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Note

Direct analysis of free fatty acids in bacteria by gas chromatography

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Fatty acids in the range $C_{4:0}$ to $C_{12:0}$ are synthesized in the mammary glands; fatty acids with longer chain-lengths are present in animal tissues, especially in brain and the nervous system, and in bacteria. In bacteria, whole cells are increasingly being used for chemotaxonomic purposes. Within bacterial cells the fatty acids may occur free or in the form of glycerides. Most acids, however, are linked to large molecules such as phospholipids, glycolipids, lipoproteins, lipopolysaccharides, lipotechoic acid. Unfortunately, there is no universally accepted method for extracting fatty acids from bacteria, and a series of techniques, some of them quite complex, are being used [1]. In extraction, a dilemma is presented to the biochemist: if the technique is gentle enough, the lipids obtained will probably have undergone relatively minor degradation; however, a certain portion of the total lipids may be missing. On the other hand, if the extraction technique is rigorous enough to obtain all or most of the total cellular lipids, most of them may have become degraded [2].

At present, "bound" fatty acids, which require acid or alkaline hydrolysis to be released, are most commonly used in chemotaxonomy. After hydrolysis, these acids are derivatized before gas chromatographic analysis. Since both the hydrolysis and derivatization procedures may produce artifacts, we feel that free fatty acids should be increasingly used for taxonomic purposes. These acids may be quite specific and differ markedly from "bound" lipids in bacteria such as *Pseudomonas*, *Alcaligenes*, *Moraxella*, and *Neisseria* [2].

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In the present study, the free fatty acids of whole cells of the taxonomically closely related *Haemophilus aphrophilus* and *Actinobacillus actinomycetemcomitans* were extracted with hexane in a Soxhlet apparatus and analyzed directly without derivatization in a gas chromatograph. In general, free fatty acids are difficult to analyze directly by gas chromatography due to the broad and often tailing peak shape. Even if this can be overcome by esterification, the derivatization step is time-consuming and in some cases may result in non-quantitative recovery [3]. So far, the best peak symmetry of free fatty acids seems to have been obtained by using glass microbeads covered with FFAP (free fatty acid phase) stationary phase, but even on this column the higher acids gave relatively broad peaks with low resolution [4].

In the present paper emphasis will be placed on methodology, while comparative fatty acid profiles of all the bacterial strains investigated will be detailed elsewhere.

MATERIAL AND METHODS

Bacteria

Two reference strains (ATCC 33389, 19415) and two laboratory strains (FDC 655, 654) of *H. aphrophilus* and two reference strains (ATCC 33384, 29523) of *A. actinomycetemcomitans* were cultivated in Brain Heart Infusion® broth (Difco Laboratories, Detroit, MI, U.S.A.) in air plus 10% carbon dioxide for 5 days at 37°C.

Synthetic fatty acids

The following synthetic fatty acids were chromatographed: caproic ($C_{6:0}$) and caprylic ($C_{8:0}$) acid (E. Merck, Darmstadt, G.F.R.), nonanoic ($C_{9:0}$) acid (Koch-Light Laboratories, Colbrook, Great Britain), capric ($C_{10:0}$) and undecanoic $C_{11:0}$) acid (Fluka, Buchs, Switzerland), and lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), and stearic ($C_{18:0}$) acid (Sigma Co., St. Louis, MO, U.S.A.).

Extraction of free whole-cell fatty acids

Bacterial cells were harvested by centrifugation, washed three times with deionized distilled water and lyophilized. Extraction of lyophilized material was performed two times with fresh *n*-hexane (Merck) in an all-glass Soxhlet-type apparatus furnished with a refluxing Liebig water condenser, each time for 3–4 h. Extended extraction times did not result in higher yields. The extracts were pooled, dried and stored at −20°C in an oxygen-free atmosphere. The reproducibility of the total analysis was determined by the reproducibility of the extraction steps, which was found to be ± 5%. By standard addition of $C_{16:0}$ to the *H. aphrophilus* 654 cell strain, a recovery of 90% was obtained.

Gas chromatography

A Carlo Erba 4200 (Carlo Erba, Milan, Italy) gas chromatograph equipped with a CP-Sil 5 glass capillary column was used. The Chrompack (Middelburg, The Netherlands) CP-Sil 5 (polydimethylsiloxane) column was 25 m × 0.22 mm

I.D., 0.14 μm film thickness and 0.25 mm HETP. Helium was used as carrier gas at 2 ml/min. The pressure at the inlet of the column was 151.5 kPa.

RESULTS

The gas chromatogram of a series of saturated and unsaturated synthetic fatty acids is shown in Fig. 1. Analogous acids exist as cellular constituents, or are produced as metabolic products by a wide spectrum of bacteria. The synthetic fatty acids were eluted successively according to increasing chain length. The $C_{16:0}$ acid followed after $C_{16:1}$. In Fig. 2 separation of these two major bacterial fatty acids are shown at various constant temperatures. The techniques described above provided excellent separation of free whole-cell fatty acids recovered from *H. aphrophilus* and *A. actinomycetemcomitans*. The fatty acid profiles of two representative strains are presented in Fig. 3. A high temperature (210°C) was chosen in order to be able to include higher fatty acids, if present.

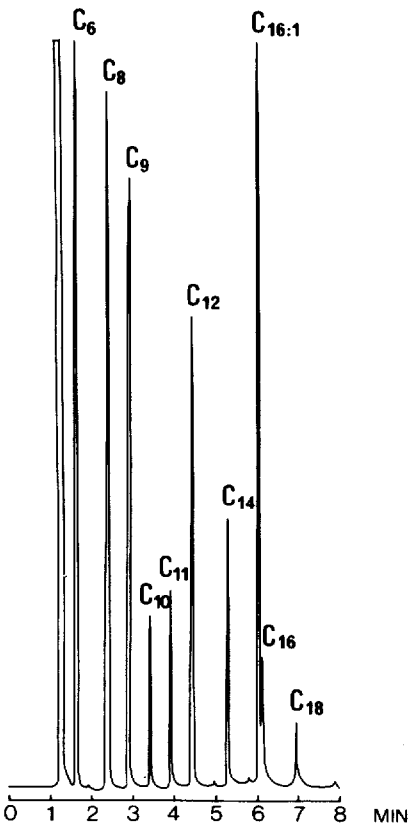

Fig. 1. Gas chromatogram of a mixture of saturated and unsaturated synthetic fatty acids. Program: hold 1 min at 110°C, then 110 to 290°C at 30°C/min. Injector, 240°C. Flame ionization detector, 320°C. Split injection, 1/100. Paper speed, 10 mm/min. Sample injected in hexane, 0.4 μl. Attenuator, 8.

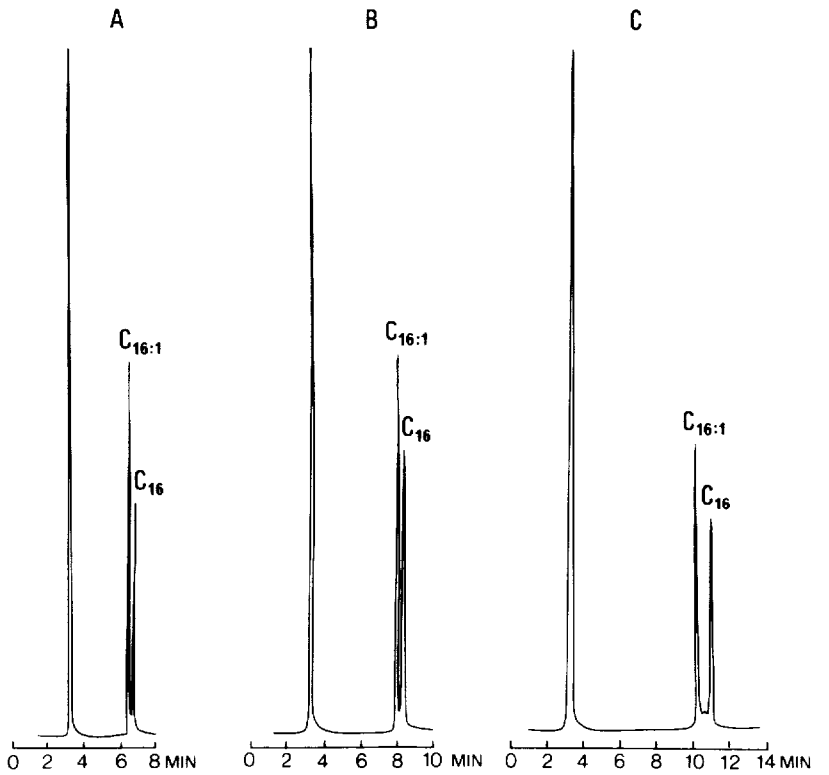

Fig. 2. Gas chromatograms showing separation of $C_{16:1}$ and $C_{16:0}$ fatty acids. (A) Isothermal, 190°C; (B) isothermal, 180°C; (C) isothermal, 170°C. Otherwise chromatographic settings were as given in Fig. 1, except the paper feed rate (5 mm/min).

DISCUSSION

In bacteriology, Soxhlet extraction has previously been performed with *Sarcina lutea* using benzene—methanol as solvent [5] and with *P. maltophilia* using chloroform—methanol [6]. The present study with *H. aphrophilus* and *A. actinomycetemcomitans* suggested that Soxhlet extraction is a simple method for removing taxonomically important long-chain fatty acids from whole bacterial cells, and that it deserves wider application in chemotaxonomy. The type of association between the lipids and the other cellular constituents determines the solvent to be used. Thus, non-polar solvents are used for extraction of neutral lipids, polar solvents for polar lipids, and after hydolysis appropriate solvents are used to extract "bound" lipids. The purpose of using a nonpolar solvent such as hexane was to provide a relatively simple extract which included the free fatty acids to be compared. The possible presence of neutral lipids in the extract did not interfere with the determination of the free fatty acids in the bacterial strains examined.

The general gas chromatographic problem of broad tailing peaks of free fatty acids was solved by using a thin film of a high-temperature non-polar stationary phase (CP-Sil 5). Extracted saturated ($C_{14:0}$—$C_{16:0}$) and unsaturated ($C_{16:1}$) whole-cell fatty acids, as well as a mixture of synthetic acids ($C_{6:0}$—

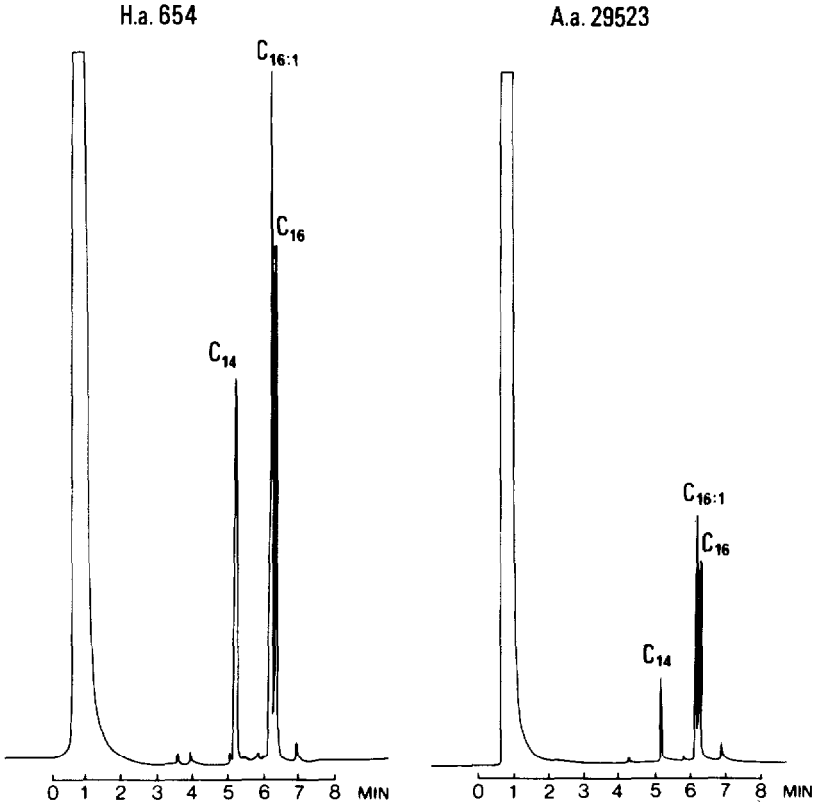

Fig. 3. Typical gas chromatograms of free fatty acids in *Haemophilus aphrophilus* (H.a.) and *Actinobacillus actinomycetemcomitans* (A.a.). Isothermal, 210°C. Injector temperature, 240°C. Flame ionization detector, 320°C. Split injection, 1/100. Sample injected in hexane, 0.4 μl. Paper speed, 10 mm/min. Attenuator, 8.

$C_{18:0}$), could be analyzed directly without derivatization. This may represent a significant improvement to or supplement current procedures of gas chromatography of fatty acids as applied in microbial taxonomy. Our method has now been used for fatty acid analysis of a series of strains of *H. aphrophilus* and *A. actinomycetemcomitans* (Brondz and Olsen, unpublished results) and should be well fitted for routine use in clinical microbiological laboratories due to its simplicity and high sensitivity. The wide spectrum of acids separated suggests that the present method is also applicable to acidic fermentation products. It may also be used to determine fatty acids in other living matter such as animals and plants.

CONCLUSIONS

Whole cells of *H. aphrophilus* and *A. actinomycetemcomitans* were Soxhlet extracted with hexane and the content of free fatty acids determined directly, without derivatization, by gas chromatography on a non-polar fused-silica column. A series of authentic standards ($C_{6:0}$—$C_{18:0}$, $C_{16:1}$) was also analyzed. Both saturated and unsaturated acids were recovered and determined with good

precision, without measurable interference. The present method may represent an important improvement on or supplement current procedures of gas chromatography of bacterial fatty acids as applied in chemotaxonomy.

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