A pyrolysis mass spectrometry study of fusobacteria

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Summary. Strains of fusobacteria (143) were examined by pyrolysis mass spectrometry (Py-MS) with a Horizon Instruments PYMS 200X. *Fusobacterium necrogenes*, *F. necrophorum*, *F. nucleatum*, *F. mortiferum*, *F. varium*, *F. gonidiaformans*, *F. naviforme*, *F. russii* and *Leptotrichia buccalis* were discriminated. Strains of fusobacteria isolated from tropical ulcers, although similar to *F. mortiferum* in conventional tests, were discriminated from each of these species in Py-MS. Identification of 416 spectra to species level agreed with conventional bacteriological methods in 91·8% of cases, was equivocal in 3·4% and disagreed in 4·8%. Classification based on pyrolysis data resolved groups largely corresponding to the recognised species. However, *F. nucleatum* strains were divided between two distinct groups. The tropical ulcer strains were resolved as a distinct homogeneous group. Py-MS is a rapid, inexpensive and convenient procedure for characterisation of bacteria, with the capacity for a high throughput of samples, although the initial cost of the apparatus is high.

Introduction

Identification of fusobacteria presents many problems for the routine diagnostic laboratory. The currently accepted classification of the genus is that of Holdeman *et al.* (1977), but classification in this area is notable for frequent revision. Conventional characterisation tests are slow and difficult to standardise (Bennett and Duerden, 1985) and the problems of identification and taxonomy of this group are due to the general lack of reactivity in the fusobacteria. Carbohydrate fermentation tests, which are the basis of most identification schemes, give few positive results. Therefore, the range of reproducible discriminatory tests available is limited.

Amongst the named species of fusobacteria, *Fusobacterium necrophorum* is known to be a significant pathogen causing necrobacillosis in animals and man (Lemierre, 1936; Finegold, 1977; Seidenfeld *et al.*, 1982; Moore-Gillon *et al.*, 1984). *F. nucleatum* is the most frequently reported species, possibly because it is the most easily recognised, being the only fusobacterium that is consistently fusiform in shape. It is commonly isolated from human sub-gingival plaque taken from healthy and diseased sites (Walker *et al.*, 1979), and has been reported as the cause of serious conditions, such as

osteomyelitis (Hall *et al.*, 1983; Templeton *et al.*, 1983). For other members of the genus, the picture is less clear. Although reports are less frequent, all species represented in the present study have been associated with serious infections (O'Grady and Ralph, 1976; George *et al.*, 1981; Hall *et al.*, 1983; Chandran and Schenfield, 1985; Reig *et al.*, 1985). The relative paucity of reports implicating these organisms as pathogens may be due, in part, to the difficulties associated with their identification. Reports of un-named anaerobic gram-negative bacilli (e.g., Mitre and Rotherham, 1974) may be of fusobacteria.

Bacterial characterisation by Py-MS was first investigated by Meuzelaar and Kistemaker (1973). A small (0·01–1 mg) sample of bacteria is coated on to a carrier and heated to a controlled temperature > 400°C in a vacuum. In the absence of free oxygen, organic materials in the specimen undergo thermal degradation to low mol. wt volatile fragments, a process termed pyrolysis. The quantitative and qualitative composition of the pyrolysate reflects the composition of the specimen and the pyrolysis conditions. Therefore, analysis of the products by mass spectrometry, with measurement of the amounts of products at each molecular mass, yields a spectrum containing information on the overall organic composition of the specimen. Py-MS of a pure chemical usually yields several products with distinct masses, and different chemicals may yield

some products of identical mass. Py-MS of complex mixtures of organic compounds, e.g., bacteria, yield spectra comprising complex, stacked data; the total intensity for each mass probably represents contributions from several different polymers. Multivariate statistical techniques are required to produce meaningful results from the data.

Considerable progress has been made in the analysis of Py-MS data (Gutteridge and Norris, 1979; Drucker, 1981; Gutteridge *et al.*, 1985; Shute *et al.*, 1985), but the high capital cost, difficulties in interfacing pyrolysers with existing mass spectrometers and lack of purpose-built equipment have hampered development. Prutech (London) developed a low cost quadrupole mass spectrometer specifically designed for automated Py-MS analysis (Aries *et al.*, 1986), marketed by Horizon Instruments (Heathfield, Sussex). We were asked to assess a prototype instrument in late 1986, and have investigated various microbiological applications, including Py-MS characterisation of bacterial genera that are difficult to characterise by conventional means. In this report we describe the application of Py-MS to the classification and identification of fusobacteria.

Materials and methods

Bacterial strains and culture conditions

A set of 119 clinical isolates and 24 reference strains of *Fusobacterium* spp. and *Leptotrichia buccalis* was examined. The reference strains were: *F. nucleatum* NCTC nos. 11326 and 10562, ATCC 25586; *F. naviforme* VPI 4877; *F. varium* VPI nos. 4234 and 0499A, ATCC 8501, NCTC 10560; *F. mortiferum* VPI nos. 5696, 4249, 0473 and 04123A; *F. necrogenes* NCTC 10723, VPI 2368, ATCC 25556; *F. necrophorum* NCTC nos. 10575 and 10576, ATCC 25286; *F. gonidiaformans* VPI nos. 11360, 4879, 4381, 0482A; *F. russii* VPI 0307; *Leptotrichia buccalis* NCTC 10249. NCTC strains were obtained from the National Collection of Type Cultures (Colindale Avenue, London), and VPI and ATCC strains from Dr T. Hofstad, Gade Institute, Bergen, with the exception of ATCC 25286, obtained from Dr M. Keaney, Hope Hospital, Salford. The clinical isolates comprised strains identified as *F. necrophorum* (18), *F. naviforme* (1), *F. nucleatum* (64, of which one was a stable L-form), *F. necrogenes* (6), *L. buccalis* (2), *F. mortiferum* (1) (Bennett and Duerden, 1985), and 27 isolates from tropical ulcers. The tropical ulcer strains were obtained from Dr B. Adriaans (Department of Dermatology, King's College Hospital, Denmark Hill, London, SE5), and had been isolated from patients in Zambia, Gambia, southern India and Papua New Guinea (Adriaans and Drasar, 1987); they resembled *F. mortiferum* in biochemical reactions, but did not hydrolyse aesculin.

Cultures were stored in 10% glycerol broth at -76°C . The organisms were grown on Columbia blood agar (Columbia Agar, Oxoid, with defibrinated horse blood, Gibco, 7.5%) incubated anaerobically at 37°C for 48 h in Whitley anaerobic jars (Don Whitley Scientific, Shipley, West Yorkshire) equipped with Engelhard D-Oxo 'D' catalyst pellets, in an atmosphere of H_2 10%, CO_2 10%, N_2 80%. The jars were evacuated to 25 mbars and refilled with the anaerobic gas mixture.

Description of apparatus

The Horizon Instruments PYMS 200X comprises an automated sample handling system, Curie-point pyrolyser, expansion chamber incorporating a 3-position valve, collimating tube, electron impact ioniser, quadrupole mass spectrometer, electron multiplier and vacuum system with Pirani and ionisation gauges. Control and data collection is via software running on a Zenith Z-200 microcomputer. The sample is coated on a 10×3 mm V-shaped ferro-nickel alloy foil in a 4×33 mm rimless glass test tube with a Viton O-ring collar (Horizon Instruments). Up to 20 tubes can be loaded on to a magazine for automatic serial processing. Tubes are collected from the magazine by a bifurcated hollow snout and inserted into the pyrolysis coil. Pressure from the snout forces the O-ring against a conical face on the expansion chamber casing creating an airtight seal (fig. 1). During this operation, the 3-position valve of the expansion chamber is in the sealed position (A), maintaining the system vacuum. After insertion, the valve moves to position B, opening a bypass line to the rotary and diffusion pumps to allow evacuation of the sample tube. The valve moves to position C, closing the bypass line and connecting the expansion chamber to a collimating tube leading to the spectrometer. A radio frequency current is passed through the pyrolysis coil, producing magnetic inductive heating within the sample-coated alloy foil. The foil and sample heat rapidly, until a temperature corresponding to the Curie-point of the alloy is reached. At this temperature, the alloy ceases to exhibit ferromagnetic properties and heating ceases; on cooling below the Curie-point, inductive heating resumes, and the foil-pyrolyser system acts as a thermostatic switch, maintaining the sample at the alloy Curie-point until current ceases to flow. The temperature rise times are generally < 1 s (0.6 s to 510°C ; Ottley and Maddock, 1986) and the Curie-point temperature is maintained for 3–4 s. In the non-oxidising environment of the vacuum, the high foil temperature causes thermolytic cleavage of covalent bonds in the organic components of the sample, releasing low mol. wt volatile fragments. The pyrolysis products diffuse from the tube into the expansion chamber and down a collimating tube to the ionisation chamber of the mass spectrometer. The expansion chamber allows flow mixing of the products, and protracts product release to allow a large number of spectrometer scans of the products. The expansion chamber and collimating tube are heated at 130°C to minimise product condensation. In the ionisation chamber, the products are bombarded with low

Fig. 1. Cross sections of the sample inlet system. The sample tube is collected from the magazine by a bifurcated hollow snout and inserted into the pyrolysis coil. Pressure from the snout forces the O-ring at the neck of the tube against the valve block, forming an airtight seal. The valve is in position A, i.e., the expansion chamber is sealed from the tube but is connected to the mass spectrometer and vacuum by-pass line. The valve moves to position B, connecting the vacuum by-pass line, expansion chamber and sample tube. The tube is evacuated and the valve moves to position C, connecting the sample tube and the mass spectrometer to the expansion chamber and sealing the by-pass line. Current then passes through the pyrolysis coil, heating the sample coated foil to the Curie point. Thermal degradation of the specimen yields volatile products which flow into the expansion chamber, mixing due to turbulence, and then through the collimating tube into the mass spectrometer. The expansion chamber and collimating tube are gold plated and heated to 130°C to minimise condensation and secondary reactions. The valve returns to position A and the solenoid opens, releasing the vacuum in the tube which is then withdrawn by the snout.

Fig. 2. Ionisation chamber and mass spectrometer. Pyrolysate molecules flow down the collimating tube and enter the ionisation chamber as a narrow beam. A crossing beam of low energy (25 eV) electrons converts the pyrolysate molecules to molecular ions which are accelerated by the repeller plate and focused by an electromagnetic lens. The quadrupole mass filter then separates the molecular ions according to mass:charge ratio, and the number of ions at each mass interval is registered by the electron multiplier tube and computer.

energy electrons (25 eV) to produce molecular ions, which are deflected into an electromagnetic lens (fig. 2). Molecules which remain un-ionised are deposited on a trap cooled with liquid nitrogen. The ionised molecules are focused in the electromagnetic lens, accelerated down a potential gradient, and directed into a quadrupole mass

spectrometer. The mass spectrometer repeatedly scans the incoming mixed molecular ions, separates them according to mass, and directs the batches of separated ions on to the lead end window of an electron multiplier tube. Interaction of the high energy ions with the lead releases a shower of electrons into the electron multiplier. Successive acceleration-target interaction steps within the multiplier amplify the electron shower to a detectable level, yielding an electrical pulse. The number of pulses detected at each mass interval of the quadrupole scan is recorded and these are summed over the repeated scans, to yield a quantitative mass spectrum (fig. 3). A magazine of 20 specimens can be processed in 45 min.

Specimen preparation

Alloy foils and acid-washed sample tubes were degreased by washing in acetone, dried at room temperature and heated at 180°C for 3 h. A foil was inserted into each tube, leaving *c.* 7 mm protruding; flamed forceps and no-touch technique were used at this and all subsequent stages to prevent contamination with extraneous organic material. The tubes containing foils were heated at 180°C for 1 h and allowed to cool. Bacterial strains were cultured on blood agar, as described above. A few colonies were picked with a heat sterilised straight wire taking care to avoid inclusion of particles of medium, and smeared uniformly over the upper surface of the foil inside a tube, to give a thin film. Tubes containing coated foils were heated at 80°C for 5 min to dehydrate the specimen, and

Fig. 3. Examples of mass spectra. The ion counts are displayed at unit mass intervals in histogram form for masses 11–200. These are “fingerprint” quantitative data of the composition of the pyrolysate, which in turn reflect the composition of the sample and the pyrolysis conditions. The data have a complex covariance structure and must be interpreted by multivariate statistical methods.

stored over P_2O_5 in a vacuum desiccator in the dark. For analysis, the foil was pushed into the tube with a flamed stainless steel tool, which positioned it 10 mm from the mouth of the tube, O-ring collars were added, and the tubes were loaded into the sample magazine for processing.

Pyrolysis mass spectrometry

The Curie-point of the alloy foils was 530°C, and current was passed through the pyrolysis coil for 4 s. The temperature of the sample inlet system was 130°C. Quadrupole scanning was initiated simultaneously with pyrolysis, and 160 scans, resolving from m/z 11 to 200, were made over the subsequent 56 s.

The replication structure of the samples was complex; several prepared replicate foils were lost in an accident and low strain representation of some species was balanced by greater replication. The replication levels were as indicated in brackets: *F. nucleatum*—NCTC

10562 (6), ATCC 25586 (6) and 4 clinical strains (6, 6, 7, 8); *F. naviforme*—VPI 4877 (6) and a clinical strain (11); *F. varium*—VPI 0449A (6), VPI 4234 (6), ATCC 8501 (6) and NCTC 10560 (11); *F. mortiferum*—VPI 5696 (11), VPI 4249 (11), VPI 0473 (11), VPI 04123A (6) and one clinical strain (5); *F. necrogenes*—VPI 2368 (5), ATCC 25556 (6), NCTC 10723 (11) and 4 clinical strains (5, 5, 6, 12); *F. necrophorum*—NCTC 10576 (6), ATCC 25206 (8), NCTC 10575 (11) and 4 clinical strains (6, 6, 6, 6); *F. gonidiaformans*—VPI 4879 (6), VPI 0482A (6) and VPI 4381 (5); *F. russii*—VPI 0307 (6); tropical ulcer strains—5 clinical strains (6, 6, 6, 6, 11); *L. buccalis*—NCTC 10249 (16) and a single clinical isolate (6). The set of replicates was used to assess ion count reproducibility and discrimination (see below).

Data analysis

Data on spectra (pyrolysis sequence number, total ion count and ion counts for masses 11–200 at unit intervals)

were recorded on floppy disk and transferred to the University of Sheffield IBM 3083X mainframe computer. Ion counts for masses 141–200 were ignored in analyses because they were <100 and, therefore, subject to large statistical fluctuations.

Normalisation of the ion counts was necessary to correct for variations in sample size; total sample ion counts varied from $c. 2 \times 10^5$ to 2×10^6 . An estimate of sample size for each spectrum was derived from the sum of ion counts over a reproducible set of masses—the normalisation set. Raw ion counts for each mass were divided by the sum of raw ion counts of the normalisation set for that spectrum, and multiplied by 10^6 to give normalised ion counts. The normalisation set was derived as follows. Reproducibility of normalised ion counts for each mass was assessed by calculating, for each strain, the within-strain standard deviation of normalised ion counts for that mass and expressing this as a percentage of the mean strain normalised ion count for that mass. The mean of this coefficient of variation was calculated for the strains of the replicate set to give a mean coefficient of variation (MCOV). Initially, all masses from 11 to 140 were included in the normalisation set, and the MCOV for normalised ion counts at each mass was calculated for the set of replicate strain spectra mentioned above. All masses for which the normalised ion count showed $\text{MCOV} > 10\%$ were eliminated from the normalisation set, the data were re-normalised, and MCOVs recalculated. After five iterations of this cycle, a stable normalisation set was obtained. This comprised masses 38, 39, 41–43, 48, 52–60, 62–87, 89–106, 109–113, 115, 118, 123–126 and 128. Spectra were normalised on this basis before further mathematical analysis. Normalisation, MCOVs and variance ratios were computed by a BASIC program.

Identification. Normalised ion counts for the selected masses were submitted to discriminant analysis using the statistical software suite SPSSX (SPSSX Users' Guide, 1983). Three analyses were undertaken. First, each spectrum was labelled as belonging to one of the recognised species (including tropical ulcer strains as a distinct "species") and the data subjected to discriminant analysis to obtain estimates of ease of discrimination between these species. In the two subsequent analyses, 50% of the spectra were labelled according to their conventional species identification (the learning set) and 50% were un-labelled (the challenge set). By reversing challenge and learning sets in the two analyses, each spectrum was identified to a species as a member of a challenge set. Identification results from this blind challenge gave first choice and second choice species, along with the probability of identification for the first choice; if this probability was $>95\%$, the result was regarded as firm, if $<95\%$, as equivocal. Py-MS identifications for strains subjected to replicate analyses were based on the consensus of firm first choice identifications.

Classification analysis was performed with the CLUSTAN 2 software suite (Wishart, 1982). Initially, data for strains with >4 replicate spectra (42) were subjected to discriminant analysis (SPSSX). This optimised discrimi-

nation between these strains, producing 41 axes (canonical discriminant functions) on which coordinates for the spectra were corrected for within strain inequality of variance between masses and for covariance. Coordinates on this axis set were then calculated for spectra from all strains; for those strains with replicate spectra, mean coordinates were calculated. Only coordinates on the first 20 axes, which accounted for 95.7% of the discrimination between strains, were used in classification. Agglomerative clustering, based on the euclidian-distance-squared coefficient and Ward's method (Ward, 1963) for hierarchical agglomeration was then applied to those coordinates.

Results

Identification

Discrimination between species. In the discriminant analysis in which each of the spectra was assigned to its conventionally determined species, clear discrimination between all species was achieved. Supra-species groupings are evident in fig. 4 where the species centroids defined by the first three discriminant functions (accounting for 83% of the total discrimination) are presented as a pseudo-three dimensional ordination diagram. Straight line distances between centroids are proportional to ease of discrimination between species. *F. gonidiaformans* and *F. naviforme*, *F. necrogenes* and *F. mortiferum*, and *F. russii* and *F. necrophorum* were those pairs of species showing poorest Py-MS discrimination. *F. nucleatum* was distinct from other species, as were the tropical ulcer strains. The discrimination of *L. buccalis* did not suggest differences at the generic level.

Blind identification. As expected from the species discrimination achieved, agreement between identification in Py-MS and biochemical methods was high (91.8%): 382 spectra were firmly identified in agreement with conventional methods; 20 spectra (4.8%) were identified in disagreement. Equivocal results were obtained for 14 spectra (3.4%), and in most cases (11 out of 14) were correct in first or second choice.

The table shows the results of strain identification, showing that 131 (91.6%) were identified in agreement with biochemical methods, 6 (4.2%) in disagreement and 6 (4.2%) yielded equivocal results in Py-MS. The L-form of *F. nucleatum* was identified incorrectly as *F. mortiferum*.

Py-MS classification

Classification on the basis of Py-MS data yielded the dendrogram shown in fig. 5. Two main groups

Table. Identification of blind challenge sets by Py-MS and conventional methods.

Species	Number of strains* for which Py-MS and conventional identification			
	Agree	Equivocal	Disagree	Total
<i>F. nucleatum</i>	61	4	2†	67
<i>F. naviforme</i>	2	0	0	2
<i>F. varium</i>	4	0	0	4
<i>F. mortiferum</i>	5	0	0	5
<i>F. necrogenes</i>	7	1	1	9
<i>F. necrophorum</i>	19	0	2	21
<i>F. gonidiaformans</i>	4	0	0	4
<i>F. russii</i>	1	0	0	1
Tropical ulcer	26	1	0	27
<i>L. buccalis</i>	2	0	1	3
Total	131	6	6	143

* Consensus results were taken for strains with replicate spectra.
† Including the L-form.

spectra of other species; the B complex, comprising B1, which included spectra from several species, and B2 containing 14 (66.7%) of the *F. necrophorum* strains; and the C complex (C1–C3) which included the tropical ulcer strains and some others, predominantly *F. necrogenes* and *F. mortiferum*.

At a dissimilarity c. 50, 14 clusters were resolved; A1–A6, B1 and B2, C1–C3 and D1–D3. The conventional species identities of the strains appearing in these clusters are shown in fig. 5. Nine clusters contained only spectra from single conventional species; A1, A2, A3, A6, D2 and D3 contained spectra of *F. nucleatum* only; B2 contained only spectra of *F. necrophorum*; C2 contained only spectra of tropical ulcer strains; and C3 contained only spectra of *F. necrogenes*. Clusters with a clear predominance of one species were: A5 comprising 31 strains including 27 strains of *F. nucleatum*, and D1 comprising 6 strains including 5 strains of *F. nucleatum*. Clusters with no clear species predominance were A4, comprising only two strains, B1 with 16 varied strains, and C1 with 15 varied strains.

The 67 *F. nucleatum* strains, the largest species group studied, were divided between several clusters. The A complex contained 50, with all the A clusters comprising spectra of this species either exclusively or predominantly. Eleven strains of *F. nucleatum* clustered in the clearly distinct D complex, together with a single strain of *F. necrophorum*. The L form of *F. nucleatum* clustered in C1, and 5 further strains were found in B1.

Of the 27 tropical ulcer strains, 25 formed cluster

Fig. 4. An ordination map indicating the ease of discrimination between species. The axes represent complex statistical quantities, derived from the inter-species discriminant analysis—the canonical discriminant functions. The mean spectrum for each species is represented by a line based at appropriate coordinates on canonical discriminant axes 2 and 3, and extending vertically to the coordinate on axis 1. The straight line distance between any pair of mean spectrum points increases with increasing ease of discrimination. The full distance structure can be represented on nine axes; the three axes shown allow representation of 83% of the inter-species discrimination achieved. Note that this diagram results from pre-imposition of the conventional species groupings and therefore does not represent a classification of the panel of test strains.
Key: nu = *F. nucleatum*; r = *F. russii*; np = *F. necrophorum*; g = *F. gonidiaformans*; na = *F. naviforme*; v = *F. varium*; ng = *F. necrogenes*; m = *F. mortiferum*; tu = tropical ulcer strains; b = *L. buccalis*.

formed at a dissimilarity value c. 700. One, the small D complex, showed a compact set of three clusters (D1, D2 and D3) comprising 12 spectra, representing 11 *F. nucleatum* strains and one *F. necrophorum*. The other contained the remaining spectra for which further divisions between dissimilarities c. 600 and 200 yielded 3 subgroups: the A complex comprising clusters A1–A6 accounting for 50 (74.6%) of the strains of *F. nucleatum*, with five

Fig. 5. A dendrogram representing the relationship between strains based on spectral data. The dissimilarity measure represents distances between spectra on a set of statistically "relaxed" axes, corrected for within-strain statistical effects. No prior species structure was imposed, and unlike fig. 4, this diagram shows an unweighted classification reflecting quantitative differences in cell composition. Conventional identities and numbers of strains are shown for each cluster. One tropical ulcer strain was an extreme outlier and is not represented in the dendrogram. Collection strains segregated as follows: *F. nucleatum* NCTC 11326 in A5, NCTC 10562 in A1 and ATCC 25586* in D2; *F. russii* VPI 0307 in A5; *F. necrophorum* NCTC 10575, NCTC 10576 and ATCC 25286* in A5; *F. gonidiaformans* VPI 11360, 4879, 4381 and 0482A in B1; *F. naviforme* VPI 4877 in B1; *F. varium* VPI 4234, VPI 0499A, ATCC 8501* and NCTC 10560* in C1; *F. necrogenes* NCTC 10723*, ATCC 25556* and VPI 2368 in C3; *F. mortiferum* VPI 5696, 4249, 0473 and 0123A in C1; *L. buccalis* NCTC 10249* in B1. Type strains are indicated by an asterisk.

C2, one clustered in C1, and one was an extreme outlier not included in fig. 5.

Spectra from the two strains of *F. naviforme* were found in the mixed cluster B1, as were spectra of the four strains of *F. gonidiaformans*. The four strains of *F. varium* were found in the mixed cluster C1 as a sub-cluster, and the five strains of *F. mortiferum* were dispersed in C1. Cluster C3 comprised five strains of *F. necrogenes*, but three strains of this species clustered in C1, and one in B1. Similarly, B2 comprised 14 strains of *F. necrophorum*, but an additional three strains clustered in A5, and one each in A4, B1, C1 and D1. The three strains of *L. buccalis* clustered in B1, and the single strain of *F. russii* clustered in A5.

Discussion

Norris (1980) aptly considered characterisation techniques to produce data on the molecules and molecular interactions at various stages in the biochemical processes of the cell: (i) the coding of the genetic material (genotypic information); which is translated to (ii) protein molecules; which act to produce (iii) other substances in metabolism or in biosynthesis of macromolecules; giving cells with (iv) specific morphology and staining characteristics. Proteins and other macromolecules are major components of the cell. New techniques such as Py-MS, which examine these components, yield 'fingerprint' quantitative data that are different from

the two-state data of conventional biochemical tests; analysis of such data has introduced new mathematical techniques to bacterial taxonomy. Therefore, studies utilising these techniques are likely to enhance understanding, not only of bacteria, but also of the processing of characterisation data.

Identification of fusobacteria by Py-MS was largely successful; most strains were allocated to the appropriate species as determined by conventional taxonomy (table). Discriminant analysis extracts and weights characters in the Py-MS data that correlate with the imposed groupings. Therefore, provided that some aspects of the Py-MS data coincide with the imposed conventional classification, spectra will be identified in agreement with conventional results. However, it must be recognised that the ordination diagram (fig. 4) represents only one, weighted, aspect of the Py-MS data. The identification results imply that the Py-MS data are congruent in some aspects with the conventional classification.

As with any identification strategy, the species characteristics resolved from Py-MS data were less representative if the number of strains contributing to them was small, although reliable discrimination and identification could be achieved if a poorly represented group was markedly different in composition from other groups.

F. necrophorum and *F. nucleatum* are easily recognised by their morphological and biochemical properties (Holdeman *et al.*, 1977; Bennett and Duerden, 1985); they were clearly distinguished in Py-MS identification. In conventional tests *F. necrogenes* is difficult to distinguish from *F. mortiferum* (Holdeman and Moore, 1977), but Py-MS indicated sufficient compositional differences for them to be distinguished. By contrast, the tropical ulcer strains differed from *F. mortiferum* in conventional tests only in failing to hydrolyse aesculin (Adriaans and Drasar, 1987), but they were readily discriminated in Py-MS. As in the classification analysis, extensive discriminatory differences were not evident between *L. buccalis* and *Fusobacterium* spp., despite the current classification of *L. buccalis* as a separate genus (Holdeman *et al.*, 1984). Generally the results showed that although Py-MS data could be extracted and weighted to allow identification to conventional species groups, the inter-species discriminatory pattern of these extracted data differed from that obtained with conventional tests. The presence or absence of specific enzymes that determine the pattern of reactions in conventional tests is unlikely to alter the overall composition of the cell significantly;

therefore dissimilarities between the two approaches might be expected.

The potential contribution of Py-MS to taxonomic studies was assessed in the classification analysis. It must be recognised that all classifications are empirical. Choices are made at each stage of any analysis—choices of tests, strains, pre-processing methods for characterisation data, similarity coefficient, clustering strategy, and method of representation of results. These decisions are often arbitrary, and each may affect the classification produced. A classification is justified by its resolution of identifiable groups of strains that share a broad range of common properties of practical importance, e.g., pathogenicity.

The results of the classification (fig. 5) confirmed the general pattern of conventional species groupings within the genus, but there were some discrepancies. *F. nucleatum* strains were predominant in two clusters (A and D) which were clearly distinct, indicating a large intra-species difference in overall cell composition. Studies of DNA–DNA homology (Love *et al.*, 1987) and electrophoretic mobility of glutamate dehydrogenases (Gharbia and Shah, 1988) also suggest that *F. nucleatum* is a heterogeneous species.

As in identification, the tropical ulcer strains were resolved as a distinct cohesive group (C2). Most *F. necrophorum* strains clustered in B2, and most *F. necrogenes* in C3. Resolution of the remaining species was poor, *F. naviforme*, *F. gonidiaformans* and *L. buccalis* strains clustered in B1, and there was no indication of the pronounced compositional differences which might be expected in a cluster including distinct genera. Strains of *F. varium*, *F. mortiferum* and 3 of the 9 *F. necrogenes* strains clustered in C1, with little indication of compositional differences. The L-form of *F. nucleatum* clustered in C1, rather than A or D, where *F. nucleatum* predominated, probably reflecting the major compositional differences inherent in loss of the cell wall.

In the present study, as in earlier pyrolysis gas-liquid chromatography studies (Magee *et al.*, 1983; Hindmarch and Magee, 1987), we have used iterative re-normalisation (Huff *et al.*, 1981) and data selection on the basis of intra-strain reproducibility and inter-strain discrimination (Eshuis *et al.*, 1977), but other groups have used different combinations of these and other approaches (Gutteridge *et al.*, 1985; Shute *et al.*, 1985). A consensus on methods of mathematical processing, based upon comprehensive analysis of several data sets is desirable.

A further problem, not specific to Py-MS, is the

difficulty of representing classification data adequately in simple diagrams. The similarity structure revealed by the analysis of the results of many tests, for many strains, is complex, and two dimensional representations, such as fig. 5, show only a fraction of this information (Hindmarch and Magee, 1987). The results of such data reduction must be interpreted with caution.

The PYMS 200X instrument is rapid and simple to operate; the data presented here were gathered within 3 days, only 8 weeks after the instrument was installed. Running costs comprise 12 pence per foil and c. £1 per day for liquid nitrogen. This prototype machine had minor design flaws, most of which were subsequently eliminated. We were unable to investigate long term stability of analyses; in the present study, the instrument was operated in batch mode, and the subsequent upgrading reduced the likelihood of achieving inter-batch

stability for further identification based on the data presented here.

The analysis of the numeric multivariate data yielded by Py-MS has led to the application of new identification and classification strategies. No single technique can provide a comprehensive description of bacteria; data from various approaches should be combined in generalised classifications, but mathematical strategies which can readily combine discrete and continuous data have yet to appear. It is clear, however, that Py-MS studies will make some contribution to systematics, enhancing our understanding and knowledge of bacteriology and of the mathematics of taxonomy.

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