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Short Communication

Intra-injector methylation of free fatty acids from aerobically and anaerobically cultured *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*

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ABSTRACT

Free fatty acids from the type strains of anaerobically and aerobically broth-cultured *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* cells were Soxhlet-extracted with hexane. The fatty acids were identified and quantified by gas chromatography and gas chromatography–mass spectrometry after intra-injector derivatization with trimethylanilinium hydroxide. This derivatization method, which we propose as suitable for routine use in clinical microbiology, is fast, accurate and sensitive, with low toxicity. Whereas the fatty acid content of *A. actinomycetemcomitans* was affected by the cultivation atmosphere, *i.e.* $C_{16:1}$, decreased under aerobic growth and $C_{16:0}$ increased, that of the closely related *H. aphrophilus* was more stable.

INTRODUCTION

Differences in the ultrastructure and surface protein profiles of aerobically and anaerobically cultured *Actinobacillus actinomycetemcomitans* cells suggest that oxygen-related variations in the cell-wall surface may play a role in the adaptation of that facultative anaerobic bacterium to different host environments [1]. The bacterial cell envelope may contain free fatty acids that exhibit a wide range of toxic activities [2] and therefore may act as virulence factors, *e.g.*, in the case of the putative periodontopathogen *A. actinomycetemcomitans* [3]. The present study was made to see if the content of free fatty acids in aerobically and anaerobically cultered *A. actinomycetemcomitans* cells is different. Cellular fatty acids are also recognized as suitable auxiliaries in the chemotaxonomy of bacteria [4,5]. The free fatty acid composition of *A. actinomycetemcomitans* was therefore compared with that of the taxonomically closely related *Haemophilus aphrophilus*, which does not seem to be associated with periodontal diseases [6].

Free fatty acids often need derivatization be-

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fore identification and quantification with gas chromatography (GC) and GC–mass spectrometry (MS). Unfortunately, current derivatization techniques for fatty acids do not seem to be quite optimal (for a review, see ref. 4). The most frequently used methods are acid-catalysed esterification, methylation with diazomethane, boron trifluoride or boron trichloride, and methylation using hydrochloric acid in methanol. None of these techniques excludes the possibility of artifacts being formed from naturally occurring lipids in a mixture. Esterification/transesterification with methanol and hydrochloric acid is a mild and useful derivatization method for lipids, but its application, which is time consuming, involves loss of volatile fatty acids [7,8]. In order to prevent such loss, alcoholysis with ethanol, propanol or butanol was proposed [9]. Base-catalysed methylation may produce unsaturated artifacts from hydroxy acids, *i.e.*, the corresponding 2-enoic acid from 3-hydroxytetradecanoic acid [10]. Artifacts produced during methylation with boron trihalides have also been reported [11]. Diazomethane reagents are carcinogenic and liable to produce artifacts [12,13]. Trimethylsilylating reagents cause isomerization of 1-glycerides into 2-glycerides and are easily hydrolysed [14]. Accordingly, there is a need for derivatization procedures in the clinical microbial laboratory that are fast, easy to handle and reproducible without causing artifacts, and which include reagents of low toxicity. Pyrolysis of tetramethylammonium salts, which requires titration of acids and a relatively high temperature, is rarely used in the microbiological field [15]. Application of other salts of acids from amines has been reported, *e.g.*, of trimethylanilinium salts [16]. Trimethylanilinium hydroxide (TMAH) has been used for quantitative methylation and detection by GC of drugs [17–21] and phenoxy acids [22], but has not been used in clinical microbiology. Trimethylsulphonium hydroxide has been applied for direct and rapid formation of bacterial fatty acid methyl esters [23,24]. Another purpose of the present study was to evaluate TMAH as a derivatization agent for free bacterial fatty acids, and to see if the derivatization procedure could be made so simple and safe as to take place inside the injector immediately before GC analysis.

EXPERIMENTAL

Bacteria

A. actinomycetemcomitans ATCC 33384^T (T = type strain) and *H. aphrophilus* ATCC 33389^T were cultured aerobically under fast shaking in a gyratory shaker (New Brunswick Scientific, New Brunswick, NJ, USA) and anaerobically under a N_2–H_2–CO_2 (80:10:10, v/v/v) gas phase in a glove-box (Forma Scientific Anaerobic System, Marietta, OH, USA) at 37°C for 5 days. The organisms had been obtained directly from the American Type Culture Collection (Rockville, MD, USA). Brain heart infusion (Difco Labs., Detroit, MI, USA) broth was used as the growth medium. Before anaerobic culture, the medium was kept overnight in the reduced conditions of the glove-box. The organisms were harvested by centrifugation, washed twice in deionized distilled water, and lyophilized over diphosphorous pentoxide (E. Merck, Darmstadt, Germany). Lyophilized cells were kept at −20°C under nitrogen. All cultures were produced in duplicate on different days.

Fatty acid extraction

Free fatty acids were extracted twice, each time for 4 h, with *n*-hexane (Merck) in a Soxhlet-type apparatus [3,25]. The extracts were evaporated with nitrogen in glass vials until dry.

Derivatization of fatty acids

MethElute "on-column" methylating agent (Pierce Europe, Oud-Beijerland, Netherlands) containing 0.2 *M* TMAH in methanol, was kept in Hypo-Vial sample storage vials. To each reaction vial, containing 0.1–0.5 mg of dried fatty acid extract, 100 μl of MethElute agent were transferred with a syringe.

GC of derivatized fatty acids

A Model 8700 gas chromatograph (Perkin-Elmer, Norwalk, CT, USA) was used for fatty acid analyses. The fused-silica capillary column (15 m

× 0.25 mm I.D.) had a film thickness of 0.25 μm. The stationary phase was methylphenylsilicone CP-Sil 5 (Chrompack, Middelburg, Netherlands). Helium served as carrier gas at a flow-rate of 2.0 ml/min. The temperature of the injector was 260°C, and the temperature of the flame ionization detector was 275°C. The program was as follows: the temperature was held for 1 min at 90°C and then brought to 290°C at 6°C/min. The attenuator was set at 8, and the paper speed was 10 mm/min. The sample (2 μl) was delivered as a splitless injection. The identities of the derivatized fatty acids were confirmed by using authentic derivatives of C_{10}–C_{24} acids. The standards had been derivatized in a way similar to the native acids, using 0.1 mg of substance.

Gas chromatography–mass spectrometry

The instrument used for GC–MS consisted of a Model 8700 gas chromatograph furnished with an ion-trap detector (Perkin-Elmer). For chromatographic conditions, see under *GC of derivatized fatty acids.*

Reference fatty acids

The FA-FAME Kit 14 (catalog No. 1039) and the bacterial acid methyl esters mixture CP 4-7080 from Supelco (Bellefonte, PA, USA) included the authentic fatty acids examined. Also National Institutes of Health reference mixture 4-7013 (Supelco) was used.

RESULTS

Derivatization with TMAH gave a recovery of 99.9% (99.0–101.0%) for fatty acids longer than C_{18}, and of 100.0% for short-chain fatty acids (C_4–C_{12}). The coefficient of variation (C.V.) was 3%, and the limit of detection was 0.1 μg/500 μl. TMAH gave single peaks for each derivatized substance. There were no indications of artifacts having been formed. MS fragmentation patterns of the TMAH-derivatized fatty acids agreed with those of authentic standards.

Recovered and derivatized free fatty acids from whole cells of aerobically and anaerobically cultered *A. actinomycetemcomitans* and *H. aphrophilus* cells are shown in Figs. 1 and 2 and Table I. $C_{16:1}$ was the major fatty acid in *A. actinomycetemcomitans* and *H. aphrophilus*, whether aerobically or anaerobically cultivated. $C_{16:0}$ was

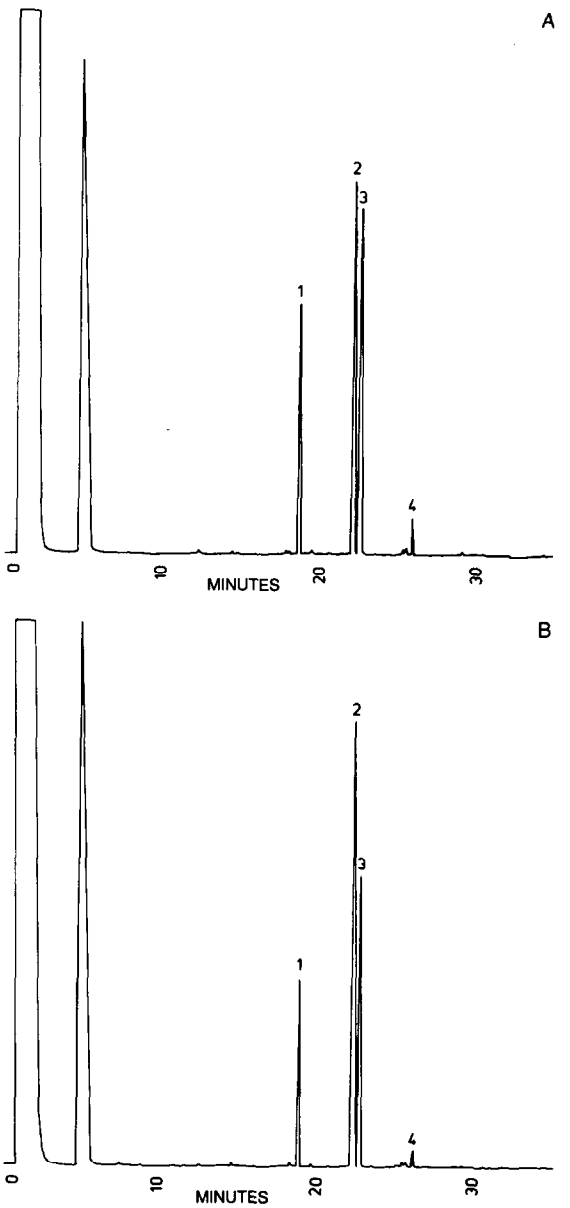

Fig. 1. Gas chromatograms of the free fatty acid compositions of whole cells from the type strain of *A. actinomycetemcomitans* (ATCC 33384) cultured aerobically (A) and anaerobically (B). Peaks: 1 = $C_{14:0}$, tetradecanoic acid; 2 = $C_{16:1}$, hexadecenoic acid; 3 = $C_{16:0}$, hexadecanoic acid; 4 = $C_{18:0}$, octadecanoic acid.

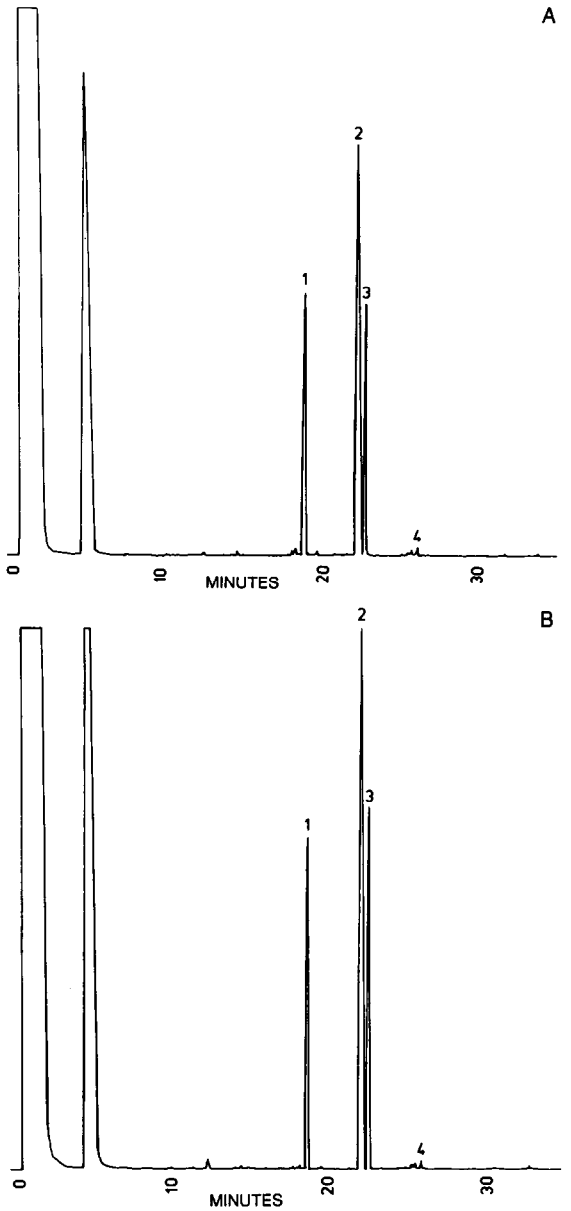

Fig. 2. Gas chromatograms of the free fatty acid compositions of whole cells from the type strain of *H. aphrophilus* (ATCC 33389) cultured aerobically (A) and anaerobically (B). Peaks as in Fig. 1.

slightly more abundant than $C_{14:0}$ in both organisms, and $C_{18:0}$ was present in minor amounts. Volatile fatty acids were not detected. *A. actinomycetemcomitans* showed the largest changes in the free fatty acid composition when the cultivation atmosphere was changed from aerobic to anaerobic. Although the amount of $C_{16:1}$ decreased considerably during aerobic culture compared with anaerobic, that of $C_{16:0}$ increased. *H. aphrophilus* showed only small changes in its free fatty acid content with a similar change in the cultivation gas phase. The difference in fatty acid composition between *A. actinomycetemcomitans* and *H. aphrophilus* was larger after aerobic than after anaerobic culture. The fatty acids contributing most to this discrepancy were $C_{16:1}$ and $C_{16:0}$.

DISCUSSION

Methylation of free fatty acids with TMAH was first described by Middleditch and Desiderio [16] and later recommended by Knapp [26]. TMAH has since been used for GC assessment of barbiturates, sedatives, xanthine bases, phenolic alkaloids, Dilantin [17–21], and phenoxy acids [22], with high accuracy (as good as or better than the thin-layer chromatography–UV method [21], high precision (C.V. 5% or less) [17], and high sensitivity (for barbiturates, down to 0.2 mg/dl) [17]. The present study confirmed that methylation of free fatty acids with TMAH is quantitative without formation of side-products [16,22]. The recovery of short- and long-chain authentic fatty acids with this method was *ca.* 100%, the C.V. was 3%, and the limit of detection was 0.1 μg/500 μl. The MethElute reagent was kept safely in a Hypo-Vial sample storage vial before reacting with the fatty acids. Toxic exposure of the operator was thereby eliminated. Since derivatization occurred immediately in the injector, the sample could be injected into the gas chromatograph without delay. This mode of derivatization is generally called "on-column derivatization", but since the derivatization actually takes place inside the injector, we suggest "intra-injector derivatization" as a more appropriate term. TMAH-derivatized fatty acids did not show any signs of degradation when stored at 4°C for 1 week. In order to test the methylphenylsilicone stationary phase, 300 injections into the column were made. No broadening of peaks or

TABLE I

FREE CELLULAR FATTY ACIDS OF FACULTATIVE BACTERIA

The relative amount of a substance is expressed as the area of its peak in a chromatogram as a percentage of the total area of the peaks of all substances that were examined. Each value is a mean (standard deviation ± 3%) derived from two independent preparations run in triplicate, with three injections being made from each preparation (n = 18). Abbreviations: $C_{14:0}$, tetradecanoic acid; $C_{16:1}$, hexadecenoic acid; $C_{16:0}$, hexadecanoic acid; $C_{18:0}$, octadecanoic acid.

Species/strain	Atmosphere	$C_{14:0}$	$C_{16:1}$	$C_{16:0}$	$C_{18:0}$
A. actinomycetemcomitans (ATCC[a] 33384[b])	Aerobic	20.0	41.6	36.4	2.0
	Anaerobic	14.3	57.3	27.7	1.0
H. aphrophilus (ATCC[a] 33389[b])	Aerobic	15.7	59.0	24.8	0.6
	Anaerobic	20.0	55.9	23.7	0.9

[a] American Type Culture Collection, Rockville, MD, USA.
[b] Type strain of the species.

changes in retention times followed. The derivatization system was easy to handle and should be suitable for routine use in the clinical microbiology laboratory, where it has not previously been applied. Currently used derivatization methods may give secondary peaks of fatty acids on the gas chromatograms, which can necessitate confirming tests, but TMAH provides a single peak response for each substance. There were no signs that artifacts had been formed, as revealed through degradation of double bonds.

Accordingly, the TMAH procedure is recommended for derivatization of free fatty acids for GC in the clinical microbiology laboratory since it is fast, accurate, and sensitive, and uses low-toxic substances. It can also be used for the derivatization of free fatty acids from other biological material, *e.g.*, blood, serum, cerebrospinal fluid, and plants, and for phenoxy acids.

It is generally difficult to maintain aerobic conditions in liquid cultures because bacterial removal of oxygen from the medium is more rapid than absorption of oxygen from the atmosphere. This exchange was optimized by using a fast shaking rate for both organisms. Examination of the free fatty acid composition in *A. actinomycetemcomitans* after aerobic and anaerobic culture revealed differences, whereas that in the closely related *H. aphrophilus* was relatively stable. For comparison, aerobic and anaerobic growth of the yeast *Saccharomyces cerevisiae* also produced marked differences in the free fatty acid composition [27]. The presence of free fatty acids in whole cells of *A. actinomycetemcomitans* and *H. aphrophilus* has also been established after culture in 10% CO_2 in air [3,25]. Although cultivation under an aerobic gas phase led to a decrease of the free hexadecenoic acid ($C_{16:1}$) fraction and an increase of the hexadecanoic acid ($C_{16:0}$) fraction in *A. actinomycetemcomitans*, the free fatty acid content of *H. aphrophilus* was found to be more stable. This observation remains unexplained. Specific information on the origin and factors influencing the free fatty acid content of microorganisms is generally lacking. To what extent, if any, autolysis may have contributed to free fatty acid formation, requires further studies. It should be realized that these bacteria live through all growth phases in the periodontal pocket. The change in the fatty acid composition of *A. actinomycetemcomitans* with the cultivation gas phase composition parallelled that observed in its ultrastructure and surface protein profiles under similar conditions [1]. All these findings indicate that *A. actinomycetemcomitans* adapts more easily to the different oxygen partial pressures (pO_2) of various ecological niches of the oral cavity than does *H. aphrophilus*. The gas space over the tongue has a pO_2 of 12–14%, but the pO_2 of the periodontal pocket is only 1–2% [28]. Both *A.*

actinomycetemcomitans and *H. aphrophilus* are indigenous to subgingival plaque but *A. actinomycetemcomitans* is able also to colonize the tongue, palate and buccal mucosa [29]. Since attachment and colonization are generally considered prerequisites for an infection to occur, *A. actinomycetemcomitans* may be more apt to cause oral infection than is *H. aphrophilus*.

Free fatty acids in the bacterial cell are generally regarded as toxic [2]. The present findings, demonstrating $C_{16:1}$, $C_{16:0}$ and $C_{14:0}$ as the most abundant free fatty acids after anaerobic growth, suggest that these substances, if released into the highly reduced periodontal pocket, or into tissues after periodontal invasion by *A. actinomycetemcomitans*, may extend the battery of potential virulence factors from this putative periodontopathogen. Free fatty acids may affect the pathogenesis of periodontal infections in several ways, *e.g.* by inhibiting host enzyme systems, glycolysis, and phagocytosis, and by inducing autolysis, adding to the deleterious effects exerted by volatile short-chain fatty acids orginating in the pocket from bacterial metabolism, such as acetic, propionic, isobutyric, butyric, and isovaleric acids [30].

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