MOLECULAR DIAGNOSIS

Differentiation of strains of *Mycoplasma fermentans* from various sources by pyrolysis mass spectrometry

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*Mycoplasma fermentans* has attracted much interest both as a cofactor for the progression of AIDS and as a pathogenic agent in non-AIDS related diseases. Previous studies with serological and genetic techniques suggest that *M. fermentans* represents a homogeneous group of organisms, with no significant differences identified among the strains examined. In this study, 25 cultures of *M. fermentans*, including isolates from human sources and tissue culture cells, were compared by pyrolysis mass spectrometry (PMS). It was possible to distinguish the ‘type’ strain PG-18 from an AIDS-associated *M. fermentans* strain ‘incognitus’ by this technique. PMS was also able to differentiate laboratory-induced aminoglycoside-resistant variants from their fully susceptible parents. Four AIDS-associated isolates were distinguished from each other, whilst five European cell culture isolates were shown to be closely related, as were six *M. fermentans* isolates from an outbreak of acute respiratory infection in Canada. PMS has proved useful in distinguishing isolates of *M. fermentans*, providing epidemiological data. In addition, PMS may help in determining the likely origin of a given isolate, and in the future may be of use in assessing the role of this micro-organism in human disease.

Introduction

*Mycoplasma fermentans* is a potential human pathogen and a recognised contaminant of tissue culture cells. It has been isolated from the urogenital tract of patients with ulcerative balanitis [1], the respiratory tract of children with community-acquired pneumonia [2], adults with acute respiratory distress syndrome [3] and the joints of patients with rheumatoid arthritis [4] by means of cell-free culture media, and from the bone marrow of leukaemic patients with the aid of cell cultures [5]. More recently, *M. fermentans* has been cultured from DNA extracts from Kaposi’s sarcoma tissue and the blood of patients suffering from AIDS [6, 7] by the use of cell cultures and from urine by means of cell-free media [8]. It has also been identified in non-AIDS patients suffering from a lethal ‘flu-like’ illness [9]. The ability of the micro-organism to cause systemic infection in immunocompromised patients has stimulated much interest in its potential role in enhancing the pathogenicity of viruses such as HIV.

The epidemiology of *M. fermentans* infections is poorly understood. At present there is no recognised typing system for these micro-organisms. A few studies have been performed with genetic or serological techniques, such as PCR [10, 11], restriction endonuclease DNA analysis [12], DNA/DNA hybridisation [13], DNA base composition [13] and immunoblotting [12], but only a few strains were compared. Data thus far suggest that isolates of *M. fermentans* form a highly conserved group of organisms.

Pyrolysis mass spectrometry (PMS) has been shown to give rapid and reliable inter-strain comparisons for a wide variety of micro-organisms [14–16]. Early studies suggested that PMS could distinguish strains of *M. pneumoniae* [17] and *M. fermentans* [18]. In this study, 25 isolates of *M. fermentans* from the UK, Europe, USA, Canada and Japan were compared by PMS.

Materials and methods

*M. fermentans* strains

The sources and antibiotic susceptibility profiles of the *M. fermentans* strains used in this study have been
documented in detail elsewhere [8, 12, 19–21]. Briefly, strain PG-18 was obtained from the National Collection of Type Cultures (NCTC), 61 Colindale Avenue, London, having been isolated in cell-free medium from a human case of ulcercative balanitis. Strains KL4 and KL8, obtained from Dr M. H. Williams, Arthur Stanley Institute of Rheumatology, London, were isolated from patients with rheumatoid arthritis. M. fermentans strains K7, E10 and Z62, from Dr W. H. Murphy, University of Michigan School of Medicine, Ann Arbor, MI, USA, were isolated with the aid of tissue culture cells from the bone marrow of leukaemic patients. A human joint isolate (GIM) and a human urethral isolate (BRO) were supplied by Professor C. Bébcar, University of Bordeaux 11, France, having been isolated in cell-free media. Strain AOU had been isolated from the blood of an AIDS patient by Professor L. Montagnier, Pasteur Institute, France. The strain of M. fermentans incognitus was obtained from Dr S-C. Lo, Department of Defense, Armed Forces Institute of Pathology, Washington DC, 20306-6000, USA; it had been isolated during transfection studies in NIH/3T3 cells with DNA from Kaposi’s sarcoma tissue from an AIDS patient [6]. Strains 2059, A6, A17, C5 and 28AC, which had been isolated from tissue culture cells from various European sources between 1982 and 1992, were supplied by Dr G. D. Windsor and Helena M. Windsor, Mycoplasma Experience Ltd, Reigate, Surrey. Strains #5 and #29 were isolated from urine deposits from AIDS patients and supplied by Dr R. B. Kundsin, Brigham and Women’s Hospital, Harvard Medical School, Boston, USA. Human respiratory isolates (M39, M51, M52, M64, M70 and M73), isolated by Dr R. Dular, Canada, were obtained from Dr J. G. Tully, Frederick Cancer and Research Facility, MD, USA. Strains F-1 and F-7 were isolated from mouse cell lines and supplied by Dr T. Sasaki, National Institute of Health, Tokyo 208, Japan.

In addition to the above, laboratory-induced amino-glycoside-resistant variants of strains PG-18 and KL4 were examined. Strep-R PG-18 and Strep-R KL4 were each resistant to streptomycin 500 mg/L, and PG-18 R⁺ and KL4 R⁺ were each resistant to kanamycin, neomycin, gentamicin and tobramycin 500 mg/L [19].

**Mycoplasma culture media**

The media used were modified Hayflick’s broth or agar [22], as described previously [18].

**Preparation of M. fermentans stock cultures**

Strains of M. fermentans were grown aerobically in modified Hayflick’s broth, incubated at 36°C until a colour shift to c. pH 6.8 occurred. Cultures were then frozen at −70°C in 2-ml portions. Culture portions of M. fermentans were thawed and subcultured on to mycoplasma agar and usually cloned once. The identity of each M. fermentans strain was checked by growth inhibition [23] with specific M. fermentans PG-18 antiserum. The strains were then stored as agar blocks at −70°C.

**Preparation of M. fermentans for pyrolysis**

A block of each M. fermentans strain to be pyrolysed was thawed and used to inoculate 3 ml of Hayflick’s broth, which was then incubated at 37°C for 3 days. The cells were harvested by centrifugation at 13 000 g, washed once in PBS and resuspended in 100 μl of PBS ready for PMS analysis. The samples were encoded and stored at −20°C before being pyrolysed. In each case, duplicate samples from every isolate were prepared and analysed separately to check the reproducibility of PMS for M. fermentans strains.

PMS analysis was performed by one worker (P.R.S.) who was unaware of the strain identity of the isolates being pyrolysed.

**Pyrolysis mass spectrometry**

The strains of M. fermentans that had been prepared as a single batch were also pyrolysed as a batch. Triplicate pyrolysis foils (Horizon Instruments Ltd, Heathfield, Sussex) were inoculated with c. 5 μl of thawed M. fermentans sample, dried at 80°C for 10 min and pyrolysed at 530°C on a Horizon Instruments PYMS 200X pyrolysis mass spectrometer. Spectra for masses 51–200 were recorded on floppy disk, together with the pyrolysis sequence number and total ion count.

**PMS data analysis**

After normalisation to correct for variations in sample size, triplicate spectra for each sample were labelled as distinct groups. On occasion, the spectral data for the duplicate subcultures of each isolate were summated before multivariate analysis. The spectral data were subjected to principal component (PC) followed by canonical variate (CV) analyses, as described previously [15]. The resulting table of Mahalanobis distances was used to construct a similarity dendrogram ordered by UPGMA (unweighted pair group method with arithmetic averages). Results of the PC and CV analyses were also represented as ordination diagrams of the first two principal component canonical variates (PCCV1 versus PCCV2).

**Results**

An initial study pyrolysing coded cloned cultures of the M. fermentans type species strain PG-18 and the AIDS-associated M. fermentans strain incognitus suggested that PMS could distinguish between these isolates. Isolates PG-18 and KL4 were similarly differentiated by PMS, as were the laboratory-induced resistant variants Strep-R PG-18, PG-18 R⁺ and Step-R KL4.
and KL4 R+ from their respective, fully susceptible parent strains PG-18 and KL4 (data not shown).

In the second experiment, four AIDS-associated isolates of *M. fermentans*, namely *M. fermentans* incognitus, AOU, #5 and #29 were distinguished from each other by PMS (Fig. 1).

*M. fermentans* incognitus and a collection of 13 *M. fermentans* isolates, which were all resistant to multiple aminoglycosides, were compared by PMS (Fig. 2). Five European cell culture isolates (strains 2059, A6, C5, 28AC and A17) were shown to be closely related by PMS, but were distinct from Japanese strain F-7 but similar to F-1. Strain AOU appeared similar on PMS analysis to the cell culture isolate F-7 from Japan, suggesting that it also may have originated from cell cultures. The other 'wild' strains (K7, E10 and Z62) did not cluster with the laboratory isolates, but the two leukaemia-associated isolates (E10 and Z62) did cluster together.

Six *M. fermentans* isolates from an outbreak of acute respiratory infection in Canada were compared with four epidemiologically unrelated isolates of *M. fermentans*. With the exception of strain M39, all of the respiratory outbreak isolates (M51, M52, M64, M70 and M73) were closely clustered by PMS but were shown to be distinct from the other strains examined (Fig. 3). All of the respiratory isolates in this experiment were fully susceptible to aminoglycosides, as were strains PG-18 and KL8.

In a final experiment, two recent French isolates, GIM and BRO, which had been isolated from cell-free media and were resistant to streptomycin, were seen to be closely related to each other, but distinct from two British isolates, Strep-R KL4 and KL8 (Fig. 4).

**Discussion**

This study confirmed the ability of PMS to distinguish between isolates of *M. fermentans*. There was good reproducibility between duplicate PMS runs with the type species of *M. fermentans* and *M. fermentans* incognitus, with identical results being obtained. Moreover, PMS was shown to distinguish laboratory-induced resistant variants of strains PG-18 and KL4 from the parent strains, suggesting a high level of discrimination for isolates of *M. fermentans*. PMS did not simply cluster the isolates according to their aminoglycoside susceptibility, which suggests that there are additional differences detectable by this technique.

![Fig. 1. Ordination diagram of spectral data from PMS analysis of four AIDS-associated isolates of *M. fermentans* (incognitus strain in duplicate).](image-url)
**Fig. 2.** Dendrogram derived from PMS analysis showing percentage similarity of 14 *M. fermentans* isolates all resistant to multiple aminoglycosides. *Mfi, M. fermentans* incognitus.

**Fig. 3.** Dendrogram derived from PMS analysis showing percentage similarity of 10 *M. fermentans* isolates, including six associated with an outbreak of acute respiratory infection (M51, M64, M73, M70, M52 and M39).

**Fig. 4.** Dendrogram derived from PMS analysis showing percentage similarity of two French (BRO and GIM) and two British (KL8 and strep-R KL4) strains of *M. fermentans.*
The four AIDS-associated isolates were differentiated by PMS, which suggests that no single strain of *M. fermentans* is responsible for this association. Three of the AIDS-associated isolates were also distinct from cell culture isolates on PMS analysis (data not shown), all of which were resistant to high levels of aminoglycosides. However, strain AOU appeared on PMS analysis to be similar to F-7, a Japanese cell culture isolate, which suggests that AOU might have originated from cell culture. Five European cell-culture isolates clustered together with a Japanese strain (F-1) isolated from a mouse cell line. As the ability of PMS to detect strain differences within *M. fermentans* has been demonstrated in this study, the clustering of cell culture isolates supports the suggestion that these isolates represent laboratory contaminants and are relatively homogeneous, i.e., they do not exhibit the strain diversity of ‘wild’ isolates of *M. fermentans*. This distinction may be very valuable in assessing the clinical significance of cell culture isolates of *M. fermentans*. Two of the leukaemia-associated isolates (E10 and Z62) were grouped together by PMS, whereas the third (K7) was distinct. As these three strains were isolated over a 5-year period, it is perhaps not surprising that there were differences in the leukaemia-associated strains. Further studies with more isolates from such patients are needed before the significance of this observation can be assessed.

PMS analysis correctly clustered isolates of *M. fermentans* associated with an outbreak of acute respiratory infection in Canada, and distinguished them from other isolates of similar susceptibility to aminoglycosides. This validates the technique as useful in epidemiological studies of such outbreaks and its further application should enhance understanding of the natural history of *M. fermentans* infections.

Two recent French strains (GIM and BRO), isolated in cell-free media and both resistant to streptomycin, were shown to be similar to each other on PMS analysis, but distinct from two British strains of *M. fermentans* isolated from the joints of patients with rheumatoid arthritis (KL4 and KL8).

In summary, PMS has been demonstrated to detect considerable strain differences within *M. fermentans* which accord with epidemiological data where that is available. Equally, PMS correctly identified a cluster of isolates that were known to be related to an outbreak of respiratory infection. *M. fermentans* isolates from AIDS patients were shown to be heterogeneous on PMS analysis. Cell culture isolates of *M. fermentans* were clustered together by this technique, which supports the hypothesis that these may be laboratory contaminants, arising from the cell lines themselves rather than from the clinical material being cultured on them. The basis of the distinctions made by PMS remains unclear, but it has been shown to be independent of the acquisition of aminoglycoside resistance.

*PMS* is a rapid, simple and inexpensive technique and its ability to characterise *M. fermentans* strains should provide useful epidemiological data as to the likely origin of a given isolate, as well as elucidating the natural history of mycoplasma disease.

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References

13. Saillard C, Carle P, Bové JM et al. Genetic and serologic relatedness between *Mycoplasma fermentans* strains and a


