Analysis of Streptococcal Cell Wall Fractions by Curie-Point Pyrolysis Gas-Liquid Chromatography

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Received for publication 20 March 1973

A streptococcal strain, classified as ZJIII was differentiated from its mutant strain, ZI, lacking the type III polysaccharide antigen, by Curie-point pyrolysis gas-liquid chromatography. Differences observed in pyrograms of whole cells or cell envelopes of both strains could be directly related to the pyrolysis pattern of the purified type III antigen. The same results were obtained when streptococci F III and its mutant were analyzed. Whereas the pyrolysis patterns of the type III antigen extracted from ZJIII and F III bacteria were identical, marked differences were found in pyrograms of the serologically identical type III antigen isolated from the culture medium. Type III antigen was also easily differentiated from the purified type I, II and IV antigens. From the above findings it was concluded that pyrolysis gas-liquid chromatography can be used as a tool for the quality control and identification of streptococcal cell wall components.

During the last 10 years pyrolysis gas-liquid chromatography (Py-GLC) has gained more importance as a technique for the analysis of nonvolatile organic substances, especially macromolecular compounds (5) such as technical polymers and bio-polymers. By pyrolysis under well-defined temperature conditions, characteristic mixtures of volatile fragments are obtained which can be separated by GLC and then provide a reproducible fingerprint of the original sample.

The value of Py-GLC in the classification and identification of microorganisms has been demonstrated by several investigations (2, 9, 10, 11, 13). Reiner has reported the identification of a large number of bacterial strains some of which differed by only one antigenic property. A similar technique has been used for rapid diagnosis of viral and fungal diseases in plant material (7).

Nevertheless, unsatisfactory interlaboratory reproducibility seems to be a major obstacle in the acceptance of Py-GLC as an analytical tool in microbiology.

Recently, however, Curie-point pyrolysis, a highly sophisticated pyrolysis technique, was adapted to the analysis of bacteriological samples (6). Rather than to apply this technique to a large number of bacterial strains, it seemed useful to study a few well-defined bacteria in order to establish more firmly the chemical basis of some of the differences observed in bacterial pyrolysis patterns.

For our investigation a minute, nonhaemolytic streptococcus, serologically classified as ZJIII, was chosen. From this strain a mutant (ZI) lacking the type III polysaccharide antigen has been isolated (14). The localization of the type III antigen in the outer layer of the cell wall of ZJIII bacteria has been established by electron microscopy by using ferritin-labeled antisera (3).

Lyophilized preparations of whole ZJIII and ZI bacteria and purified cell envelope fractions and purified cell wall antigens have been used in the model studies described in this paper.

MATERIALS AND METHODS

Bacterial strains. Strains ZJIII (MG 216) and F III (NCTC 8096) were obtained from C. A. de Moor (National Institute of Public Health, Utrecht, The Netherlands). A mutant strain, ZI, lacking the type III antigen was isolated by Willers and Alderkamp (14) by cultivation in anti-type III serum.

An analogous mutant of F III was obtained by treatment with a mutagenic agent before cultivation in anti-III serum. One milliliter of an 18-h culture was incubated in Todd-Hewitt broth at 37 C for 1 h with 1
ml of a 1:25 dilution of ethylmethane sulfonate (EMS; Koch-Light Laboratories, Colnbrook, England) in water. The reaction was stopped by washing with 5% sodium thiosulfate, and the treated culture was subcultured for 18 h. One drop of this culture was used for the incubation procedure with antiserum (14). After 10 sequential incubations the culture generally consisted of only mutants lacking the type III antigen.

**Sample preparation.** Unless otherwise stated, bacteria were grown 16 h in Todd-Hewitt broth containing in grams per liter: Todd-Hewitt broth (Difco), 15; glucose, 10; and sodium bicarbonate, 5. Other Difco media used were: proteose-peptone, 1%; Casamino Acids, 1%; bactocasinone, 1%; and tryptose, 1%, all supplemented with: yeast extract, 0.5%; glucose, 1%; and NaHCO₃, 0.5%. Cells were harvested by centrifugation, washed twice in saline, once in a solution of 0.15 M (NH₄)₂CO₃, and then freeze-dried. Cell envelope fractions were prepared according to the method described elsewhere (4).

Isolation and purification of formamide-extracted antigens has been described earlier (14). Formamide-extracted types I, II, and IV, and the antigen isolated from the culture medium were a gift from J. M. N. Willers, Department of Immunology, Utrecht.

**Pyrolysis technique.** The Curie-point technique used is based on high-frequency induction heating of a ferromagnetic wire, or hollow cylinder, coated with a thin layer of sample material (1, 12).

The pyrolysis reactor used in the present study is essentially the same as the one described by Meuzelaar and in't Veld (6). Freeze-dried samples were suspended in purified CS₂ by mild sonic treatment and small drops of this suspension were brought on ferromagnetic wires by a micropipette. In this way 30 to 40 μg of the sample was applied to each wire. The wires were then inserted into specially made boro-silicate glass reaction tubes (6).

Wire and reaction tubes were thoroughly cleaned before use. The wires were heated for several hours at 600 C in a H₂ atmosphere saturated with water, cooled by flushing with pure N₂, and stored in a dry place. Used wires were discarded. The boro-silicate reaction tubes were boiled in concentrated HNO₃, rinsed several times with distilled water, and dried.

All samples were pyrolyzed on Fe wires (Curie temperature, 770 C). The high-frequency power supply (Fischer Labortechnik, 1.5 kW, 1.1 μCi) achieved a temperature rise-time of 80 ms with these wires. Total pyrolysis time was 0.6 s.

The pyrolysis reactor was directly coupled to a (32-m, 0.5-mm inner diameter) stainless-steel capillary column (SCOT column, Perkin Elmer) coated with 10% Carbowax 20 M.

Purified N₂ was used as the carrier gas at an inlet pressure of 3 pounds per square inch (psi) per g., providing a column flow of 4 ml per min. The gas chromatograph (Becker, Delft, model 409) was equipped with a matrix temperature programmer and flame ionization detectors. The temperature program is shown in Fig. 1. Pyrolyzer base temperature was 175 C; detector temperature was 225 C. After each analysis the column was conditioned at 180 C for 12 min. Peaks were numbered according to order of appearance in a model pyrogram obtained by superposition of several pyrograms of whole streptococcal cells, as well as cell fractions, after correcting for variation in retention time.

**RESULTS**

The pyrograms of whole Z₃,III and Z₃ bacteria were compared. The mutant strain as compared with the parent strain shows a marked decrease in the height of peaks 56, 57, 61, and 75 relative to the height of peaks 70 and 72 which are essentially unchanged and only reflect the influence of sample size (Fig. 1).

These differences proved to be consistent
peaks in the pyrogram of the type III antigen correspond directly to the differences observed in the pyrogram of whole Z₃,III and Z₃ cells (Fig. 1). Subtraction of the latter two pyrograms, after correcting for sample size, produces a pattern (Fig. 4) clearly resembling that of the purified type III antigen. This procedure, how-

when various isolates of these strains were analyzed. Growing the microorganisms in four different media also did not influence these fingerprints. In pyrograms of cell envelope fractions (Fig. 2) the differences between the parent and mutant strain were even more pronounced with the exception of peak 75.

As Z₃III differs from Z₃ by the presence of type III polysaccharide in the cell wall, the differences in the pyrogram should be confirmed by pyrolysis of the purified type III polysaccharide. The pyrograms of this polysaccharide are shown in Fig. 3.

The upper two pyrograms (a and a') are successive Py-GLC analysis of the same sample demonstrating the high degree of reproducibility achieved. Identical fingerprints of the type III antigen are also obtained when the isolation procedure is repeated on different batches of bacteria (Fig. 3b). It is apparent that the major peaks in the pyrogram of the type III antigen correspond directly to the differences observed in the pyrogram of whole Z₃,III and Z₃ cells (Fig. 1). Subtraction of the latter two pyrograms, after correcting for sample size, produces a pattern (Fig. 4) clearly resembling that of the purified type III antigen. This procedure, how-

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ever, must be regarded with some caution as will be discussed below.

In ZIVIII bacteria a large amount of type III antigen is present. According to quantitative isolations performed, the type III antigen amounts to about 6% of the dry weight of the cell. It was interesting to know whether the typical pattern of the type III antigen would be preserved in strains in which the type III antigen is present in lower amounts. For this purpose a strain classified as F III was chosen. The type antigen of this strain was shown to be serologically identical with that of ZH1 (Huis in't Veld, Thesis, Utrecht, 1973) and accounts for 1 to 2% of the dry weight of these bacteria. The F III strain was also compared to a mutant strain, lacking the type III antigen, which can be classified as an F strain.

The pyrograms of F III and F bacteria are shown in Fig. 5. The differences between F III and F correspond with the differences between ZIII and ZI. Again peaks 56, 57, 61, and 75 are involved, but as expected, the differences are less pronounced.

Furthermore, the pyrogram of the purified type III antigen of F III (Fig. 6) is almost identical to the pyrograms of the corresponding antigen of ZIVIII (Fig. 3), which suggests the chemical identity of these two type III polysaccharides. Independent structural investigations such as GLC-MS after acid hydrolysis of the methylated polysaccharide are in agreement

![Figure 7](http://aem.asm.org/fig7.png)

**Fig. 7. Pyrogram of the type III polysaccharide antigen isolated from the culture medium.**

![Figure 8](http://aem.asm.org/fig8.png)

**Fig. 8. Pyrograms of extracted streptococcal polysaccharide type antigens I, II, and IV. Compare also with pyrograms of the type III antigen as shown in Fig. 3 and 6.**

TABLE 1. Chemical composition (%) of (formamide) extracted streptococcal polysaccharide type antigens I, II, III, and IV, and the type III antigen isolated from the culture medium (IIIm).

| Type | Glucose | Galactose | Rhamnose | Glucosamine | Galactosamine | Fucose |
|------|---------|-----------|----------|-------------|---------------|-------|
| I    | 22      | 46        | 23       | 13          | 16            |       |
| II   | 26      | 12        | 24       |             |               |       |
| III  | 33      | 52        | 15       |             |               |       |
| IV   | 35      | 31        | 26       | 8           | 8             |       |
| IIIm | 17      | 55        | 10       | 17          |               |       |

*a Unpublished results of Willers and Michel.
with these findings (Huis 'n't Veld, Thesis, Utrecht, 1973). Strains carrying the type III antigen in the cell wall also excrete a substance into the culture medium which reacts with anti-type III serum but has a different chemical composition (Table 1). The pyrogram in Fig. 7 strongly supports these findings. The pattern of this pyrogram is completely different from that in Fig. 3. Peak 56 is nearly absent, whereas peaks 51, 57, 58, and 67 are prominent. Among these latter peaks only 57 and 61 are typical for the extracted type III antigen. A remarkable feature is also the low intensity of peak 75 which is a major peak in all extracted antigens thus far analysed (see also Fig. 8).

To decide whether the observed pyrolysis pattern of the type III antigen was only typical for this polysaccharide or represented a general polysaccharide pattern, we also analyzed other streptococcal type antigens. Three more related type antigens have been identified by serological methods. These were designated as type I, II, and IV (8). The pyrograms of these antigens are shown in Fig. 8. In spite of the close chemical relationship between some of these antigens (Table 1), marked differences exist in the pyrolysis pattern.

Typical features of the type III antigen (Fig. 3) are a high peak 56 and, to a lesser extent, a prominent peak 61. Although a large peak 61 is also present in type II antigen, this pyrogram is characterized by a high peak 67. Moreover, at the beginning of the pyrogram further differences with the other antigens are also observed. At a first glance the pyrograms of type I and IV look rather similar, but a more close inspection shows a reversal in the ratio of peaks 61 and 62 and the absence of peak 59 in the pyrogram of type I.

**DISCUSSION**

The experiments described in this paper show that, for well-defined bacterial systems, the differences observed in bacterial pyrolysis patterns can be explained on the basis of known chemical variations. Moreover, a high degree of reproducibility was obtained by using Curie-point pyrolysis in direct combination with high resolution GLC. Different batches, isolated from various culture media over a period of more than six months, did not notably influence the pyrograms.

Streptococcus ZIII could be easily differentiated from its mutant, lacking the type III polysaccharide antigen. The observed differences were even more pronounced when pyrograms of cell envelopes of these strains were compared.

The analysis of the purified type III polysaccharide indicates that this antigen is completely responsible for the observed differences in the pyrolysis patterns of these bacterial strains, which is concluded from qualitative aspects of the pyrograms as well as from direct subtraction of typical pyrograms of whole bacteria. Although the additivity of pyrograms of relatively simple substances has been reported in literature (12), the observation of this phenomenon for complex biological samples was not described until now.

The feasibility of the subtraction, however, is probably restricted to a situation where relatively large amounts of characteristic material are present, since some of this material will undoubtedly be involved in secondary pyrolysis reactions, leading to nonspecific pyrolysis products. In line with this consideration, subtraction of the pyrograms of F from F III, a strain containing minor amounts of the type III antigen (1-2%) as compared with ZIII, tended to give less reliable results. Nevertheless, the presence of the type III antigen could be clearly inferred from a qualitative comparison of the pyrograms. In this respect, especially peak 56 proved to be informative, because this peak was highly specific for the type III antigen as compared with the other known streptococcal polysaccharide type antigens (I, II, and IV). Whereas the pyrograms of the type III polysaccharides extracted from ZIII and F III bacteria were identical, major differences existed between the chemically closely related type I and II antigens. These findings suggest that Py-GLC can be used to discriminate between polysaccharides differing only in structural aspects. Preliminary results obtained from Py-GLC of structurally different glucans seem to confirm this. Further work on this subject is in progress and will include Py-GLC in direct combination with mass spectrometry.

Analysis of type III antigen isolated from the culture medium is a good illustration of the fact that serological identity is not necessarily correlated with chemical or structural identity. Chemical analysis of the medium antigen showed a different sugar composition and structure (Huis 'n't Veld, thesis, Utrecht, 1973), which is reflected in the pyrogram.

Finally, the use of purified bacterial compounds responsible for variation in bacterial strains offers an attractive possibility for defining the optimum Py-GLC conditions for the detection of these compounds in whole bacteria, which may enable a more rational approach to the problem of classification of bacteria by Py-GLC.
The technique described in this paper could be of general value in monitoring the isolation and purification procedures of microbiological compounds as well as in the identification of these compounds. Moreover, the technique might be used for the prompt recognition of mutant strains lacking specific cell wall components.

ACKNOWLEDGMENTS

We thank J. M. N. Willers for his valuable support and advice. We also gratefully acknowledge the expert technical assistance of B. Dohmen.

The work of H. L. C. M. and A. T. was supported by the Dutch Foundation for Fundamental Research on Matter and the Dutch Ministry of Health.

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