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DIFFERENTIATION OF *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* FROM *HAEMOPHILUS APHROPHILUS* BY GAS CHROMATOGRAPHY OF HEXANE EXTRACTS FROM WHOLE CELLS

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SUMMARY

In the present study, differentiation of the closely related bacteria *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* has been made possible through gas chromatography of hexane extracts from their whole cells. While the investigated strains of *H. aphrophilus* were homogeneous, *A. actinomycetemcomitans* could be divided into three clusters of strains according to their gas chromatographic fingerprints. Myristic, palmitic and palmitoleic acid were major fatty acids in both *A. actinomycetemcomitans* and *H. aphrophilus*. In strain cluster III of *A. actinomycetemcomitans*, 13-methyltetradecanoic acid occurred as a fourth major fatty acid. Essentially no minor components were detected in cluster I of *A. actinomycetemcomitans* and in *H. aphrophilus*. In cluster II, the presence of minor components was moderate, in cluster III, marked.

INTRODUCTION

Actinobacillus actinomycetemcomitans and *Haemophilus aphrophilus* are morphologically very similar Gram-negative facultative rods, indigenous to dental plaque [1]. Such plaque has been the source of infection in cases where these organisms have caused endocarditis [2]. *A. actinomycetemcomitans* has recently attracted much attention due to its suspected role as a major pathogen in periodontosis [3]. *H. aphrophilus* has not been implicated to the same extent, haemophili being considered by most authors

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to have a low pathogenic potential in the periodontal pocket [4]. Taxonomic differentiation between these species is difficult and is based only on a few physiological characters [5]. In Bergey's Manual of Determinative Bacteriology [6], *A. actinomycetemcomitans* is designated as a species incertae sedis, uncertainty especially existing in the relationship to *H. aphrophilus*. A close phenotypic similarity has been found between *A. actinomycetemcomitans* and *H. aphrophilus* [7, 8], and inclusion of *A. actinomycetemcomitans* in the genus *Haemophilus* has been suggested [7].

Gas chromatography of cellular fatty acids is generally considered a useful tool in the differentiation between facultative bacteria (reviews: refs. 9 and 10). Usually, bound fatty acids, which are released by acid or alkaline hydrolysis and then derivatized before gas chromatographic analysis, are used for this purpose. Unfortunately, both hydrolysis and derivatization may cause artifacts. We have developed a direct method of gas chromatographic analysis of free, non-derivatized, whole-cell fatty acids from bacteria which circumvents these problems [11]. The present paper deals with the application of this method in the taxonomic differentiation of *A. actinomycetemcomitans* and *H. aphrophilus*.

MATERIAL AND METHODS

Bacteria

The strains of *A. actinomycetemcomitans* and *H. aphrophilus* investigated, and the sources from which they were obtained, are shown in Table I. Strain ATCC 29522 was first obtained from Forsyth Dental Center, and later, for comparison, through the American Type Culture Collection, strains ATCC 33389, 33384 and 19415 through the American Type Culture Collection, and the remaining strains through Forsyth Dental Center. The organisms, which were maintained on blood agar plates kept in an atmosphere of 80% nitrogen, 10% carbon dioxide and 10% hydrogen, were transferred weekly. Mass cultivation occurred in Brain Heart Infusion® (Difco, Detroit, MN, U.S.A.), broth in air plus 10% carbon dioxide for five days at 37°C. All mass cultivations were made in duplicate, on different days.

Fatty-acid extraction

Lyophilized material from each of the two series of bacterial cultures was extracted twice with fresh *n*-hexane (E. Merck, Darmstadt, F.R.G.) in an all-glass Soxhlet apparatus furnished with a refluxing Liebig water condenser, each time for 3—4 h [11]. The two extracts from one culture were pooled, dried and stored at —20°C in an oxygen-free atmosphere.

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 (polydimethylsiloxane) capillary column used was 20 m × 0.22 I.D. with film thickness 0.14 μm and height equivalent to 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

TABLE I

LIST OF BACTERIA INVESTIGATED

Organism	Strain	Source	Site of origin
Actinobacillus actinomycetemcomitans	33384 (9710)	ATCC* (NCTC**)	Lung abscess
	29524	ATCC	Chest aspirate
	29523	ATCC	Blood
	29522§	ATCC	Mandibular abscess
	29522§§	ATCC	Mandibular abscess
	2112	FDC***	Periodontitis
	2097	FDC	Periodontitis
	2043	FDC	Periodontitis
	511	FDC	Periodontitis
	N27	FDC	Periodontosis
	Y4	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.
§Obtained through Forsyth Dental Center.
§§Obtained through American Type Culture Collection.

151.5 kPa. From the duplicate bacterial cultures, twenty runs were made on the gas chromatograph, ten from each pooled extract. The gas chromatrographic profiles of the duplicate cultures differed by 1—2%. The bacterial fatty acids were identified tentatively by comparing their retention times with those of authentic standards, i.e. $C_{14:0}$ acid (myristic acid, Sigma, St. Louis, MO, U.S.A.), $C_{15:0}$ acid (pentadecanoic acid, Sigma), iso-$C_{15:0}$ acid (13-methyltetradecanoic acid, Larodan Fine Chemicals, Malmö, Sweden), anteiso-$C_{15:0}$ acid (12-methyltetradecanoic acid, Larodan), $C_{16:0}$ acid (palmitic acid, Sigma), $C_{16:1}$ acid (palmitoleic acid, Sigma). The identity of the bacterial fatty acids was confirmed by gas chromatography—mass spectrometry (GC—MS), cochromatography of authentic standards, proton nuclear magnetic resonance (NMR) spectrometry, and infrared spectroscopy.

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 methylsilicone column (20 m × 0.3 cm I.D.). Helium served as carrier gas. The column temperature was programmed from 100°C to 250°C at 10°C/min. Electron impact (EI) 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. Chemical ionization (CI) mass spectra were recorded with isobutane

as a reactant gas and with an ionizing energy of 50 eV. The other experimental conditions were as for EI ionization. High-resolution mass spectra were obtained at 70 eV from an MS902 double-focus spectrometer connected to an AEI computer (Scientific Apparatus, Manchester, U.K.).

Proton nuclear magnetic resonance spectrometry

For proton NMR analysis, a Varian EM360A spectrometer (Varian, Palo Alto, CA, U.S.A.) was used at 60 MHz with $Si(CH_3)_4$ (Fluka, Buchs, Switzerland) as internal standard.

Infrared spectroscopy

Infrared spectra were obtained with a Jasco IRA-1 diffraction grating infrared spectrophotometer. Samples, prepared by mixing dried extract onto infrared grade KBr (Merck), were pressed into 10-mm diameter pellets.

RESULTS

Yield of hexane-extractable material

The amount of hexane-extractable material from all the bacterial strains investigated is shown in Table II. The yield was highest in strains of *H. aphrophilus*. Oral strains of *A. actinomycetemcomitans* and of *H. aphrophilus* usually yielded more hexane-extractable material than did non-oral strains.

TABLE II

HEXANE-EXTRACTABLE MATERIAL

Values are expressed as percentage (w/w) of dried cells.

Actinobacillus actinomycetemcomitans		*Haemophilus aphrophilus*	
ATCC 33384	1.3	ATCC 33389	2.0
ATCC 29524	1.0	ATCC 19415	2.0
ATCC 29523	1.7	FDC 655	10.0
ATCC 29522*	2.5	FDC 654	6.0
ATCC 29522**	2.1		
FDC 2112	4.0		
FDC 2097	3.6		
FDC 2043	2.4		
FDC 511	4.0		
FDC N27	4.0		
FDC Y4	1.0		

*Obtained through Forsyth Dental Center.
**Obtained through American Type Culture Collection.

Gas chromatography

The gas chromatograms of the hexane extracts from the examined bacteria revealed major components only, or major components together with minor ones. Based on the distribution of all the components, the *A. actinomycetemcomitans* strains could be divided into three clusters. In the main cluster, i.e. cluster I, which consisted of strains ATCC 33384, 29524, and FDC 2112,

2043, 511, and N27, the major components were $C_{14:0}$, $C_{16:0}$ and $C_{16:1}$ acids. Minor components were present in negligible amounts, i.e. they constituted less than 0.1% (Fig. 1), which was confirmed when a ten-fold higher concentration of test substance was injected into the gas chromatograph (attenuator 2).

Cluster II of *A. actinomycetemcomitans* comprised strains ATCC 29523 and FDC 2097. The major components of these strains were the same as those detected in cluster I. In addition, a series of minor substances were present in moderate amounts (Fig. 2).

Cluster III of *A. actinomycetemcomitans* included strains ATCC 29522, obtained first through Forsyth Dental Center, and later directly through the American Type Culture Collection, and strain FDC Y4. The fatty-acid profiles of the two ATCC 29522 strains did not differ qualitatively. Characteristic of strain cluster III was the presence of a fourth major fatty acid: iso-$C_{15:0}$ acid. A series of minor components were also detected (Fig. 3). The iso-$C_{15:0}$ acid could be separated from anteiso-$C_{15:0}$ acid. At 130°C, the difference in retention time between these two isomeric acids was 0.16 min, at 133°C it was 0.13 min, and at 134°C, 0.12 min. The $C_{14:0}$, $C_{15:0}$, iso-$C_{15:0}$, and anteiso-$C_{15:0}$ acids were well separated at 133°C (Fig. 4). The difference in retention time between $C_{14:0}$ acid and $C_{15:0}$ acid was 1.92 min, between $C_{14:0}$ acid and anteiso-$C_{15:0}$ acid 1.25 min, and between $C_{14:0}$ and

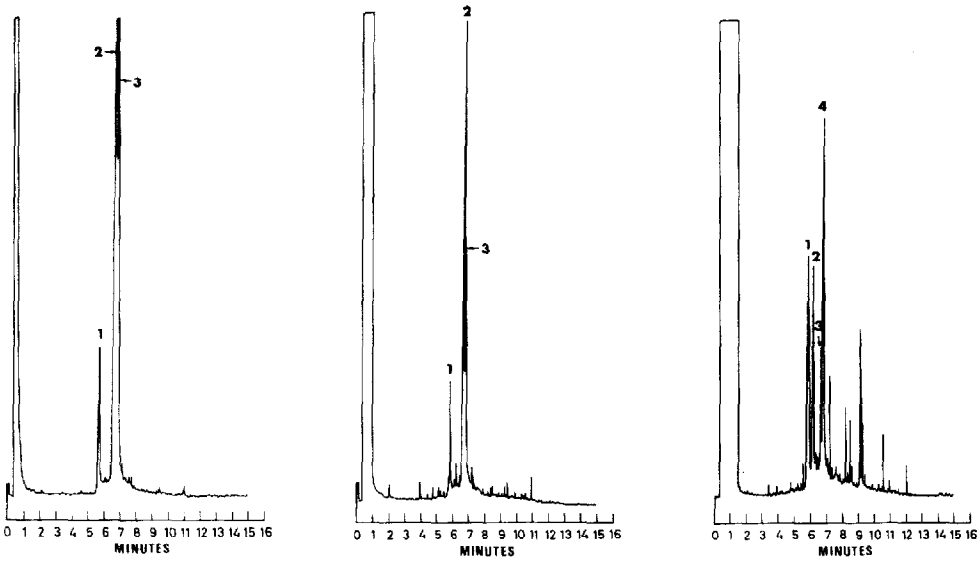

Fig. 1. Gas chromatogram of *A. actinomycetemcomitans*, strain FDC 511, characteristic of cluster I. Program: hold 1 min at 110°C, then 110 to 340°C at 15°C/min. Injector temperature 210°C. Flame ionization detector temperature 210°C. Splitless injection. Paper speed 5 mm/min. Sample injected in hexane 0.1 µl. Attenuator 8. 1 = $C_{14:0}$, 2 = $C_{16:1}$, 3 = $C_{16:0}$.

Fig. 2. Gas chromatogram of *A. actinomycetemcomitans*, strain FDC 2097, characteristic of cluster II. Settings as in Fig. 1. 1 = $C_{14:0}$, 2 = $C_{16:1}$, 3 = $C_{16:0}$.

Fig. 3. Gas chromatogram of *A. actinomycetemcomitans*, strain FDC Y4, characteristic of cluster III. Settings as in Fig. 1. 1 = $C_{14:0}$, 2 = iso-$C_{15:0}$, 3 = $C_{16:1}$, 4 = $C_{16:0}$.

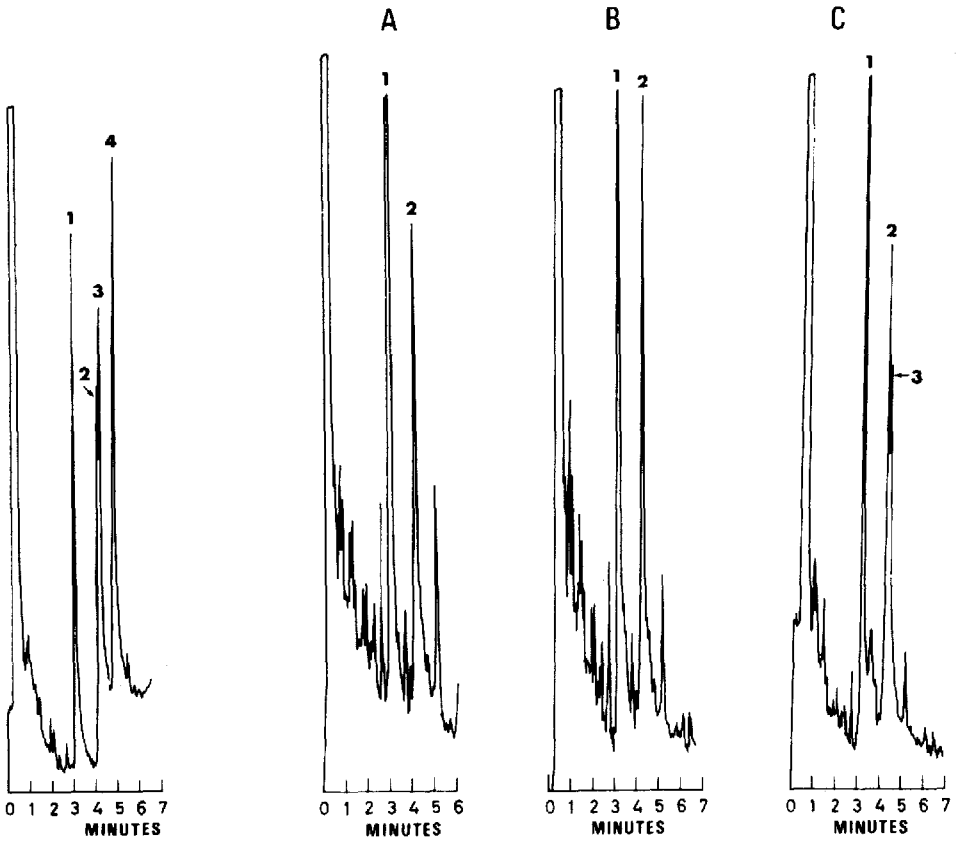

Fig. 4. Gas chromatogram of a mixture of saturated and unsaturated, branched and unbranched synthetic fatty acids. 1 = $C_{14:0}$, 2 = iso-$C_{15:0}$, 3 = anteiso-$C_{15:0}$, 4 = $C_{15:0}$. Isothermal, 133°C. Otherwise settings as in Fig. 1.

Fig. 5. *A. actinomycetemcomitans*, strain FDC Y4, chromatographed alone (A) and cochromatographed with iso-$C_{15:0}$ acid (B) and anteiso-$C_{15:0}$ acid (C). 1 = $C_{14:0}$, 2 = iso-$C_{15:0}$, 3 = anteiso-$C_{15:0}$. Isothermal, 133°C. Otherwise settings as in Fig. 1.

iso-$C_{15:0}$ acid 1.14 min. In Fig. 5, the hexane extract from strain FDC Y4 has been chromatographed alone or cochromatographed with iso-$C_{15:0}$ or anteiso-$C_{15:0}$ acid.

The fatty acid profiles of the strains of *H. aphrophilus* investigated were similar to those of the strains in cluster I of *A. actinomycetemcomitans* (Fig. 6).

The quantitative distribution of the major fatty acids in each bacterial strain is shown in Table III. It was similar in the strains of cluster I and cluster II of *A. actinomycetemcomitans*. The quantitative distribution of these strains differed from that in the strains of cluster III of *A. actinomycetemcomitans* and in *H. aphrophilus*. There was further a marked difference in the fatty-acid content of cluster III of *A. actinomycetemcomitans* and *H. aphrophilus*. In cluster I and cluster II of *A. actinomycetemcomitans*, the $C_{16:1}$ acid was more abundant than the $C_{16:0}$ acid. The $C_{14:0}$ acid was lowest. In cluster III of *A. actinomycetemcomitans*, the $C_{16:0}$ acid was higher than the $C_{16:1}$ and

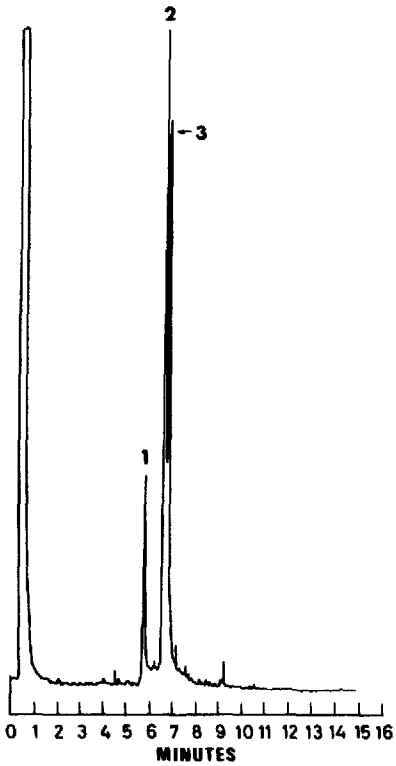

Fig. 6. Gas chromatogram of *H. aphrophilus*, strain FDC 654. 1 = $C_{14:0}$, 2 = $C_{16:1}$, 3 = $C_{16:0}$. Setting as in Fig. 1.

$C_{14:0}$ acids. Whereas iso-$C_{15:0}$ acid was a major fatty acid in all strains of cluster III, it constituted less than 0.1% in the other strains. In *H. aphrophilus*, the $C_{16:1}$ acid was higher than the $C_{16:0}$ acid, and $C_{14:0}$ was lowest.

Gas chromatography—mass spectrometry

The four major components of the hexane extracts were examined further by GC—MS (EI and CI mass spectra). In the spectra from $C_{14:0}$, $C_{16:1}$, $C_{16:0}$, and iso-$C_{15:0}$ acids, the fragment with *m/e* 60 is characteristic of fatty acids and a result of the McLafferty rearrangement [12]. The abundance of this fragment in the spectrum of $C_{14:0}$ acid was 62.8%, of $C_{16:1}$ acid 20.3%, of $C_{16:0}$ acid 62.1%, and of iso-$C_{15:0}$ acid 50.2%. With EI, the molecular ions were approximately 12% of the base peak in all the acids.

Proton nuclear magnetic resonance spectrometry

Non-saturated and saturated carboxylic acids were present in a mixture. The major peaks represented protons in a CH_3 group (δ 0.9) and CH_2 group (δ 1.25). Olefin protons occurred at δ 5.3. The signal from the acid proton (δ 8.5) was weak with a frequency of 550 Hz.

Infrared spectroscopy

A broad band between 3300 and 2500 cm^{-1} suggested O—H deformation

TABLE III

FREE FATTY ACIDS

Data are expressed as a percentage (w/w) of the total in the strain and are means of a total of twenty runs on two extracts obtained from cultures grown at different days.

Cluster	*Actinobacillus actinomycetemcomitans*	$C_{14:0}$	Iso-$C_{15:0}$	$C_{16:1}$	$C_{16:0}$
I	ATCC 33384	13.8		55.3	30.9
	ATCC 29524	17.8		57.8	24.3
	FDC 2112	10.3		45.1	44.6
	FDC 2043	16.4		58.6	24.9
	FDC 511	8.4		51.5	40.1
	FDC N27	9.8		46.4	43.8
II	ATCC 29523	9.3		45.4	45.2
	FDC 2097	14.0		55.5	30.5
III	ATCC 29522*	20.8	9.0	26.8	43.4
	ATCC 29522**	23.7	7.9	30.4	38.0
	FDC Y4	21.9	16.8	17.8	43.6
	Haemophilus aphrophilus				
	ATCC 33389	20.8		41.2	38.1
	ATCC 19415	21.6		46.1	29.9
	FDC 655	19.8		52.4	27.8
	FDC 654	24.2		44.4	30.8

*Obtained through Forsyth Dental Center.
**Obtained through American Type Culture Collection.

in the carboxylic acid. In the areas 2950 to 2920 cm^{-1}, and at 2850 cm^{-1}, three bands were seen suggesting C—H deformation. Intense bands in the 1715 cm^{-1} area represented stretching frequencies in dimerized C=O carboxylic acid groups. Moderate bands at 1280 cm^{-1} indicated deformation in the carboxylic acid C—O dimer. The absorption at 725 to 730 cm^{-1} was characteristic of CH_2 groups in long-chain ($C \geqslant 16$) fatty acids.

DISCUSSION

Fatty acids of whole bacterial cells can be extracted either as bound fatty acids or as free acids [13]. In the present study, free fatty acids were chosen since previous investigations had revealed no difference in the content of bound fatty acids in *A. actinomycetemcomitans* and *H. aphrophilus* [14—16]. With our methods, *A. actinomycetemcomitans* proved to be rather heterogeneous and could usually be separated from the more homogeneous *H. aphrophilus*. These findings supported the idea of maintaining *A. actinomycetemcomitans* and *H. aphrophilus* in separate species, but suggested at the same time that *A. actinomycetemcomitans* might benefit from being divided into subspecies. This particularly applied to the cluster III strains. The heter-

ogeneity of *A. actinomycetemcomitans* demonstrated in the present study on cellular fatty acids parallelled its serological variety. Rabbit immune sera against *A. actinomycetemcomitans* contained two serotypes: (a) which was represented by ATCC 29523, and (b) represented by ATCC 29524, 29522, and FDC Y4 [17].

By means of GC, GC—MS, proton NMR, infrared spectroscopy, and by comparison of our results with existing literature data on fatty acids [18—20], we found the major fatty acids of *H. aphrophilus* to be myristic ($C_{14:0}$), palmitic ($C_{16:0}$), and palmitoleic ($C_{16:1}$) acid. This was in complete agreement with the observations made by Braunthal et al. [14]. In *A. actinomycetemcomitans* the same acids were found, but we also detected 13-methyltetradecanoic acid (iso-$C_{15:0}$) as a fourth major acid in strain cluster III. Calhoon et al. [15] and Jantzen et al. [16], studying bound whole-cell fatty acids of *A. actinomycetemcomitans* and *H. aphrophilus*, detected 3-hydroxymyristic acid as a fourth major acid, but this acid was not a major distinguishing character in these bacteria. It is well known that in bacteria free fatty acids may differ markedly from bound fatty acids [9]. It is also recognized that a number of factors, e.g. growth medium and atmosphere, which differed in our study from that used by the above cited authors may have a profound influence not only on the quantitative, but also on the qualitative distribution of fatty acids in bacteria. Care must therefore be taken when comparing the gas chromatographic profiles of cellular fatty acids from bacteria cultured under different conditions. The extraction technique may also have a strong impact on the yield of fatty acids recovered from bacteria. Contrary to free fatty acids extracted with hexane, hydroxy-acids are covalently bound in the bacterial cell and may not be released but rather require hydrolysis with base or acid. Hydroxy-fatty acids could not be detected in the lipids extractable from bacterial cells with solvents such as ethanol and methanol—chloroform [21].

In cluster III of *A. actinomycetemcomitans*, iso-$C_{15:0}$ was a major fatty acid, while it was present only in trace amounts in the other bacterial strains investigated. It was high in strain ATCC 29522 whether it was obtained from Forsyth Dental Center or directly through the American Type Culture Collection. Recently, iso-$C_{15:0}$ acid was found as a major fatty acid in another CO_2-loving group of bacteria in dental plaque, viz. *Capnocytophaga*, where it constituted between 61 and 78% of the total [22]. Other gliding bacteria (the *Cytophaga—Flexibacter* group, the myxobacteria) also contain a significant amount of 13-methyltetradecanoic acid [23]. This acid is also a major fatty acid in oral and non-oral species of *Bacteroides* [24]. It is not possible at the moment to decide why the cluster III strains of *A. actinomycetemcomitans* were so high in iso-$C_{15:0}$ acid. Synthesis of branched-chain fatty acids in bacteria is believed to occur via α-keto acids, derived from endogenous precursors or from exogenous amino acid substrates, through acyl-CoA esters [25]. Mutant strains may produce less iso-branched acid than their origin. In *Bacillus subtilis* a mutant strain requiring L-leucine, which serves as the precursor for iso-acids, produced iso-$C_{15:0}$ and iso-$C_{17:0}$ fatty acids in much smaller proportions than did the parent strain [25].

At the moment, there is no universally accepted method for extracting lipids from whole bacterial cells, and the number of techniques currently used are legion [13]. The present study demonstrated that hexane extraction from lyophilized bacteria in a Soxhlet apparatus is a useful method for removing long-chain saturated and non-saturated, straight-chain as well as branched-chain whole-cell fatty acids from bacteria for taxonomic purposes. By standard addition of acid, this method recovered free fatty acids with an efficiency of 90% [11]. It was not necessary to increase the volatility of the recovered fatty acids through derivatization before gas chromatographic analysis. In fact, our direct method of gas chromatography made it possible not only to differentiate between the examined bacteria according to the major components present in their hexane extracts, but also according to the minor substances recovered. This meant that each bacteria could be grouped according to the total chemical fingerprint it left on the gas chromatogram. The simplicity, reproducibility and sensitivity of the present procedures suggested that they could be useful in future chemotaxonomic work on microorganisms, either as separate techniques or in combination with more well-established methods.

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