Effect of Different Growth Conditions on the Discrimination of Three Bacteria by Pyrolysis Gas-Liquid Chromatography

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High-resolution pyrolysis gas-liquid chromatography was applied to three bacteria (Escherichia coli NCTC 9001, Pseudomonas putida NCIB 9494, and Staphylococcus aureus NCTC 8532) grown under a variety of conditions. Changing the culture medium drastically altered the quantitative aspects of the pyrograms of all three organisms, but the effects of culture time and incubation temperature were less severe. Mathematical analysis of the relative peak heights showed that four peaks could be used to discriminate the three bacteria however they were cultured.

The discrimination of microorganisms by pyrolysis gas-liquid chromatography or pyrolysis mass spectrometry is based upon differences in peak heights (10), peak areas (15), or mass intensities (8). Often the relevant quantitative differences between pyrograms or spectra are small, are masked by redundant and irreproducible data (5), and need to be amplified by appropriate mathematical methods (1, 5, 6, 9). Thus it has been assumed that careful control of the microbiological growth conditions is essential and that any other approach would introduce an unacceptable amount of variation into the quantitative data (7).

The isolation of a representative flora cannot usually be achieved by a single procedure, and it is advisable to use a selection of media, incubation temperatures, and culture times (3). Thus, in any development of a routine pyrolysis gas-liquid chromatography or mass spectrometry system it would sometimes be advantageous to be able to compare organisms isolated in different ways, without recourse to a standard subculturing technique. This possibility has been investigated by mathematical analysis of the pyrograms of three bacteria grown under a variety of conditions.

MATERIALS AND METHODS

Organisms and growth conditions. Escherichia coli NCTC 9001, Pseudomonas putida NCIB 9494, and Staphylococcus aureus NCTC 8532 were cultured on agar plates using nutrient agar (NA) (Oxoid), plate count agar (Oxoid), or blood agar made as 10% (wt/vol) horse blood in blood agar base (Oxoid CM55), at pH 7.0 under various conditions (Table 1).

Pyrolysis gas-liquid chromatography. All samples were pyrolyzed at 610°C for 2 s using a Curie-point pyrolysis reactor (11) powered by a 1.5 kW, 1.1 MHz high-frequency generator (Fischer Labotechnik, Bad Godesberg, West Germany), and the pyrolysis products were separated in a Perkin-Elmer F17 gas chromatograph using a 40-m Carbowax 20M support coated open tubular column (Scientific Glass Engineering Ltd., London, U.K.) and nitrogen carrier gas at a flow rate of 4 ml/min. The temperature program was 70°C for 6 min, rising at 5°C/min to 180°C, and then held isothermally until analysis was complete. Eluted products were detected by flame ionization (attenuation, 4; gas flow rates: air, 300 ml/min; hydrogen, 20 ml/min), recorded on a Servoscribe 1S chart recorder (Smiths Industries Ltd., London, U.K.) at 1 mV sensitivity and with a speed of 1 cm/min, and digitized by an Infotronics 304/50 computing integrator (Laboratory Data Control, Shannon, Ireland) in the form of peak heights. A typical pyrogram produced by this system is shown in Fig. 1.

Preparation of samples for pyrolysis. Whole cells (from either a single colony or a few small colonies) were transferred with a platinum loop directly from the surface of the agar to a flame-cleaned Curie-point wire which was then positioned within a cleaned glass reaction tube (12) and inserted into the reactor. Apart from the exceptions noted in Table 1, each set of growth conditions was sampled at least twice, and data from 98 pyrograms were analyzed mathematically.

Data analysis. Each pyrogram was represented by the heights of 35 peaks (Fig. 1), identified by their retention times from the integrator output. This set of peaks was chosen arbitrarily and was not the total available information but contained the largest fragments and was sufficient to indicate the major trends in the data.

The peak heights were normalized to remove the effect of sample size (6), punched on cards, and analyzed, off-line, in two ways: (i) by calculation of overall similarity, using a proportional similarity coefficient (14, 23) which produces similarities between replicates in the region of 80% (14, 22); and (ii) by discriminant analysis using canonical variates analysis and stepwise discriminant analysis to select peaks and test their ability to discriminate between groups (9, 21).
Similarity calculations and canonical variates analysis were programmed in GENSTAT on a System 4 computer at Rothamsted Experimental Station, and stepwise discriminant analysis was part of the BMDP package (4) run on a similar computer at the University of Wales Institute of Science and Technology.

RESULTS

Examination of the overall variation. A 98 × 98 similarity matrix was computed using all 35 peak heights. To test the effect of altering growth conditions, pyrograms of samples grown on nutrient agar at 30°C for 18 h were taken as a reference and were compared, on the basis of overall similarity, with pyrograms of samples grown on nutrient agar at 30°C for 12, 24, 36, and 60 h for time; on nutrient agar at 35, 25, and 20°C for 18 h for temperature; and on plate count and blood agars at 30°C for 18 h for medium. The ability to discriminate between the three organisms was assessed by comparing their respective reference pyrograms. The cumulative results are plotted in Fig. 2, where ranges of percentage of similarity represent the difference between the two most similar and the two least similar pyrograms for a particular comparison.

Pyrograms of all replicates (i.e., more than one analysis of a single organism grown under one set of conditions) were 77 to 86% similar. These absolute values are not significant because they depend on the method of calculation (see above), but the range (9%) represents the reproducibility of the pyrolysis gas-liquid chromatography system from analysis to analysis. The similarities of the reference pyrograms for the three bacteria were 69 to 75%, which gives an additional 2 to 8% of overall variation compared with the replicates, showing that pyrolysis gas-liquid chromatography could discriminate between the three organisms.

Alteration either the culture time or the incubation temperature did not introduce any significant extra variation for E. coli but did for P. putida and S. aureus (although this was based on fewer samples). Variation between pyrograms of the same organism grown on different media was always greater than between pyrograms of different organisms grown on the same medium (Fig. 2).

TABLE 1. Culture conditions

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Organisms*</th>
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<tbody>
<tr>
<td>Medium*</td>
<td>Temp (°C)</td>
</tr>
<tr>
<td>NA</td>
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<tr>
<td>NA</td>
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<td>30</td>
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<tr>
<td>BA</td>
<td>30</td>
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</tbody>
</table>

* *, Tested; NT, not tested.
*b, NA, Nutrient agar, PCA, plate count agar; BA, blood agar.

Single pyrogram.

FIG. 1. High-resolution pyrogram of E. coli grown on nutrient agar at 30°C for 18 h, showing the 35 peaks selected for mathematical analysis.
The use of two different manufacturers' batches of nutrient agar did not produce any significant variation (not shown in Fig. 2).

**Discriminant analysis.** Canonical variates analysis of the 35 normalized peak heights for all 98 samples (Fig. 3) shows that the three organisms could be discriminated irrespective of how they were cultured. This observation was explained by stepwise discriminant analysis, which showed that a subset of four peaks (16, 17, 30, 8; Fig. 1) could discriminate the organisms. Figure 4 shows that peak 16 was always characteristic for *E. coli*, but that peaks 17, 30, and 8 were required in combination to distinguish all the *P. putida* and *S. aureus* samples.

**DISCUSSION**

The need for standardization of microbiological growth conditions was recognized in some of the earliest pyrolysis studies (2, 18), and this approach has been adopted for pyrolysis mass spectrometry (13) as well as for pyrolysis gas-liquid chromatography. Changing the culture medium has always been shown to affect the pyrogram (6, 14, 17) or mass spectrum (13) drastically, and the use of three different media in
this study confirmed these observations. It is important to stress that growth on different media produces no unique products but changes the peak height ratios of the pyrolysate which are used for the discrimination of microorganisms (9, 22).

The consequences of changing culture age or incubation temperature seem to depend on the organism under study; they were minimal for *E. coli*, but severe for *P. putida* and *S. aureus* (Fig. 2). These differences are assumed to reflect variations in the growth rate and metabolic activity of the three bacteria. Similar observations for culture age have been made with other organisms (2, 13, 16, 19), but, to our knowledge, incubation temperature has not been considered.

The application of canonical variates analysis and stepwise discriminant analysis to these data shows that when growth conditions are changed, some peaks remain characteristic for particular bacteria. In this example, the organisms were unrelated and easily differentiated, but a similar observation, albeit based on visual inspection, has been made for serotypes of *E. coli* (20). All this suggests that it will be possible to prepare a single data base for the identification of unknowns isolated in different ways, provided (i) a few characteristic peaks can be selected by appropriate mathematical methods, and (ii) the differences reflected by those peaks are stable over a period of time.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**