Discrimination of Some Gram-negative Bacteria by Direct Probe Mass Spectrometry

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The technique of direct probe mass spectrometry is described together with its application to the analysis of 50 strains of Gram-negative bacteria representing seven genera. Thirty-six of these strains were analysed in duplicate, and the 72 spectra held in a computer library. The intensities of 63 ions from each of the spectra were analysed by discriminant analysis techniques and all seven groups could be distinguished by as few as six ions. A set of 29 strains, 15 of which were already in the data base, were used as unknowns to challenge the library on two separate occasions. The success rate of these challenges was 97% and 90% using the full spectra, but only 72% and 62% using the selection of six ions. Possible explanations for this are discussed as well as the scope and limitations of the method as a means of characterizing micro-organisms.

INTRODUCTION

The combination of analytical pyrolysis with gas chromatography (Py-GC) or mass spectrometry (Py-MS) produces data which can be used to discriminate micro-organisms (for reviews, see Irwin, 1979; Gutteridge & Norris, 1979). With several permanent modifications to a standard mass spectrometer (Meuzelaar & Kistemaker, 1973), Py-MS can be used for the rapid characterization of bacteria and fungi at a rate of one sample every 5 min (Meuzelaar *et al.*, 1974; Weyman, 1977). Puckey *et al.* (1980), following some earlier research by Anhalt & Fenselau (1975) and Risby & Yergey (1976, 1978), proposed the use of a simpler technique, direct probe mass spectrometry (DPMS), in which samples are thermally degraded adjacent to the ion source of a mass spectrometer using a standard accessory available on most instruments.

The purpose of this paper is to describe the DPMS method in detail and to show its application to the differentiation of bacteria using seven well-defined groups of Gram-negative bacteria. Furthermore, an attempt has been made to establish a data base and match unknowns against it.

METHODS

Organisms and growth conditions. The 50 strains used in the study are listed in Table 1. All the strains of *Citrobacter freundii*, *Serratia liquefaciens*, *Pseudomonas* sp., *Hafnia alvei* and *Klebsiella pneumoniae* E58 and E62 were isolates from meat. The remaining *K. pneumoniae* strains and *Acinetobacter calcoaceticus* 4406 and 44100 were provided by Dr G. L. French, St. Thomas' Hospital, London. All other cultures were obtained from the relevant collection.

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Table 1. *List of strains*

Group	Name	Strains*
1	<i>Citrobacter freundii</i>	E23 ^{ab} ; E26 ^{ab} ; E59 ^b ; E82 ^a
2	<i>Acinetobacter calcoaceticus</i>	NCTC 7363 ^a ; NCTC 7364 ^a ; NCTC 7461 ^a ; NCTC 7462 ^a ; NCTC 10290 ^{ab} ; NCTC 10291 ^{ab} ; 4406 ^b ; 44100 ^b
3	<i>Serratia liquefaciens</i>	E14 ^a ; E15 ^b ; E19 ^{ab} ; E20 ^{ab} ; E21 ^b ; E43 ^b
4	<i>Klebsiella pneumoniae</i>	R334 ^{ab} ; R524 ^a ; R1096 ^{ab} ; R1109 ^a ; R1113 ^a ; KA3 ^{ab} ; KA6 ^a ; E58 ^b ; E62 ^b
5	<i>Pseudomonas</i> sp.	DC5 ^{ab} ; DC12 ^a ; NT5 ^{ab} ; NT11 ^a ; MB23 ^a ; MB24 ^a ; P58 ^b ; P59 ^b
6	<i>Proteus morganii</i>	NCTC 232 ^a ; NCTC 235 ^b ; NCTC 1707 ^a ; NCTC 2815 ^a ; NCTC 5845 ^b ; NCTC 10041 ^{ab} ; DSM 30117 ^{ab} ; CCM 680 ^b
7	<i>Hafnia alvei</i>	E4 ^a ; E5 ^a ; E44 ^a ; E46 ^a ; E57 ^b ; E80 ^{ab} ; E86 ^{ab}

* a, in data base; b, used as unknown.

Fig. 1. Direct probe system layout: A, probe; B, valve; C, mass spectrometer housing; D, probe tube; E, ion source; F, ionization region; G, lenses; H, quadrupole rods; I, electron multiplier.

The identification of all the strains was checked using the API 20E system (API Laboratory Products, Farnborough, U.K.). The strains were maintained on nutrient agar (Oxoid) slopes at 4 °C but cultured for DPMS on plate count agar (Oxoid) at 25 °C for 24 h.

Preparation of samples for mass spectrometry. Approximately 50 µg of whole cells from a single large colony or a few small colonies was taken from the surface of a culture plate using a platinum wire and placed inside a clean quartz-glass tube which was inserted into the stainless steel probe.

Mass spectrometry. The analyses were carried out on a Finnigan 4000 quadrupole instrument coupled to a 2100 data system. The layout of the direct probe insertion system is illustrated schematically in Fig. 1. The mass spectrometer was operated at an electron energy of 25 eV, with a multiplier voltage of 1800 V and a source temperature of 270 °C. After insertion into the high vacuum of the instrument, the probe was temperature programmed at 60 °C min⁻¹ from ambient to 300 °C. Spectra were recorded on to a magnetic disc over the mass range *m/e* 33–400 using a 2 s scan cycle. A full analysis, including the time taken to cool the probe to ambient temperature, took 15 min per sample.

Data analysis. Only 36 of the 50 strains were used to establish a data library but each was analysed in duplicate and the library consisted of 72 'fingerprints'. On two separate occasions the library was challenged by a set of 'unknowns', which contained the remaining 14 strains and 15 that were already included in the data base (see Table 1). Each unknown was analysed once.

A set of intensities for 63 ions (*m/e* 53–61, 67–74, 81–89, 91, 95–99, 108–115, 117, 124–126, 128, 129, 135, 136, 151, 154, 158, 166, 167, 185, 236, 237, 239, 264, 310–313, 339) was derived for each of the library spectra. The ions chosen were those with the widest range of intensity values across the library. The data were normalized and subjected to canonical variates analysis (MacFie *et al.*, 1978), stepwise discriminant analysis (Emswiler & Kotula, 1978; Gutteridge *et al.*, 1980) and jackknifing (O'Donnell *et al.*, 1980) to test for discrimination of groups, to select the ions responsible for the differentiation, and to test the stability of the classification. This set of ions represented less than 20% of the available data, but data reduction was essential to comply with the following numerical constraint necessary for a statistically valid analysis (Dixon, 1975): $N_v < N_s - N_g - 1$, where N_v is the number of variables (ions) per sample, N_s is the number of samples and N_g is the number of groups.

Identification of unknowns was attempted using two methods: (i) by comparison of the full spectrum of an unknown with each library spectrum using the library search routine (Finnigan Corporation, 1978) available on the mass spectrometer data system – this matching procedure reduces the spectrum by a complex algorithm to a set of 'chemically significant' peaks before comparison with similarly abbreviated library entries; (ii) by the use of classification functions derived during stepwise discriminant analysis (Dixon, 1975).

RESULTS

Ion current profiles and spectra

As the direct probe was temperature programmed, the total number of ions detected by the mass spectrometer was recorded and displayed as a function of time in an ion current profile. Figure 2 shows typical profiles for duplicate analyses of *C. freundii* E23 and *P. morganii* NCTC 10041. The general shape was the same for each of the organisms studied, with thermal degradation occurring at temperatures above 220 °C resulting in a large pulse of ions which only subsided when the programme was halted at 300 °C. The probe system has a theoretical limit of 350 °C but, in practice, 300 °C is the highest temperature that can be regularly obtained without damaging the vacuum seals of the mass spectrometer. Some profile shapes were complex (e.g. *P. morganii* NCTC 10041 in Fig. 2*b*) but they could not be used to characterize organisms because they seemed to be affected by the size and positioning of the sample within the probe tube.

By trial and error it was found that the most reliable method for obtaining a single spectrum (fingerprint) was to average the total number of ions between the beginning and the amplitude of the pulse (i.e. 220–300 °C) and then to subtract a ten-scan background taken from before the pulse. Thus, the actual number of scans per sample varied but the averaging procedure was reproducible from sample to sample because it was applied to the same section of each ion current profile.

Typical examples of averaged spectra are shown in Figs 3 and 4. Ions were detected up to m/e 400 but were generally of low intensity above m/e 200, except for clusters around m/e 236 and 313. There were no detectable qualitative differences, as is usually the case with pyrolysis of micro-organisms (Irwin, 1979), so the application of methods for handling quantitative data was essential.

Canonical variates analysis

Table 2 gives the matrix of Mahalanobis D^2 distances between the seven group means. Given normality in the statistical sense, 95 % of a population should lie within 5.99 D^2 units of its mean (MacFie *et al.*, 1978), thus Table 2 indicates that all seven groups can be discriminated using the full set of 63 ions.

Stepwise discriminant analysis and jackknifing

Analysis of the data by stepwise discriminant analysis showed that the seven groups could be distinguished by considering as few as six ions (m/e 117, 82, 185, 264, 70 and 89 in order of inclusion). The clarity of this data is illustrated in Fig. 5 which is the scatter diagram obtained by plotting the intensity values of m/e 117 against m/e 82 for each of the library spectra. The *P. morganii* group was completely distinguished by m/e 117 and the *Pseudomonas* group by m/e 82 with the exception of some overlap with one analysis of *A. calcoaceticus*. Figure 5 also illustrates differences in the variability of intensity values from strain to strain within a group. For example, for *P. morganii* m/e 82 varies from 14.3–14.9 but for *Pseudomonas* it varies from 22.5–32.0.

The stability of the discrimination obtained using the six ions is shown by Table 3 which lists the Mahalanobis D^2 values for each analysis from each group mean after jackknifing. One analysis (*S. liquefaciens* E14 2) was mis-classified into the *K. pneumoniae* group but the other 71 samples were reallocated to their original groups. There was no obvious explanation for the

Fig. 2. Typical ion current profiles for duplicate analyses of (a) *Citrobacter freundii* E23 and (b) *Proteus morganii* NCTC 10041.

Fig. 3. Mass spectrum of *Citrobacter freundii* E23.

mis-classification of E14 2 and it was not deleted from the data base. Examination of the within-group distances reveals that *C. freundii*, *S. liquefaciens* and *H. alvei* are homogeneous in contrast to *A. calcoaceticus* and *Pseudomonas*, whilst *K. pneumoniae* R1096 and *P. morganii* NCTC 10041 were outliers of their respective groups.

Computer matching of unknown spectra

The results of the two challenges are presented in Table 4. The use of the full spectrum produced better results (28/29 and 26/29) than using classification functions based on the six selected ions (21/29 and 18/29). Challenge 1 was presented 7 weeks after the creation of the

Fig. 4. Mass spectrum of *Proteus morganii* NCTC 10041.

Table 2. Matrix of Mahalanobis distances between all seven groups

<i>C. freundii</i>	(1)	—					
<i>A. calcoaceticus</i>	(2)	53	—				
<i>S. liquefaciens</i>	(3)	56	62	—			
<i>K. pneumoniae</i>	(4)	88	74	56	—		
<i>Pseudomonas</i> sp.	(5)	127	119	124	121	—	
<i>P. morganii</i>	(6)	73	72	75	95	143	—
<i>H. alvei</i>	(7)	48	63	61	75	115	70
		(1)	(2)	(3)	(4)	(5)	(6)

Fig. 5. Scatter diagram of the relative intensities of ions of m/e 117 versus m/e 82: 1, *Citrobacter freundii*; 2, *Acinetobacter calcoaceticus*; 3, *Serratia liquefaciens*; 4, *Klebsiella pneumoniae*; 5, *Pseudomonas* sp.; 6, *Proteus morganii*; 7, *Hafnia alvei*.

Table 3. *Jackknifed classification using six selected ions*

Group, strain and duplicate	Jackknifed Mahalanobis D^2 from group						
	1	2	3	4	5	6	7
1. <i>C. freundii</i>							
E23 1	6	60	11	26	173	72	12
E23 2	1	79	19	23	214	93	17
E26 1	2	99	27	43	194	84	7
E26 2	3	112	38	48	228	94	11
E82 1	1	77	22	28	214	92	15
E82 2	2	93	29	29	239	107	18
2. <i>A. calcoaceticus</i>							
NCTC 7363 1	81	20	31	47	132	113	104
NCTC 7363 2	142	25	105	117	147	185	178
NCTC 7364 1	63	11	26	38	158	105	87
NCTC 7364 2	74	14	29	52	127	98	89
NCTC 7461 1	110	5	77	93	162	139	137
NCTC 7461 2	100	2	65	80	166	129	127
NCTC 7462 1	69	24	52	64	222	122	90
NCTC 7462 2	64	31	50	59	244	119	85
NCTC 10290 1	107	9	75	76	227	158	148
NCTC 10290 2	181	52	159	121	454	294	270
NCTC 10291 1	106	8	75	96	183	100	134
NCTC 10291 2	125	16	85	127	153	89	137
3. <i>S. liquefaciens</i>							
E14 1	20	42	9	13	172	75	39
E14 2	22	57	14	4	224	104	51*
E19 1	37	57	4	29	139	77	40
E19 2	28	59	2	24	142	84	32
E20 1	28	57	4	29	131	83	30
E20 2	22	53	1	23	142	76	28
4. <i>K. pneumoniae</i>							
R334 1	37	66	20	1	237	151	74
R334 2	43	92	25	3	270	163	81
R524 1	39	43	17	5	229	128	74
R524 2	39	59	18	2	245	136	76
R1096 1	20	99	25	14	257	139	52
R1096 2	29	126	41	28	283	160	64
R1109 1	33	55	21	4	231	147	69
R1109 2	40	52	20	3	235	150	77
R1113 1	35	47	14	3	225	129	68
R1113 2	51	51	29	7	271	166	95
KA3 1	41	73	23	3	267	143	82
KA3 2	27	59	8	2	209	121	55
KA6 1	22	71	14	2	234	135	53
KA6 2	50	100	25	8	251	186	84
5. <i>Pseudomonas</i> sp.							
DC5 1	223	173	161	248	1	171	185
DC5 2	211	160	156	245	2	156	171
DC12 1	211	191	177	282	13	148	159
DC12 2	249	235	216	342	20	164	185
NT5 1	212	168	154	245	1	144	171
NT5 2	199	154	138	223	2	139	163
NT11 1	359	346	336	422	52	332	316
NT11 2	201	175	151	225	12	177	171
MB23 1	213	163	141	227	6	154	177
MB23 2	170	134	110	190	12	133	139
MB24 1	190	140	131	222	9	127	149
MB24 2	226	180	152	245	7	160	182
6. <i>P. morganii</i>							
NCTC 232 1	61	99	58	114	144	5	44
NCTC 232 2	109	111	84	156	130	4	85
NCTC 1707 1	81	113	78	141	146	2	61

Table 3—continued

Group, strain and duplicate	Jackknifed Mahalanobis D^2 from group						
	1	2	3	4	5	6	7
NCTC 1707 2	58	91	54	111	135	10	40
NCTC 2815 1	124	126	104	176	146	4	101
NCTC 2815 2	79	97	66	130	127	4	59
NCTC 10041 1	210	270	206	270	307	36	196
NCTC 10041 2	154	205	145	212	223	15	134
DSM 30117 1	73	121	77	128	193	5	62
DSM 30117 2	79	85	58	117	125	6	64
7. <i>H. alvei</i>							
E4 1	17	97	26	65	144	64	3
E4 2	10	110	35	66	182	62	2
E5 1	9	100	23	55	163	65	1
E5 2	14	103	28	65	153	63	1
E44 1	16	142	46	78	187	96	3
E44 2	15	110	35	73	159	67	1
E46 1	10	100	29	62	161	70	1
E46 2	10	109	37	68	179	74	1
E80 1	15	120	37	73	163	77	1
E80 2	12	112	31	63	165	80	1
E86 1	11	111	31	64	162	67	1
E86 2	13	136	50	80	205	77	4

The distances in bold type indicate group allocation on jackknifing.

* Indicates sample mis-identified on jackknifing.

data base and challenge 2 after a further 2 weeks, which suggests that the instrumentation was stable for this time.

DISCUSSION

The DPMS system used in this research is a substantial improvement on the method we have previously described (Puckey *et al.*, 1980) which was based on an LKB 9000 magnetic sector instrument. The use of a rapid scanning quadrupole and an integrated data system confer major advantages in the processing of the fingerprints, although the discriminant analysis procedures still have to be carried out on an off-line main frame computer. The use of lower electron energies (25 eV rather than 70 eV) produced an improvement in the specificity of the spectra and thus in their usefulness for discriminating micro-organisms, an observation which agrees with results from Curie-point Py-MS (Posthumus *et al.*, 1974). The only possible disadvantage of the Finnigan-based DPMS system compared with the magnetic sector machine is the reduced size of high mass fragments ($>m/e$ 400) which were found by both Anhalt & Fenselau (1975) and Puckey *et al.* (1980). Since the limiting step with these methods is data processing, a reduction in the available information in favour of a more integrated and rapid approach to data handling is a sensible compromise.

The seven groups of Gram-negative bacteria that were differentiated did not represent a real identification problem because they can be easily distinguished by conventional techniques, but most pyrolysis studies have concentrated on the species or sub-species levels (e.g. Borst *et al.*, 1978; Emswiler & Kotula, 1978) and it is important to show that these techniques can operate successfully at higher levels. Most published studies have only considered discrimination of micro-organisms (e.g. Cone & Lechowich, 1970; Stern *et al.*, 1979), but Wieten *et al.* (1981) have shown that Py-MS can be used for identification of clinical mycobacteria and this data base has been stable for more than 1 year. Although our study was only conducted over 2 months, it shows that recognition of a wider variety of organisms

Table 4. *Results of matching unknowns against library*

Strain	Challenge 1		Challenge 2	
	Full spectra	Classification functions	Full spectra	Classification functions
<i>C. freundii</i>				
E23	+	+	+	—
E26	+	+	+	—
E59	+	+	+	—
<i>A. calcoaceticus</i>				
NCTC 10290	+	+	+	+
NCTC 10291	+	+	+	+
4406	+	+	—	+
44100	+	—	+	—
<i>S. liquefaciens</i>				
E15	+	—	+	—
E19	+	+	+	+
E20	+	+	+	+
E21	+	+	+	+
E43	—	+	+	+
<i>K. pneumoniae</i>				
R334	+	+	+	+
R1096	+	+	+	+
KA3	+	—	+	—
E58	+	—	—	—
E62	+	+	—	+
<i>Pseudomonas</i> sp.				
DC5	+	+	+	+
NT5	+	+	+	+
P58	+	+	+	—
P59	+	+	+	+
<i>P. morganii</i>				
NCTC 235	+	+	+	+
NCTC 5845	+	+	+	+
NCTC 10041	+	+	+	+
DSM 30117	+	—	+	+
CCM 680	+	+	+	+
<i>H. alvei</i>				
E57	+	—	+	—
E80	+	—	+	—
E86	+	—	+	—
<i>Total</i>	28/29	21/29	26/29	18/29

is possible through the use of computerized data libraries. It remains to be proven that this is possible over long periods (many years) and the reduction in the number of successful matches during the second challenge suggests that the performance of the data base did deteriorate with time. The use of dedicated instrumentation and regular monitoring of mass spectrometer performance by the use of standard samples may provide a solution to this important problem.

The data analysis methods used here have been applied mainly to Py-GC data (Emswiler & Kotula, 1978; O'Donnell *et al.*, 1980) where there are usually fewer variables per sample than in DPMS or Py-MS. Consequently, with mass spectrometry, pre-processing methods for data reduction are essential. In this study, ions were selected purely on the basis of overall variability, and a more statistically based approach to data reduction, such as those of Eshuis *et al.* (1977) and Blomquist *et al.* (1979), may prove beneficial. Nevertheless, there was enough information in the set of 63 ions to distinguish the seven groups.

Further reduction of this data set using stepwise discriminant analysis showed that six ions could distinguish the groups but classification functions based on these ions could not be used to recognize unknowns, in contrast to library searching of the full spectra. This indicates that the six ions selected provided only one solution to the problem of discriminating the seven groups and this six ion solution was not sufficiently stable to allow identification of unknowns. This implies that either (i) ions with good discriminating properties were not included in the set of 63 presented for discriminant analysis or (ii) that classification functions are too data-specific to be useful for identification purposes. Despite these problems there is a role for stepwise discriminant analysis for testing the stability of data bases and the 'goodness of fit' of individual strains, but the development of improved methods for recognition using this type of quantitative data will require further investigation.

Comparison of the canonical variates analysis results (Table 2) with a similar exercise conducted with Py-GC (MacFie *et al.*, 1978) shows that DPMS produces better discrimination at the genus level, which is further evidence for the technical superiority of Py-MS techniques over Py-GC techniques (Meuzelaar & Kistemaker, 1973). It would be wrong to regard DPMS as competitive with Curie-point Py-MS since they produce essentially the same type of data, but some comparisons are necessary to assess their potential usefulness.

The pyrolysis methods employed in the two techniques are radically different, and although the DPMS temperature programme is reproducible the starting temperature and the point at which the analysis is halted are not easily controlled. It would be a considerable advantage to be able to programme the probe to temperatures well in excess of 300 °C until no breakdown products could be detected, but this is not possible with the mass spectrometer used. Consequently, the short term, day to day, reproducibility of DPMS data (10–12%, unpublished results) is not as good as for equivalent Py-MS data (6–8%, Windig *et al.*, 1979) although the higher mass fragments which can be observed with DPMS may make the spectra more characteristic of particular micro-organisms. The analysis times per sample are longer than for Py-MS (Meuzelaar *et al.*, 1977) and thus the automated Curie-point pyrolysis mass spectrometer is the preferred method for routine fingerprinting of large numbers of samples. However, until purpose-built instrumentation is commercially available the use of DPMS will make pyrolysis techniques more accessible and will encourage a rapid development of data processing methods and procedures for recognizing unknown strains.

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