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Note

Determination of bound cellular fatty acids in *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* by gas chromatography and gas chromatography—mass spectrometry

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In some bacterial cells, fatty acids occur as free acids. Most bacterial acids, however, are linked to larger molecules, such as phospholipids, glycolipids, lipoproteins, lipopolysaccharides, and lipotechoic acids [1]. Unlike free lipids, which can be extracted with appropriate solvents, bound lipids require acid or alkaline hydrolysis to be released. Bound lipids have been found to differ markedly from free lipids in bacteria such as *Pseudomonas*, *Alcaligenes*, and possibly also *Moraxella* and *Neisseria* (review: ref. 1), and it has been suggested that many other bacteria will appear with similar differences as more data are accumulated, particularly among Gram-negative organisms. We have previously reported on the free fatty acid content as a tool in the chemotaxonomic differentiation between the closely related facultative Gram-negative rods *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* [2, 3]. The present study, which deals with the composition of bound cellular acids in *A. actinomycetemcomitans* and *H. aphrophilus*, establishes the usefulness of differentiating between free and bound acids in these bacteria, and suggests such differentiation as a routine in chemotaxonomic studies.

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MATERIAL AND METHODS

Bacteria

The strains of *A. actinomycetemcomitans* and *H. aphrophilus* investigated, and the sources from which they were obtained, are listed in Table I. Strains ATCC 33389, 33384, 29522, and 19415 were obtained directly from the American Type Culture Collection, the remaining strains through Forsyth Dental Center. The organisms were maintained and mass cultivated as described previously [3].

TABLE I

LIST OF BACTERIA INVESTIGATED

Bacteria	Strain	Source	Site of origin
Actinobacillus actinomycetemcomitans	33384 (9710)	ATCC* (NCTC**)	Lung abscess
	29524	ATCC	Chest aspirate
	29523	ATCC	Blood
	29522	ATCC	Mandibular abscess
	2112	FDC***	Periodontitis
	2097	FDC	Periodontitis
	2043	FDC	Periodontitis
	511	FDC	Periodontitis
	N 27	FDC	Periodontosis
	Y 4	FDC	Periodontosis
Haemophilus aphrophilus	33389 (5906)	ATCC (NCTC)	Endocarditis
	19415 (5886)	ATCC (NCTC)	Endocarditis
	655	FDC	Periodontitis
	654	FDC	Periodontitis

*American Type Culture Collection, Rockville, MD, U.S.A.
**National Collection of Type Cultures, London, U.K.
***Forsyth Dental Center, Boston, MA, U.S.A.

Removal of free fatty acids from whole cells

Lyophilized material from each of two series of bacteria, cultured on different days, was Soxhlet-extracted twice with fresh *n*-hexane [2, 3].

Removal of bound fatty acids from hexane-extracted cells

Lyophilized, hexane-extracted bacterial cells (200 mg) were suspended in 2—3 ml of deionized distilled water and sonicated for 10 min under ice cooling. The suspension was diluted to 10 ml with deionized distilled water and centrifuged (27,000 *g*, 15 min, 4°C). The recovered pellet was freeze dried until constant weight, suspended in 2 ml of 2 *M* hydrochloric acid in anhydrous methanol and incubated in a PTFE-sealed tube with screw cap for 24 h at 85°C. The methanolysis tube was cooled to 20°C, shaken, and its content transferred to a centrifuge tube. After two washings of the methanolysis tube, each time with 2 ml of absolute ethanol, centrifugation was carried out at 48,200 *g* for 15 min at 4°C. The supernatant was pipetted off and filtered through a 0.22-μm Millex®-GS filter (Millipore, Molsheim, France) and recentrifuged. The supernatant was diluted with 4 ml of deionized distilled

water and extracted twice with chloroform, 4 ml each time. The organic phase was washed twice, each time with 2 ml of deionized distilled water, and dried with 400 mg of magnesium sulphate for 30 min, filtered through a filtration paper, and evaporated by a stream of nitrogen while kept on ice. The residue was solubilized in hexane, filtered and analysed by gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS).

Reference compounds

The ammonium salt of 3-deoxy-D-manno-2-octulosonic acid (KDO) was obtained from Sigma (St. Louis, MO, U.S.A.). Its methyl derivative was synthesized by incubation of KDO in a PTFE-sealed tube in the presence of 2 *M* hydrochloric acid in anhydrous methanol at 85° C for 24 h. The methyl ester of iso-$C_{15:0}$ acid (Larodan Fine Chemicals, Malmö, Sweden) was synthesized by methylation of the acid with BF_3 in methanol. Methyl esters of lauric, myristic, palmitic, and palmitoleic acid were obtained from Supelco (Bellefonto, PA, U.S.A.). The methyl ester of racemic 3-hydroxymyristic acid was synthesized by Reformatsky reaction [4]. After fractional distillation, the ester was saponified for 1 h with 2 *M* potassium hydroxide in aqueous methanol at refluxing temperature. The solution was chilled in water and the crystalline potassium salt of the hydroxy acid recovered by filtration, washed with ethanol at 0° C and dissolved in hot deionized distilled water at 25° C. The aqueous solution was acidified with hydrochloric acid until pH 2 and cooled to 2° C. The insoluble 3-hydroxymyristic acid was recovered by filtration and its methyl ester synthesized by heating the 3-hydroxymyristic acid in anhydrous methanol with 0.2 *M* hydrochloric acid for 3 h at 75° C. The methanol solution was concentrated to 0.2 ml, diluted with 2 ml of deionized distilled water and extracted three times with chloroform. The chloroform-containing solutions were pooled, dried for 30 min with 40 mg of anhydrous magnesium sulphate and filtered through a paper filter which afterwards was washed with chloroform. The chloroform solutions were pooled and evaporated by a stream of nitrogen while kept on ice. GC—MS was used to control the synthesized product.

Gas chromatography

A Sigma 3 gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) furnished with an electronic integrator (Sigma 10) was used. The Chrompack (Middelburg, The Netherlands) CP-Sil 5 CB (polydimethylsiloxane) capillary column used was 25 m × 0.22 mm I.D. with film thickness 0.14 μm and height equivalent of a theoretical plate (HETP) 0.25 mm. Helium served as carrier gas at a flow-rate of 2 ml/min. The pressure at the inlet of the column was 151.5 kPa, and the temperature of the injector and flame ionization detector 220° C. The gas chromatograph was programmed from 120° to 260° C at 5° C/min. The attenuator of the gas chromatograph was set at 8, that of the Sigma data system at —1. Paper-speed was 10 mm/min. The identity of the bacterial fatty acids (methyl esters) was established by direct cochromatography and by GC—MS. Tentative identification was made by comparing their retention times with those of authentic standards.

Gas chromatography—mass spectrometry

The instrument used for combined GC—MS consisted of a Carlo Erba 4200 gas chromatograph, a Micromass 7072F (Vg Micromass, Cheshire, U.K.) mass spectrometer, and a Vg data system 2200. The gas chromatograph was equipped with a glass capillary OV-1 methyl silicone column (20 m × 0.3 mm I.D.). Helium served as carrier gas. The column temperature was programmed from 120°C to 250°C at 5°C/min. Electron-impact ionization spectra were recorded under the following conditions: ionizing energy 70 eV, ionizing current 200 μA, ion-source temperature 240°C, and accelerating voltage 4 kV.

RESULTS

Gas chromatography

The composition of bound acids (methyl esters) of previously hexane-extracted whole cells from *A. actinomycetemcomitans* and *H. aphrophilus* is given in Table II. The major acids included $C_{14:0}$, 3-OH-$C_{14:0}$, $C_{16:1}$, $C_{16:0}$, and KDO. All strains, except *A. actinomycetemcomitans* strain ATCC 29522, yielded more $C_{16:0}$ than $C_{16:1}$ acid. A typical gas chromatogram of the bound acids is shown in Fig. 1. The fatty acid profiles were not so characteristic as to allow differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*.

Gas chromatography—mass spectrometry

The fragmentation pattern of the methyl esters of $C_{14:0}$, $C_{16:1}$ and $C_{16:0}$ was identical with that of the synthetic analogues and in accordance with the McLafferty rearrangement [5] with formation of a 74 *m/e* fragment. Fragmen-

TABLE II

MAJOR BOUND CELLULAR FATTY ACIDS IN *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* AND *HAEMOPHILUS APHROPHILUS*

Values, which are expressed as relative percentage (w/w) of total substances present, are means of four runs on the gas chromatograph. S.D. = 5%.

Bacteria		$C_{12:0}$	$C_{14:0}$	3-OH-$C_{14:0}$	$C_{16:1}$	$C_{16:0}$
Actinobacillus actinomycetemcomitans						
ATCC	33384	0.1	21.7	9.2	21.4	26.6
ATCC	29524	0.1	19.6	21.2	17.0	27.8
ATCC	29523	0.3	21.9	18.4	19.6	26.0
ATCC	29522	0.8	30.8	24.7	16.1	13.2
FDC	2112	0.4	18.3	32.6	13.9	16.7
FDC	2097	0.8	20.1	14.2	21.9	28.5
FDC	2043	0.5	32.8	17.8	16.4	24.9
FDC	511	0.1	20.7	15.5	22.5	30.0
FDC	N 27	0.1	18.0	27.1	14.9	27.0
FDC	Y 4	0.4	24.1	21.8	23.3	29.6
Haemophilus aphrophilus						
ATCC	33389	0.5	19.5	10.0	25.4	31.6
ATCC	19415	0.5	19.7	16.1	16.5	36.0
FDC	655	0.6	26.5	18.0	13.8	31.5
FDC	654	0.7	19.8	20.2	23.4	24.8

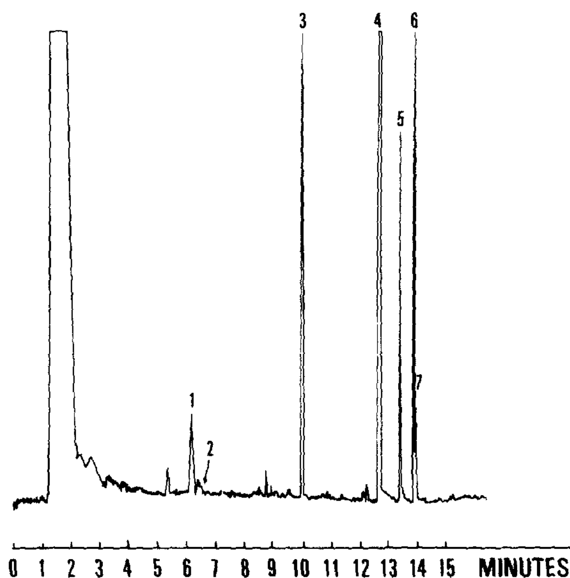

Fig. 1. Typical gas chromatogram of bound acids (methyl esters) from *A. actinomycetemcomitans* and *H. aphrophilus*, as respresented by *A. actinomycetemcomitans* strain FDC 2112. Programme· 120°C to 260°C and 5°C/min. Temperature of the injector and flame ionization detector, 220°C. Attenuator of gas chromatograph, 8, that of Sigma 10 data system, −1. Paper-speed, 10 mm/min. 1 = KDO, 2 = $C_{12:0}$, 3 = $C_{14:0}$, 4 = 3-OH-$C_{14:0}$, 5 = $C_{16:1}$, 6 = $C_{16:0}$, 7 = substance with M^+ 278.

tation of the methyl ester of β-hydroxymyristic acid occurred through formation of the 103 *m/e* fragment [6]. A minor unknown substance with M^+ 278, possibly of chain length C_{17}, was detected in all the bacterial strains investigated.

DISCUSSION

In previous studies on cellular fatty acids in bacteria, fatty acids removed by organic solvents were designated as free lipids, those that remained after this procedure, as bound lipid [7, 8]. The same concepts have been adopted in the present study. After removal of free fatty acids with hexane, four major bound fatty acids, i.e. myristic, palmitic, palmitoleic, and 3-hyroxymyristic acid, were detected in whole cells of *A. actinomycetemcomitans* and *H. aphrophilus* by means of GC, GC—MS, and by comparing our results with existing literature data on fatty acids [5, 6, 9, 10]. The present results agreed with those of Calhoon et al. [11] and Jantzen et al. [12], but varied with those of Braunthal et al. [13] who detected little or no 3-hydroxymyristic acid. The distribution of bound fatty acids was not so specific as to allow differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*. In our GC study on free fatty acids from the very same species, differentiation between *A. actinomycetemcomitans* and *H. aphrophilus* was possible to some extent [3]. It should be emphasized that free fatty acids had been removed completely beforehand, as checked by reextraction of the hexane-extracted cells with chloroform—methanol. Interestingly, iso-$C_{15:0}$ acid, which appeared as a free major fatty

acid in *A. actinomycetemcomitans* strain ATCC 29522 and FDC Y 4, could not be detected among the bound fatty acids. It was also noteworthy that the amount of bound $C_{16:0}$ acid was higher than that of $C_{16:1}$, except in one strain. A reverse relationship prevailed among free $C_{16:0}$ and $C_{16:1}$ acids [3]. Among the bound acids recovered were also KDO, 3-hydroxymyristic acid and an unidentified substance, probably acid of chain length C_{17}. None of these substances were detected as free acids. Exact figures for KDO were not given in the present study due to possible interference during quantification by methanolysis and derivatization products. These aspects have been discussed during quantification of KDO in lipopolysaccharide (LPS) from *A. actinomycetemcomitans* and *H. aphrophilus* [14]. In LPS, KDO links the polysaccharide to lipid A in a ketosidic linkage [15]. Hydroxylated fatty acids in Gram-negative bacteria are largely found in LPS, ornithine lipid, and the polymer poly-β-hydroxybutyrate [1] where they are covalently bound. Hydroxy fatty acids could not be detected in lipids extractable from bacterial cells with lipophilic solvents such as ethanol and methanol—chloroform [7, 8]. Certain species of *Alcaligenes* containing both free and bound hydroxy acids [1] may be exceptions.

Our studies on cellular fatty acids in *A. actinomycetemcomitans* and *H. aphrophilus* demonstrated that free and bound acids differ markedly in these bacteria. Such differences may be significant in the identification and characterization of bacterial cultures. In some extraction methods for bacterial lipids of whole cells, e.g. methanolysis, free and bound acids are removed simultaneously without any differentiation being made. In the currently investigated bacteria, free fatty acids accounted for approximately 90—70% of the total extractable fatty acids, bound fatty acids for about 10—30%. When the content of free fatty acids is that high, these acids may tend to mask bound acids when free and bound acids are examined simultaneously. To provide accurate determination of all the cellular acids present in bacteria, it would appear reasonable as a routine first to screen for the presence of free fatty acids, then for bound acids.

It was recently demonstrated that in patients with localized juvenile periodontitis, *A. actinomycetemcomitans* strains ATCC 29522 and FDC Y 4, which constituted serotype b, were approximately twice as frequent as serotype a or c strains, suggesting a particularly high periodontopathic potential of the serotype b strains [16]. This observation coincided with our demonstration of an extra free major fatty acid, iso-$C_{15:0}$ acid, in the serogroup b strains [3]. It seems possible that screening for free fatty acids in whole cells may also provide information as to potential pathogenic mechanisms in bacterial disease.

Other significant features of the present study were that it allowed methanolysis and methylation to be performed simultaneously, and that additional derivatization of the formed products, including the methyl ester of KDO, was not necessary before GC analysis. Also 3-hydroxymyristic acid (methyl ester) was sufficiently volatile and stable to allow direct analysis. This was in contrast to free β- or α-hydroxy acids which are thermally unstable [17]. We did not detect any myristoleic (*cis*-9-tetradecenoic) acid by means of the present method. β-Hydroxy acids may give αβ-unsaturated acids when boiled with base or acid in the presence of water [17]. When Hase et al. [18]

examined fatty acids of *Fusobacterium nucleatum* LPS, they discovered that alkaline hydrolysis with 4 *M* potassium hydroxide resulted in the production of Δ^2-tetradecenoic acid as an artifact resulting from 3-hydroxytetradecanoic acid. It should also be kept in mind that the fatty acid content and composition determined after acidic hydrolysis of lipids containing 3-hydroxyalkanoic acid may be seriously in error due to acid-catalysed polymerization of 3-hydroxy acid and esterification between hydroxy and non-hydroxy acids [19].

CONCLUSIONS

Bound cellular acids extracted from the bacteria *A. actinomycetemcomitans* and *H. aphrophilus* were determined by GC and GC—MS after free fatty acids had been removed. Bound acids included myristic, palmitic, palmitoleic, 3-hydroxymyristic, and 3-deoxy-D-manno-2-octulosonic acid and possibly its methanolysis products. In chemotaxonomic studies on bacteria it would be reasonable as a routine to differentiate between free and bound fatty acids since they may differ markedly.

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