueno. They produce butyric acid, and their main fatty acids are *iso*-15:0 and 3-OH-*iso*-17:0, and consequently similarity in their values is high, but low between them and *B. fragilis* groups. FINEGOLD and BARNES (19) proposed that the asaccharolytic strain at present known as *B. melaninogenicus* var. *asaccharolyticus* should be reclassified in a separate species, *B. asaccharolyticus*, by reason of the difference of its DNA base ratio and so on from those of other two *B. melaninogenicus* subspecies. In case the presence of sphingosine bases is confirmed, it seemed to be proper to leave three strains in our study in the member of genus *Bacteroides*.

B. succinogenes or *B. amylophilus* formed a cluster distinct from that of the groups centering around *B. fragilis* or *B. splachnicus* in the dendrogram based on similarity values. JANTZEN *et al.* (20) have reported that the results of grouping by clustering based on the technique like this were in good accordance with those obtained by the conventional techniques in *Moraxella*, *Acinetobacter*, and others. IKEMOTO *et al.* (21) made use of this technique in the genus *Pseudomonas* and obtained the results that agreed fairly well with those in the past. However, it may not be correct to judge immediately this technique as being valid generally in grouping of bacteria. For example, even though similarity value between *B. amylophilus* and *Fusobacterium* species is high, it cannot be considered that they belong to the same genus if a great difference in the mode of energy metabolism is taken into consideration. This is the same fact it does not always follow that they belong to the same genus, even if a very similar value in GC % is obtained between two species of bacteria. Therefore, at present we consider that it is useful to calculate the correlation coefficient by the method like this to find a clue for comparing an unknown bacterium with a known bacterium. First, a semiquantitative-quali-

tative analysis of products is especially essential to the diagnosis of an anaerobe. Then, as a technique on an extra frame of this analysis, the method mentioned above will be easily accepted to a laboratory which deals with an anaerobe.

Results of field isolates will be reported in the next paper. The accumulation of a knowledge like this in many bacteria will enable their arrange more rationally than ever, including other information, as well as the knowledge based on tests needed to arrange more rationally.

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