High-Performance Liquid Chromatographic Analysis of Bacterial Fatty Acid Composition for Chemotaxonomic Characterization of Oral Streptococci

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A high-performance liquid chromatographic method was developed to analyze the fatty acid composition of bacterial lipids. After saponification of lipids extracted from bacteria, the liberated fatty acids were labeled with a fluorescence reagent, 4-bromomethyl-7-acetoxycoumarin, followed by reversed-phase high-performance liquid chromatographic separation and fluorescence detection. All bacterial fatty acids were simultaneously separated within 30 min and sensitively determined. This method was applied to the chemotaxonomic characterization of oral streptococci. The fatty acid composition of phospholipids and total lipids distinguished *Streptococcus mutans* from any other species examined and showed that *Streptococcus sanguis* had a close taxonomic relationship with *Streptococcus salivarius*.

Lipids are the major components of the bacterial membrane; in particular, phospholipids play important roles in the maintenance of membrane functions. Because bacteria grow to suit different environments, they show characteristic forms and functions, and thus their lipid components or composition should be specific for each individual bacterium. Characteristic phospholipids and their fatty acid composition seem to be important indicators in bacterial chemotaxonomy (7, 11).

Gas chromatography (GC) of cellular fatty acids and metabolic products has generally been used for the identification and classification of bacteria in recent years (1, 3, 13, 16). Although high-performance liquid chromatography (HPLC) has been widely used in biological fields, as has GC, it has not been successfully applied to bacterial chemotaxonomy.

In this study, the utility of HPLC as a bacterial chemotaxonomic tool was investigated. This report describes HPLC analysis of the fatty acid composition of bacterial phospholipids and its application to several oral streptococci whose chemotaxonomic phospholipid characterization is not apparent despite increasing interest in their cariogenicity.

MATERIALS AND METHODS

Preparation of bacterial lipids. Five species of oral streptococci (Table 1) were incubated at 37°C in brain heart infusion medium. The stock cultures in our laboratories were used for this study. The cells were harvested at late logarithmic phase by centrifugation and washed twice with sterile saline. They were broken by sonication (200 W, 60 min) and lyophilized. Lipids were extracted from the lyophilized samples by the method of Bligh and Dyer (2). The extract was used to analyze the fatty acid composition of total lipids. The lipid extract was spotted on a silica gel 60

thin-layer chromatographic plate (E. Merck AG, Darmstadt, Federal Republic of Germany). Phosphatidylglycerol (PG) and cardiolipin (CL) were purified by two-dimensional chromatography (solvent system: development 1, chloroform:methanol:ammonia [65:35:8]; development 2, chloroform:methanol:acetic acid:water [80:20:12:5]). Spots corresponding to these two phospholipids were revealed by coloration with iodine, and then they were scraped. The lipids were identified by comparing their R_f values with standards. PG and CL were extracted with a mixture of chloroform and methanol (6:1). Phosphorus in the lipid extracts was determined by the method of Rouser et al. (15).

Fatty acid derivatization. The lipid extract corresponding to phosphorus (100 pmol) was put into a siliconized glass ampoule together with 100 μ l of a nonadecanoic (19:0) acid in benzene solution (internal standard, 2 nmol/ml). The solvent was evaporated to dryness, and then 500 μ l of 1 M KOH in 50% aqueous methanol solution was added to the residue. The sealed ampoule was heated at 95°C for 30 min. The fatty acids liberated by saponification were extracted with 1.0 ml of a mixture of chloroform and *n*-heptane (1:1) after acidification with 2 M HCl. The solvent was evaporated to dryness, and then 3 mg of a mixture of KHCO_3 and anhydrous Na_2SO_4 (1:1) and 200 μ l of a solution (250 nmol/ml) of 4-bromomethyl-7-acetoxycoumarin (Br-Mac) in dimethylsulfoxide (DMSO) were added to the residue. Br-Mac was prepared as reported previously (17). The mixture was allowed to stand at room temperature for 5 min. After centrifugation, 50 μ l of the supernatant was subjected to HPLC analysis.

HPLC analysis of fatty acid composition. An HPLC system was constructed in a manner similar to the one used in our previous study (18). Fatty acids labeled with Br-Mac were separated with a column (250 by 4.0 mm) packed with LiChrosorb RP-8 (particle size, 5 μ m; Merck) by a balanced-density slurry-packing method. Eluates from the column were successively hydrolyzed as reported previously (17, 18), followed by detection of fluorescent hydrolysates (365

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TABLE 1. Fatty acid composition of lipids obtained from oral streptococci

Culture	% Total fatty acids ^a												Un ^c
	8:0 ^b	10:0	12:0	14:0	16:0	18:0	20:0	14:1	16:1	18:1	20:1		
PG													
<i>S. mutans</i> E-49	0.9 ± 0.5	2.7 ± 1.1	3.7 ± 0.5	9.6 ± 0.3	35.4 ± 0.9	14.9 ± 0.9	1.2 ± 0.2	1.2 ± 0.1	6.2 ± 0.8	15.5 ± 1.0	4.6 ± 0.4	4.3 ± 0.4	
<i>S. milleri</i> NCTC 10708	5.4 ± 3.0	6.2 ± 2.3	5.9 ± 1.4	11.0 ± 0.9	24.4 ± 2.6	9.3 ± 1.8	0.6 ± 0.2	4.0 ± 0.8	14.0 ± 1.7	10.0 ± 1.3	ND ^d	8.0 ± 1.4	
<i>S. mitis</i> ATCC 9811	1.8 ± 0.3	6.0 ± 1.3	5.2 ± 1.3	10.9 ± 2.0	27.7 ± 2.3	13.0 ± 1.2	0.9 ± 0.1	3.9 ± 1.6	11.3 ± 1.9	10.9 ± 1.6	ND	8.0 ± 0.8	
<i>S. sanguis</i> ATCC 10556	4.0 ± 1.4	6.8 ± 1.1	5.1 ± 0.6	10.8 ± 0.9	27.4 ± 2.7	11.5 ± 1.6	0.7 ± 0.1	4.2 ± 1.0	10.0 ± 1.2	10.8 ± 0.8	ND	8.1 ± 0.5	
<i>S. salivarius</i> ATCC 7073	3.9 ± 2.4	6.5 ± 1.3	5.4 ± 1.8	11.3 ± 1.7	27.6 ± 3.6	11.6 ± 0.6	0.8 ± 0.4	2.3 ± 0.7	9.6 ± 1.7	10.1 ± 1.2	ND	7.7 ± 0.7	
CL													
<i>S. mutans</i> E-49	2.2 ± 1.4	5.9 ± 3.9	6.8 ± 2.3	11.4 ± 1.0	30.9 ± 3.2	13.4 ± 1.5	1.1 ± 0.1	1.6 ± 0.2	7.0 ± 1.5	9.8 ± 1.9	2.6 ± 0.5	7.3 ± 0.7	
<i>S. milleri</i> NCTC 10708	2.4 ± 1.1	3.8 ± 0.6	5.4 ± 0.4	10.5 ± 1.3	31.6 ± 4.3	15.3 ± 1.9	0.9 ± 0.1	2.4 ± 0.6	10.0 ± 1.1	8.9 ± 1.3	ND	7.8 ± 0.7	
<i>S. mitis</i> ATCC 9811	0.8 ± 0.5	3.1 ± 0.9	5.9 ± 0.2	11.3 ± 1.4	31.3 ± 2.5	15.6 ± 1.4	1.0 ± 0.1	3.1 ± 0.6	8.5 ± 1.2	9.4 ± 0.1	0.8 ± 0.2	8.0 ± 0.5	
<i>S. sanguis</i> ATCC 10556	0.6 ± 0.2	2.7 ± 0.4	5.9 ± 0.9	11.6 ± 0.5	31.1 ± 0.8	15.8 ± 1.8	1.0 ± 0.2	1.8 ± 0.3	10.0 ± 1.2	11.1 ± 0.3	ND	8.6 ± 0.2	
<i>S. salivarius</i> ATCC 7073	1.0 ± 0.5	2.9 ± 0.1	5.3 ± 0.9	11.1 ± 0.4	30.9 ± 1.4	17.8 ± 0.5	1.2 ± 0.2	1.9 ± 0.2	8.6 ± 0.7	10.6 ± 0.9	ND	8.7 ± 0.4	
Total lipid													
<i>S. mutans</i> E-49	2.1 ± 0.9	4.3 ± 1.2	5.0 ± 1.6	10.7 ± 1.3	32.5 ± 0.6	13.9 ± 1.9	1.1 ± 0.1	1.3 ± 0.2	7.2 ± 1.6	11.9 ± 1.7	3.8 ± 0.8	6.3 ± 0.7	
<i>S. milleri</i> NCTC 10708	0.4 ± 0.1	1.1 ± 0.3	9.9 ± 0.4	14.5 ± 0.5	36.8 ± 1.2	12.2 ± 0.4	1.3 ± 0.1	2.1 ± 0.2	8.4 ± 1.6	8.5 ± 0.2	ND	3.9 ± 0.2	
<i>S. mitis</i> ATCC 9811	0.3 ± 0.1	1.4 ± 0.6	3.4 ± 0.5	10.8 ± 0.4	29.3 ± 0.9	13.2 ± 0.4	1.4 ± 0.1	3.1 ± 1.7	9.1 ± 0.7	17.1 ± 0.6	5.3 ± 0.4	5.7 ± 0.3	
<i>S. sanguis</i> ATCC 10556	0.3 ± 0.2	1.2 ± 0.2	3.0 ± 0.3	6.5 ± 0.2	26.6 ± 0.3	11.4 ± 0.3	0.7 ± 0.1	1.2 ± 0.2	15.7 ± 0.4	27.3 ± 0.4	0.8 ± 0.1	5.3 ± 0.1	
<i>S. salivarius</i> ATCC 7073	0.5 ± 0.3	0.9 ± 0.5	2.6 ± 0.4	9.7 ± 0.1	37.7 ± 1.5	11.3 ± 0.5	0.7 ± 0.2	1.7 ± 0.7	9.5 ± 0.4	18.2 ± 0.4	2.9 ± 0.1	4.3 ± 0.1	

^a Values are averages of five different determinations and standard deviations.^b Numbers refer to number of carbon atoms, and "colon 1" refers to unsaturated fatty acid.^c Un, Unidentified fatty acid. Percentages were obtained based on the peak height of linoleic (18:2) acid eluted at the same retention time.^d ND, Not detected.

FIG. 1. High-performance liquid chromatogram obtained from standard fatty acids. Peaks: 1, 8:0; 2, 10:0; 3, 12:0; 4, 14:1; 5, 14:0; 6, 16:1; 7, 18:2; 8, 16:0; 9, 18:1; 10, 18:0; 11, 20:1; 12, 20:0. IS, Internal standard (19:0). Chromatographic conditions: Column, LiChrosorb RP-8 (250 by 4.0 mm; particle size, 5 μ m); column temperature, 40°C; mobile phase, solvent 1, methanol: acetonitrile:water (35:35:30), and solvent 2, 90% aqueous methanol solution. The gradient was prepared by adding solvent 2 to solvent 1. The dotted lines show the concentrations of organic solvents in the mobile phase. Flow rate of the mobile phase, 1.2 ml/min; detector, spectrofluorometer (excitation at 365 nm and emission at 460 nm).

nm for excitation and 460 nm for emission). All solvents used for preparation of a mobile phase were of liquid chromatographic grade (Nakarai Chemicals, Kyoto, Japan). Detailed chromatographic conditions are shown in Fig. 1. Fatty acid composition was calculated after determination of each fatty acid in lipids based on a calibration graph which was prepared by using standard fatty acids. All standard fatty acids were purchased from Serdary Research Laboratories, London, Canada.

RESULTS

HPLC of fatty acids. Fatty acids were labeled with a fluorescence reagent, Br-Mac, so that they could be sensitively determined. DMSO, *N,N*-dimethylformamide (DMF), and *N,N*-dimethylacetamide (DMA) were examined for derivatization solvents. Reactivity of Br-Mac with representative fatty acids in DMSO is shown in Fig. 2. Their

FIG. 2. Reaction of Br-Mac with fatty acids in DMSO. Standard fatty acids (100 pmol of each) were derivatized at room temperature and then subjected to HPLC analysis. The peak heights were obtained under the same conditions as those described in the legend to Fig. 1.

derivatization was completed at room temperature for 5 min, whereas DMF and DMA needed reaction for a longer time. The relative peak height of palmitic (16:0) acid after complete reaction was as follows: 100, DMSO; 91, DMF; and 15, DMA. Derivatization in both DMF and DMA gave a major interfering peak eluted at about 8 min on chromatograms. DMSO was chosen for its superiority with respect to shortness of reaction time and fluorescence intensity of the derivatives.

A reversed-phase octyl silane column was used to achieve the simultaneous separation of the fatty acid derivatives in a short time. Their retention times and resolution factors were remarkably influenced by both the column temperature and the solvent composition of the mobile phase. After detailed examination of HPLC conditions, the column temperature was maintained at 40°C, and a gradient elution method in which the methanol and acetonitrile concentrations, respectively, increased and decreased with time was used. Thirteen fatty acids found in the lipids of oral streptococci, including the internal standard, were separated within 30 min (Fig. 1). A calibration graph prepared by plotting the peak height ratio of each fatty acid to that of the internal standard was rectilinear in the range of 1,000 pmol to 1 order of magnitude of pmol.

HPLC separation of bacterial fatty acids. Representative high-performance liquid chromatograms obtained from *Streptococcus mutans* are shown in Fig. 3. Five streptococci were characterized by chromatographic profiles of their fatty acids in each lipid, i.e., the presence or absence and the relative peak height of each individual peak. Fatty acid composition was determined based on the HPLC analysis. The results are summarized in Table 1.

PG fatty acid composition. Palmitic (16:0) acid showed the highest composition ratio of all fatty acids. *S. mutans* contained this fatty acid in the highest concentration (35%)

of the five streptococci. Each of the other major fatty acids, myristic (14:0), palmitoleic (16:1), stearic (18:0), and oleic (18:1) acids, was contained at about 10%. These five major fatty acids accounted for 70 to 80% of the total fatty acids. The remaining 20 to 30% included caprylic (8:0), capric (10:0), lauric (12:0), myristoleic (14:1), and arachidic (20:0) acids. In addition to these minor fatty acids, eicosenoic (20:1) acid at 4.6% was found only in *S. mutans*. The total unsaturated fatty acids accounted for more than 30% of the total fatty acids in all the streptococci except *S. mutans*.

CL fatty acid composition. CL contained the five major fatty acids of kinds similar to those found in PG. They accounted for 70 to 80% of the total fatty acids. Palmitic (16:0) acid showed the highest value of the five fatty acids, and it was uniformly about 30% in the five streptococci. Stearic (18:0), myristic (14:0), oleic (18:1), and palmitoleic (16:1) acids followed in this order. Eicosenoic (20:1) acid was contained in the CL of *S. mutans*, although its composition ratio was not so high as that in PG. In addition, *Streptococcus mitis* contained a very small amount of this fatty acid. The total unsaturated fatty acids accounted for about 30% in all the streptococci except *S. mutans*.

Total lipid fatty acid composition. Total lipids contained the major fatty acids of kinds similar to those of both phospholipids, although there was no apparent similarity in their composition among the five streptococci. The five major fatty acids accounted for 80 to 90% of the total fatty acids. *Streptococcus sanguis* characteristically contained a large amount of oleic (18:1) acid, which showed the highest value of the five major fatty acids. All the streptococci except *Streptococcus milleri* contained eicosenoic (20:1) acid. The presence of a relatively large amount of this fatty acid was specific to *S. mitis*. No apparent similarity among the five streptococci was observed in the occupation ratio of the total unsaturated fatty acids.

FIG. 3. High-performance liquid chromatograms obtained from *S. mutans* lipids. (A) PG; (B) CL; (C) total lipid. Peak numbers, except for peak 7 (unidentified fatty acid), and chromatographic conditions were as described in the legend to Fig. 1. IS, Internal standard (19:0).

DISCUSSION

Pre-column labeling reagents related to Br-Mac were generally allowed to reflux with fatty acids in aprotic solvents (5, 9). To shorten reaction time, we examined DMSO, DMF, and DMA, which were expected to have solvent effects. Even at room temperature, derivatization in DMSO proceeded more rapidly than expected. In a previous study (18), derivatization in acetone needed a reaction at 50°C for 30 min in the presence of dibenzo-18-crown-6. Not only was the reaction time remarkably shortened, even in the absence of a catalyst, but the concentration of Br-Mac required for complete derivatization also was reduced to 10% that of the previous method. Such enhancement of reactivity in DMSO seems to be explained by the donor-acceptor theory (8).

Relatively short- to long-chain fatty acids (8:0 to 20:0) were analyzed simultaneously. Shortness of the separation time for bacterial fatty acids was comparable to that of GC. The sensitivity of the HPLC method was at least 500 times that of GC with a flame ionization detector, which has been widely used. We tried HPLC separation of various fatty acids other than those shown in Fig. 1. They included hydroxy and branched-chain fatty acids. A mixture of their derivatives was separated; the HPLC conditions were different from those described in Fig. 1, and their simultaneous separation took longer. However, no major peak corresponding to such fatty acids was observed on chromatograms obtained from oral streptococci. It was decided that hydroxy and branched-chain fatty acids were not so important, at least for the chemotaxonomy of our streptococci. Thus, we dealt only with straight-chain fatty acids in this

study to simplify HPLC conditions and shorten analytical time.

Lipid composition varied with the age of the bacterial cells. The ratio of saturated fatty acids in total fatty acids tended to increase with bacterial incubation time. We obtained the growth curve of each streptococcus and then set the harvesting time on late logarithmic phase to avoid variation of fatty acid composition with time.

Lambert and Moss (12) reported the cellular fatty acid composition of *S. mutans* and related streptococci. They hydrolyzed whole bacterial cells and analyzed total liberated fatty acids by GC. Fatty acids detected by the HPLC method agreed with their results, whereas there were some differences in fatty acid composition. We found eicosenoic (20:1) acid in the PG and CL of *S. mutans* and in the total lipids of all the streptococci except *S. milleri*. On the other hand, it was reported that the cellular fatty acids of *S. mutans* and *Streptococcus salivarius* contained a relatively large amount of this fatty acid, but *S. sanguis* and *S. mitis* did not (*S. milleri* was not examined) (12). Such discrepancies may have been due to differences in bacterial strains or cultivation conditions.

The three lipid samples offered a peak eluted at the retention time of linoleic (18:2) acid. To our knowledge, this fatty acid has not previously been reported in bacterial lipids. Several cyclopropane acids have been described in various bacteria (3, 10), and the one commonly found in streptococci is lactobacillic (19 cyc) acid (7, 13, 14). The peak eluted at about 18 min may be ascribed to such a fatty acid, and studies are under way to identify it.

Other workers have reported the absence of eicosenoic

(20:1) acid or the presence of significant amounts of branched-chain fatty acids and odd-numbered fatty acids in streptococci (4, 6, 13, 14). Disagreements between our report and those of other workers may be accounted for by differences in preparation conditions for bacterial fatty acids, as suggested by Lambert and Moss (12).

The profiles of PG fatty acid composition characterized *S. mutans*. There was PG similarity among *S. mitis*, *S. sanguis*, and *S. salivarius*. The profiles of CL, as well as of PG, were characteristic of *S. mutans*. *S. sanguis* resembled *S. salivarius* with respect to CL profiles. The fatty acid composition of total lipids was considerably different among the five streptococci, although *S. sanguis* was relatively similar to *S. salivarius*. These two streptococci were characterized by a high degree of unsaturation in total fatty acids. From these results, it appears that *S. mutans* has no taxonomic similarity to the other four oral streptococci and that *S. sanguis* has a close taxonomic relationship with *S. salivarius*. Because the number of strains used in this study was limited, the application of our method to more strains is needed to support such taxonomic conclusions.

This study suggests that the presence of eicosenoic (20:1) acid, the degree of unsaturation, and the composition of relatively short-chain fatty acids are important indicators for the differentiation of oral streptococci. When the fatty acid composition of each individual lipid is not strikingly different, comparison of fatty acid profiles among the three lipids may be useful for chemotaxonomic purpose.

To confirm the reliability of our method, its wider application to more strains and more kinds of fatty acids and a comparison with GC are now under way.

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