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Note

Chemotaxonomy at a crossroads?

Gas chromatographic analyses of a single colony from the bacterium *Haemophilus aphrophilus*

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Bacteria, after having been recovered by cultivation on blood agar plates from clinical samples, are usually subcultured and then transferred to liquid media for identification. Occasionally, these time-honoured procedures are saddled with problems. A number of bacterial isolates exist today that have not been identified because they cannot be cultured in liquid media. It is also known that serial transfer techniques have several disadvantages: risk of contamination, selection of variants or mutants and possible loss of culture. Further, serial transfers for identification in liquid media may delay microbial diagnosis and specific antimicrobial treatment. In chemotaxonomy, analyses of the composition of bacterial cells are generally performed with organisms cultured in liquid media. After mass cultivation in such media, washing of the bacterial cells may remove extracellular materials. To overcome these problems, taxonomy should be based on monoclonal bacterial cells as they occur in the primary colony on the agar plate.

In the present study, a system has been developed that makes it possible to perform chemotaxonomy on a single bacterial colony. This report emphasizes methodological aspects. Data from a series of bacterial strains will be published later.

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MATERIAL AND METHODS

Bacterium

The type strain of *Haemophilus aphrophilus* (ATCC 33389, NCTC 5906) was cultured anaerobically (80% nitrogen, 10% hydrogen, 10% carbon dioxide) on blood agar plates for seven days. A solitary colony for analysis was picked up with a precleaned, glass capillary tube, care being taken to prevent agar sampling. All tubes with colonies were examined for agar under a stereomicroscope. Furthermore, agar was sampled in separate capillary tubes as a control. The sampled material was lyophilized over diphosphorus pentoxide (Merck, Darmstadt, F.R.G.). Eight agar plates were inoculated, each at seven-day intervals, for the sampling of one solitary colony on each.

Methanolysis

That part of the capillary tube containing the dried colony, 2—3 mm in length, was partially cut off with a diamond knife. The partially cut tube was broken off in a 100-μl screw-capped vial furnished with a septum (Hewlett-Packard Models). A micropipette with a glass tip was used to transfer 100 μl of 2 *M* hydrochloric acid in methanol to the microvial. The screw cap was furnished with an extra PTFE liner, 8 mm in diameter, to prevent contamination with rubber during hydrolysis. Methanolysis took place overnight at 85°C, after which the vial was cooled on ice plus sodium chloride. Drying of the methanolysate occurred with chromatographic-grade nitrogen. The gas was transported through an active coal filter and a dust filter. After gassing, the methanolysate was dried further in a vacuum pump for 1 h.

Derivatization

The methanolysate was derivatized in a mixture consisting of trifluoroacetic anhydride (TFAA; Fluka, Buchs, Switzerland) and acetonitrile (Rathburn, U.K.) in a ratio of 1:3. An aliquot of 10 μl of the reagent was transferred to the 100-μl screw-capped vial. The flask was closed and its contents heated to 85°C for 3 min. A continuous temperature-monitoring device [1] and a stop-watch were used to maintain the conditions inside the flask.

Reference compounds

The reference compounds of the sugars and fatty acids used have been described elsewhere [2].

Gas chromatography and quantitation

A type 5840A Hewlett-Packard (Avondale, PA, U.S.A.) gas chromatograph furnished with an electronic integrator was used. A Chrompack (Middelburg, The Netherlands) CP-Sil 5 (polydimethylsiloxane) glass capillary column (30 m × 0.22 mm I.D.) with film thickness 0.13 μm was used. Helium served as the carrier gas at a flow-rate of 2 ml/min. The pressure at the inlet of the column was 151.5 kPa. The temperature of the injector was 200°C and that of the flame ionization detector was 220°C. Programme: hold 2 min at 90°C, then from 90 to 190°C at 9°C/min with the attenuator set at 0. The chart paper speed was 10 mm/min. The sample (0.2 μl) was delivered by splitless

TABLE I

PERCENTAGE DISTRIBUTION OF IDENTIFIED SUGARS AND FATTY ACIDS FROM THE TYPE STRAIN OF *HAEMOPHILUS APHROPHILUS*

$n = 32$; S.D. $\leq 15\%$.

Compound	Distribution (%)
Galactose	17.4
Glucose	57.8
L-Glycero-D-mannoheptose	11.8
Myristic acid	1.9
β-Hydroxymyristic acid	6.5
Palmitoleic acid	0.7
Palmitic acid	3.9

injection. The identities of the methanolysed and derivatized sugars and fatty acids were determined tentatively with gas chromatography [3]. The identities of these derivatives had previously been established with gas chromatography—mass spectrometry [4—6]. From each of the eight derivatized colonies, four runs were made on the gas chromatograph. The data presented in Table I are therefore mean values of 32 runs. Quantitation of sugars and fatty acids was performed as described previously [3].

RESULTS

The distribution of identified sugars and fatty acids in a single colony from *H. aphrophilus* is given in Table I. The quantity of sugars was much higher than that of fatty acids. Among the identified sugars (galactose, glucose and L-glycero-D-mannoheptose), glucose was the dominant substance. The gas

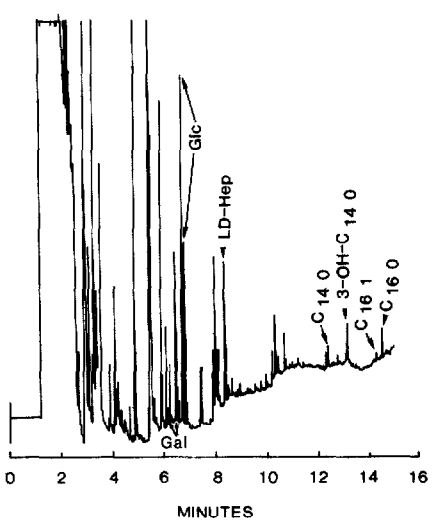

Fig. 1. Gas chromatogram of a single methanolysed and TFAA-derivatized colony from *Haemophilus aphrophilus* (type strain). Peaks: Gal = galactose; Glc = glucose; LD-Hep = L-glycero-D-mannoheptose; $C_{14:0}$ = myristic acid; 3-OH-$C_{14:0}$ = β-hydroxymyristic acid; $C_{16:1}$ = palmitoleic acid; $C_{16:0}$ = palmitic acid.

chromatogram obtained for a single colony from *H. aphrophilus* is shown in Fig. 1. A number of the unidentified peaks were probably methanolysed and derivatized extracellular polymeric material.

DISCUSSION

The present study demonstrated that it is feasible to perform chemotaxonomy on a single colony. The small biomass of solid cultures has previously been considered a disadvantage for their application in microbiology [7]. Although surface colonies of *Haemophilus* are smooth, flat, convex, and attain a diameter of only 0.5—2.0 mm within 48 h at 37°C [8], the biomass (in ng) was sufficient for chemical analyses. Well separated peaks, providing a multitude of information on the chemical composition of monoclonal bacterial cells, were obtained on the chromatograms. All analysed colonies were free from agar, as checked by examination under a stereomicroscope. Whereas dried bacterial material appeared as a translucent film on the inner wall of the capillary, dried blood agar particles were heavily coloured and could easily be detected. Agar contains D-galactose residues and 3,6-anhydro-L-galactose residues with a half-ester sulphate on about every tenth galactose unit [9]. In cases of contamination with agar, the source of galactose might therefore be difficult to trace. For safety's sake, galactose should probably not be used as a chemotaxonomic marker in this system. Nevertheless, we believe that the present method may be important to microbiology. There are several reasons for this optimism.

(1) Diagnosis of primary isolates evades the disadvantages of multiple transfer, such as risk of contamination, selection of variants and mutants, and loss of culture. Loss of isolates can be considerable during multiple transfer of anaerobic bacteria, e.g. *Bacteroides*.

(2) Classification and identification of the primary colony ensure rapid diagnosis of the infectious disease, and thereby immediate and specific treatment. In cases of serious infections, this may be life-saving.

(3) Taxonomy performed directly on the primary isolate will probably allow an increase in the working load of the clinical laboratory.

(4) Chemotaxonomy on the primary colony may also enable classification and identification of bacteria hitherto stored undiagnosed in clinical laboratories because it has been impossible to grow them in liquid media.

(5) The present technique allows the microbiologist to select colonies with specific chemical properties, which is of current importance in biotechnology. A striking feature of the present study was the greater quantitative variation (S.D. $\leqslant$ 15%) of sugars and fatty acids in solid-grown cells than in liquid-grown cells (S.D. $\leqslant$ 5%) [2, 3]. The variation was higher for fatty acids than for sugars. Thus, the relationship between glucose and L-glycero-D-mannoheptose was 4.0 in whole liquid-grown cells [2, 3] and 4.8 in solid-grown cells. The quantitative variation of cellular components in solid-media-grown cells suggested that each colony is rather specific in its chemical composition [7]. During growth of a multitude of bacterial cell clones in liquid media, such differences may tend to be levelled out. The low level of fatty acids in the colonies may be due to their consumption as storage material during the prolonged growth period (seven days).

(6) Physiological associations are maintained among cells in solid cultures.

(7) Solid cultures are completely free from particulate components of the nutrient medium, and relatively free from small molecular nutrients and their own metabolic products because these tend to be diluted into the large volume of the medium [7].

(8) Gas chromatography of colonies provided more detailed information on cellular components than on cultures grown in liquid media [2, 3]. Not all components on the chromatograms have yet been identified, but it is clear that the colonies of *H. aphrophilus* contained a number of extracellular polysaccharides not detected in liquid-grown cells. Holt et al. [10], using transmission and scanning electron microscopy, found that agar-surface-grown cells of *H. aphrophilus* were, for the most part, covered with adhering exopolymeric material. Liquid-grown cells, however, appeared to lack significant surface-associated or adhering exopolymeric material. There is an increasing number of observations on surface exopolymers of Gram-negative bacteria recovered from clinical infections: bacterial endocarditis, pharyngitis, pneumonia, gastroenteritis and dental caries (for review, see ref. 10). Bacterial surface polymers are probably involved in bacterial—host interactions.

(9) The present technique offers a unique opportunity to define the chemical composition of these possible virulence factors. A parallel technique to the present one is pyrolysis—mass spectrometry, which is more complicated and requires more sophisticated analytical equipment [11].

It is our hope that the present method has brought chemotaxonomy to a crossroads, from which large efforts should be directed at making the primary colony a base unit for future taxonomy. Efforts are now in progress in our laboratories to develop a manual in which chemotaxonomic characters of single colonies from clinically important bacteria are visualized on gas chromatograms. Such a manual may become a useful guide for the microbiologist in his efforts to rapidly classify and identify isolates from clinical infections.

CONCLUSIONS

(1) A method based on chemical analysis of a single bacterial colony by means of gas chromatography has been developed.

(2) Galactose, if present as a contaminant from agar, should not be used as a taxonomic marker in this system.

(3) In *H. aphrophilus*, the same sugars (galactose, glucose and L-glycero-D-mannoheptose) and fatty acids (myristic, β-hydroxymyristic, palmitoleic and palmitic acid), which previously had been demonstrated in whole liquid-grown cells, were detected in a single colony together with a number of additional unidentified substances.

(4) Analyses of a single colony may offer a number of advantages to established procedures based on liquid-grown cells, and seem to have a high potential in future chemotaxonomy.

(5) Single-colony analysis even enables diagnosis of bacteria in mixed culture.

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