Differentiation between Gram-negative Anaerobic Bacteria by Pyrolysis Gas Chromatography of Lipopolysaccharides

By GUNNAR DAHLÉN1* AND INGER ERICSSON2

1 Department of Oral Microbiology, Institute of Medical Microbiology, University of Göteborg, Guldhedsgatan 10, S-413 46 Göteborg, Sweden
2 Department of Analytical Chemistry, University of Lund, P.O. Box 740, S-22007 Lund, Sweden

(Received 23 March 1982; revised 25 August 1982)

Lipopolysaccharides extracted by phenol-water from nine strains of Gram-positive anaerobic bacteria (Bacteroides, Fusobacterium and Veillonella), have been examined by means of pyrolysis gas chromatography. Lipopolysaccharides were fragmented into a group of low molecular weight components and four characteristic high molecular fractions probably consisting of hydrocarbons from the lipid part of the material. The latter fractions were specific for each of the genera tested. At the species level characteristic differences were also found although a limited number of strains were tested. Due to the high reproducibility of the technique, the potential of using the method in differentiating Gram-negative anaerobic bacteria was indicated.

INTRODUCTION

Gram-negative anaerobic non-sporeforming bacteria represent a major group of the normal flora on the mucous membranes of man (Rosebury, 1962) and they have been frequently isolated from clinical material (Finegold, 1977). The identification of anaerobic bacteria is often time consuming and does not always lead to a definite result due to an incomplete taxonomy and lack of enough numbers of stable and specific tests for differentiation.

Pyrolysis gas chromatography (Py-GC) has been developed as a method for analysis of complex organic polymers (Drucker, 1981; Ericsson, 1975, 1977). Py-GC of whole microorganisms has proved useful in the differentiation of several bacterial genera (Haddadin et al., 1973; Quinn, 1974; Oxborrow et al., 1976; Needleman & Stuchbery, 1977; Emswiler & Kotula, 1978; Stack et al., 1978; Gutteridge & Norris, 1979). Several of these authors have noted that there are problems in the reproducibility and evaluation of the complex pyrolysates that are formed from micro-organisms. More reliable information may be obtained by using optimal conditions for the pyrolser (Ericsson et al., 1977) but the pyrograms are still very complex.

One way of producing simpler, but diagnostic, pyrograms is to use a macromolecule complex having genus, species or serological group specificity. A potential macromolecule is the lipopolysaccharide (LPS) present in the outer membranes of Gram-negative bacteria. The polysaccharide part expresses the O-antigenic specificity that constitutes the basis for differentiation into chemotypes in the serological classification of species within Enterobacteriaceae (Kauffmann, 1961, Lüderitz et al., 1966). Strains of Gram-negative anaerobic genera such as Veillonella and Fusobacterium have also been shown to be divided into chemotypes based on the sugar content of LPS (Fredriksen & Hofstad, 1978, Hofstad, 1978). The lipid part (lipid A) of the LPS also may contain specific differences between genera or even species of Gram-negative anaerobic bacteria (Hofstad & Skaug, 1980, Wollenweber et al., 1980). LPS from Gram-negative anaerobic bacteria are genotypically stable macromolecules potentially suitable for pyrolysis differentiation of micro-organisms. The present study evaluates the possible use of Py-GC of LPS for the differentiation of Gram-negative anaerobic bacteria.


METHODS

**Bacteria.** Gram-negative anaerobic bacteria of the genera *Bacteroides*, *Fusobacterium* and *Veillonella* were obtained from a variety of sources. *Bacteroides orallis* (Bact-MC3), *Fusobacterium necrophorum* (Fus-MC4) were originally isolated together in an infected root canal in a monkey (Fabricius et al., 1982). *Fusobacterium nucleatum* (NCTC 10562), *F. mortiferum* (VPI 8365) and two strains of *Veillonella* (Ve 5, Ve 9) were kindly provided by T. Hofstad (Bergen, Norway). Another two strains of *Veillonella* (Lim 14 : 4 an and Lim 14 : 4 cpr) were obtained from teeth with infected necrotic pulps in humans (Dahlén & Bergenholtz, 1980) and one strain of *Veillonella* (Veil-H3) was obtained from human dental plaque. The *Veillonella* strains were further classified into species according to the VPI Manual (Holdeman & Moore, 1975) and *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibbons, 1974).

**Cultivation and cell preparations.** The bacteria were grown at 37 °C in batch cultures, using screw-cap bottles filled to the top. Brain Heart Infusion Broth (Difco 0037-01) with 0-03% (w/v) cysteine HCl and 5% (w/v) yeast autolysate supplemented with 0-01% (w/v) hemin and 0-01% (w/v) menadione according to the VPI Manual (Holdeman & Moore, 1975), was used for *Bacteroides* and *Fusobacterium* strains. Cultivation of *Veillonella* strains was performed in a medium containing proteose peptone (Difco 0122-01), 40 g; Na2HPO4·2H2O, 1-0 g; KH2PO4, 0-4 g; 50% (w/v) sodium lactate, 25 ml and redistilled water, 965 ml. After sterilization, 10 ml of a salt stock solution was added to the latter medium according to Linder et al. (1974). Cultivation was performed anaerobically and the cells harvested by centrifugation in late-exponential phase of growth. The cells were finally washed twice with phosphate-buffered saline (pH 7-2).

**Preparation of lipopolysaccharides (LPS).** Bacterial cells were treated with equal volumes of phenol-water (Westphal et al., 1952) (1 g cells to 100 ml liquid) at 20 °C for 15 min. The water phase was removed after centrifugation and dialysed against tap water. LPS preparations were then lyophilized or further purified by ultracentrifugation (100000 g) and treated with DNase and RNAse (Hofstad & Kristoffersen, 1970).

**Chemical analysis.** The amount of protein in the LPS preparation was estimated by the Lowry method using tyrosin as standard.

**Preparation of Py-GC samples.** Lyophilized LPS preparations were suspended in distilled water (10 μg μl⁻¹) and gently ultrasonicated for 30 s.

**Py-GC equipment.** The detailed equipment of the pyrolyser was described previously (Tydén-Ericsson, 1973; Ericsson, 1977). Briefly, a pyrolyser with a platinum foil (2-6 mm wide, 15 mm long and 0-012 mm thick) was heated by two current pulses. One of the pulses heated the foil to the temperature desired when the other compensated for the cooling. The amplitude and time of the first pulse also determined the temperature rise time of the pyrolyser. The platinum foil was placed in a glass cell maintained at a temperature of 175 °C. The pyrolyser was installed in a flame ionization gas chromatograph (Varian 1860) fitted with a 2 m stainless steel column (id. 1-9 mm) packed with 10% Apiezon L on Chromosorb 750. The nitrogen carrier gas and hydrogen flow rates were both held at 20 ml min⁻¹, and that of the air, approximately at 200 ml min⁻¹. The temperature of the detector was 275 °C and the column temperature programmed from 60–250 °C at 15 °C min⁻¹.

**Py-GC test conditions and analysis.** Prior to pyrolysis, 5 μl of the sample was placed on the foil of the pyrolyser and evaporated to dryness. Samples were pyrolysed to final temperatures of 600, 700 or 800 °C during 0-1, 2 or 10 s with a temperature rise time of 8 ms. Most of the pyrolys es were performed at a temperature of 700 °C for 2 s if not stated otherwise.

RESULTS

**Chemical analysis of LPS.** The protein content of all LPS preparations used was lower than 10%.

**Pyrolysis of LPS.** The pyrolysis of LPS material showed a general pattern of peaks at very short retention times, corresponding to volatile low molecular weight fragments and a few characteristic peaks with longer retention times corresponding to fragments of higher molecular weight. These latter peaks (designated C12-C15) had the same retention times as normal saturated hydrocarbons with 12–15 carbon atoms. Other characteristic peaks were numbered 1–8. Typical pyrograms of LPS of anaerobic bacterial species are shown in Figs 1 and 2.

**Precision.** Veillonella (Veil-H3, 50 μg) was pyrolysed four times at 700 °C for 2 s. The standard deviations for the heights of the four characteristic peaks are shown in Table 1.

**Temperature and time.** Pyrograms of LPS extracted from Veillonella strain Veil-H3 at three different temperatures and three different times showed a similar pattern and only small quantitative differences could be seen among the characteristic peaks. Maximal peak height was not seen in the pyrogram of 600 °C and 2 s. The peaks eluting first (low molecular
Pyrolysis of lipopolysaccharides

Fig. 1. LPS from one Bacteroides and five Veillonella strains (50 μg) pyrolysed at 700 °C for 2 s. The characteristic peaks for each genus are indicated. C₁₂-C₁₆ are peaks with the same retention times as for hydrocarbons with 12-16 carbon atoms, respectively. Other characteristic peaks are numbered 1-8. A complex of small peaks (×) and peak no. 2 are present in the pyrogram of Ve 5 and Veill-H3.

Table 1. The precision obtained for the heights of the characteristic peaks of LPS from Veillonella (Veill-H3) pyrolysed on four different occasions during 2 d

Samples of 50 μg LPS were used, the end temperature was 700 °C and the pyrolysis time was 2 s.

<table>
<thead>
<tr>
<th>Pyrolysis</th>
<th>C₁₂</th>
<th>C₁₃</th>
<th>C₁₄</th>
<th>C₁₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>89</td>
<td>154</td>
<td>43</td>
<td>140</td>
</tr>
<tr>
<td>2</td>
<td>73</td>
<td>126</td>
<td>40</td>
<td>115</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
<td>127</td>
<td>41</td>
<td>118</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>138</td>
<td>37</td>
<td>106</td>
</tr>
</tbody>
</table>

Mean value 72 136 40 120
s.d. (%)

fragments) increased with increasing temperature and time, probably depending on a more complete degradation to volatile fragments of the polysaccharide part of the LPS. The peaks from the high molecular fragments remained more constant in height indicating the more stable character of these components probably derived from lipids. We used 700 °C and 2 s in the following experiments if not otherwise stated. Dose-response studies revealed that 20–50 μg was suitable for analysis.
Fig. 2. LPS (50 μg) from three strains of *Fusobacterium* pyrolysed at 700 °C for 2 s. The characteristic peaks are indicated. C_{12}-C_{16} are peaks with the same retention times as for hydrocarbons with 12–16 carbon atoms, respectively. Other characteristic peaks are numbered 1–8.

**Genus differences.** Pyrograms of nine strains belonging to three different genera were recorded and a distinct pattern of characteristic peaks for each genus was found (Figs 1 and 2). *Bacteroides oralis* always showed C_{15}, C_{16}, 5, 6 and 7 as characteristic peaks; *Fusobacterium* strains always showed C_{13}, C_{14}, C_{15} and C_{16} and *Veillonella* C_{12}, C_{13}, C_{14} and C_{15} with no C_{16}.

**Strain differences.** Five strains of *Veillonella* were tested to examine the homogeneity of the patterns of characteristic peaks in the pyrograms of LPS (Fig. 1). They all showed the four characteristic peaks C_{12}, C_{13}, C_{14} and C_{15} that were common to the *Veillonella* genus. Two of the strains (Ve 5 and Veill-H3, both catalase negative indicating a designation as *V. parvula*) showed a complex of small peaks in the figure marked with × and a small peak (2) close to the C_{12} peak which were not found in LPS of the three other strains (Ve 9, Lim 14:4 cpr and Lim 14:4 an, all catalase positive indicating a designation of the strains as *V. alcalescens*). Three strains of different species of *Fusobacterium* were tested (Fig. 2). They all showed the characteristic C_{13}, C_{14}, C_{15} and C_{16} peaks. *Fusobacterium nucleatum* (NCTC 10562 and *F. necrophorum* (Fus-MC4) showed a rather similar pattern, although there were differences that might indicate strain or even species differences. The strain *F. mortiferum* (VPI 8365) showed a more complex pattern with a quantitative lower amount of C_{16} and higher of C_{13}.

**DISCUSSION**

Pyrolysis procedures have been used in attempts to characterize a variety of products including micro-organisms. Whole micro-organisms give rise to complex patterns of peaks that
Pyrolysis of lipopolysaccharides

561

can be identified by gas chromatography and/or mass spectrometry but problems are found in identification and reproducibility (Quinn, 1974; Gutteridge & Norris, 1979). Pyrolysis of cell fragments such as cell walls, flagella or DNA give less complex pyrograms (Emswiler & Kotula, 1978). The present study shows that the use of LPS gives pyrograms with a limited number of characteristic peaks which may be more readily identified. The technique with the use of a foil pyrolyser has been improved for characterization of complex organic materials (Ericsson, 1977; Ericsson et al., 1977). The pyrolyser used in this study is a prototype but it is commercially available from Dr. I. Ericsson (Lund, Sweden).

The present study showed that patterns and also quantitative results were quite constant within certain limits with varying times and temperatures and peak heights were proportional to doses. Specific fragments were therefore obtained over a broad range of conditions. The optimal conditions seemed to be 700–800 °C in 0.1–2 s. Using other types of column or column material it might be possible to increase the diagnostic power by resolving the profiles into a greater number of characteristic peaks. The tendency of LPS to form aggregates in water solution required efficient ultrasonication before analysis. The presence of small amounts of salts or other low molecular weight compounds such as phenol residues did not seem to interfere with the results significantly. Samples pyrolyzed after a 1 day interval showed exactly the same pattern. A slow decrease in sensitivity could be seen with five subsequently pyrolysed samples. The foil should be regularly cleaned after each sample because of the accumulation of salt residues from the samples.

The preparation and purification of LPS also seems to have little effect on the pyrolysis results. It may therefore be possible to use the water phase after the phenol-water extraction directly as a sample for pyrolysis, thus simplifying the procedures considerably.

LPS seemed to be characteristically fragmented into two distinct groups of fractions. The low molecular weight fraction group was suggested to contain volatile fragments from sugars (Gutteridge & Norris, 1979; unpublished observations). The yield of these fragments seems to be more dependent on the pyrolysis conditions and the content of different sugars and probably does not give useful characteristic pyrolysis profiles with the chromatographic conditions used. The high molecular weight group of fractions are probably hydrocarbons formed from the lipid part of the LPS. Based on the quantitative estimation of the peak heights mentioned, it may be possible to indicate the sugar/lipid ratio of the LPS. It seems that the pyrolysis products formed from the lipid part of the LPS are characteristic for each genus.

Hydroxy-fatty acids occur in Gram-negative bacteria where they are constituents of LPS. Preparations from strains of the genus Bacteroides differ from LPS of other genera in the lipid part by containing 3-OH-14:0 (3-hydroxymyristic acid) only as a minor component (Wollenweber et al., 1980). In a gas chromatographic analysis, three major groups of cellular fatty acids were found on examination of 31 strains of B. oralis and B. ruminicola and others (Miyagawa & Suto, 1980), indicating the potential for chemotaxonomy in Bacteroides strains. Hase et al. (1977) and Hofstad & Skaug (1980) showed that 3-OH-14:0 and 3-OH-16:0 were present as characteristic fatty acids in the LPS of F. nucleatum. The latter authors found that LPS preparations from F. necrophorum and F. mortiferum did not contain 3-OH-16:0 and that this was a useful taxonomic difference in identification of F. nucleatum. Jantzen & Hofstad (1981) examined fatty acids of 31 Fusobacterium strains which confirmed the species differences. In the present study differences in characteristic peaks were found between the three strains (species) of Fusobacterium, however, additional investigations are needed before pyrolysis of LPS can be considered for subgrouping of Fusobacterium strains. It was, however, easy to distinguish between Fusobacterium and other genera. The Veillonella group contains only two species, but subgrouping has been suggested (Berger’s Manual of Determinative Bacteriology, Buchanan & Gibbons, 1974). Hofstad (1978) has shown four different chemotypes based on the serological patterns of their LPS. No studies of the fatty acids of LPS or the cell walls have been performed on Veillonella. The present study shows that Veillonella can be easily distinguished from Bacteroides and Fusobacterium by pyrolysis of phenol-water extracted LPS. Furthermore, it may be suggested that the strains used fell into two groups, indicated by the pattern of the pyrograms. These groups corresponded to the two species V. alcalescens and V. parvula.
At present, gas chromatography of metabolic end-products is widely used as a taxonomical aid for anaerobic bacteria (Holdeman & Moore, 1975; Sutter et al., 1975), to the genus level. Analysis of cell envelope components is being included in taxonomical studies to an increasing extent, indicated by the reports of the GLC of fatty acids (Fritsche, 1974; Miyagawa et al., 1979; Miyagawa & Suto, 1980; Jantzen & Hofstad, 1981). This method includes methanolysis and extraction of methyl esters from the micro-organisms. Pyrolysis of phenol-water extracted LPS is no more laborious although limited in application to Gram-negative bacteria. In conclusion this study gives evidence that pyrolysis of LPS gives useful information less complex than that obtained by pyrolysis of whole cells. Pyrolysis of LPS can be used to complement other methods used in differentiating for these and other Gram-negative bacteria.

REFERENCES


