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RAPID IDENTIFICATION OF ANAEROBIC BACTERIA USING MATRIX ASSISTED LASER DESORPTION IONISATION TIME-OF-FLIGHT MASS SPECTROMETRY

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Abstract

Developments in a number of mass spectrometry techniques over recent years have made it possible to analyse cell extracts from bacteria to aid their identification. With the advent of MALDI-TOF-MS crude bacterial proteins can be analysed without the need for extensive separation. Furthermore, intact bacterial cells can be taken directly from a colony and analysed by MALDI-TOF-MS providing within minutes, a unique spectral fingerprint of moieties desorbed from the cell. This spectral fingerprint can then be used to identify the bacterium by matching it to a database of spectral fingerprints of well-characterised bacteria. We have built a quality-controlled database of bacterial spectral fingerprints in collaboration with NCTC and in collaboration with Micromass UK software has been developed for automatically acquiring the data and searching the database. Mass spectral data from 96 well plates were collected in less than 30 seconds per sample. The best match, with a probability score, genus, species and strain of the bacteria was reported within minutes. The results were also subjected to cluster analysis. An anaerobic database of 165 anaerobic bacteria was challenged by isolates of *Prevotella* some strains of which had been identified by ribotyping. The four ribotype 4 strains of 8 *P. intermedia* isolates clustered together suggesting that the method has great potential as a rapid method that can discriminate up to strain level.

Itroduction

Matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF-MS) of intact cells pioneered in 1996 by Claydon *et al.*, and Holland *et al.*, is currently the most popular method of mass spectrometry used for the rapid identification of bacteria. In this process, bacteria are applied to a target directly from an agar plate and after co-crystallisation with a matrix, and soft ionisation with a laser, characteristic bacterial spectral fingerprints are produced. Intact cell MALDI-TOF-MS produces reproducible results provided the culture medium, temperature, time and atmosphere are carefully controlled (Bright *et al.*, 2001, Dare *et al.*, 2001). Phenotypic characteristics reflected in the mass fingerprints resulting from intact cell MALDI-TOF-MS can however, vary with type of growth media (Walker *et al.*, 2002) or, the age of the culture (Arnold *et al.*, 1999) and are most marked if this results in morphological change as in sporulation (Bright *et al.*, 2002a). The relationships of intact cell MALDI-TOF-MS profiles of bacteria can be expressed in dendrograms (Bright *et al.*, 2002b).

There have been very few MALDI-TOF-MS studies to date on intact anaerobic bacteria (Fenselau and Demirev 2001). It is often very difficult to distinguish between anaerobic species and strains because of a lack of phenotypic markers. The potential of MALDI-TOF-MS was therefore accessed in this study. The aims of the study were: i) to investigate the potential of MALDI-TOF-MS to differentiate between 8 clinical isolates of *Prevotella* species and ii) to investigate the potential use of an NCTC type strain database to identify these isolates.

Methods

Bacterial strains, Growth conditions and Ribotyping

- Freeze dried NCTC strains (**Table 1**) were revived by suspension in nutrient broth and 0.1 ml was streaked onto Columbia Blood (5% v/v) agar (CBA) and, or, Fastidious Anaerobe Agar (FAA).
- The PHLS accredited laboratories in Chester supplied the media.
- Incubation: the minimum of 24, 48, or 72 hours as recommended by NCTC to produce good growth at 37°C in an anaerobic atmosphere.
- Two further sub-cultures were made before ICM-MS analysis.
- Culturing and ribotyping of clinical *Prevotella intermedia* isolates (as supplied by D. A. Devine, Leeds Dental Institute) was carried out as described by Pearce *et al.*, 1996.

Genus	Number of Species	Entries[a]
Actinomyces	8	14
Anaerococcus	1	1
Arachnia	1	1
Arcanobacterium	4	4
Bacteroidies	8	15
Bifidobacterium	3	3
Campylobacter	3	3
Clostridium	20	74
Collinsella	1	1
Eggerhella	1	1
Filifactor	1	2
Finegoldia	1	1
Fusobacterium	4	8
Garderella	1	1
Helicobacter	1	1
Micromonas	1	1
Mitsuokella	1	1
Mobiluncus	2	6
Peptococcus	1	1
Peptoniphilus	3	3
Peptostreptococcus	1	1
Porphyromonas	3	3
Prevotella	11	12
Propionibacterium	4	4
Ruminococcus	1	1
Tissierella	1	1
Veillonella	1	2
Totals 27	88	166

[a] Different species, strains, or, growth conditions

Table 1 Numbers of genera, species and entries in the anaerobic database

MALDI target preparation

- Bacteria from several colonies were applied to 12 replicate target plate wells.
- Samples were air-dried for at least 1 hour.
- Samples were overlaid with 1µL of matrix solution and air-dried.
- Gram-positive bacteria matrix: a saturated solution of 5-chloro-2-mercaptobenzothiazole (Sigma-Aldrich Chemical Company).
- Gram-negative bacteria matrix: a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich).
- Matrix solvent: acetonitrile:methanol:water 1:1:1 with 0.1% (v/v) formic acid and 0.01M 18-crown-6 (5).

Instrumentaion

- M@LDI-Linear time of flight mass spectrometer (Micromass UK Ltd., Manchester, UK).
- A nitrogen laser giving a 337nm output of 3ns pulse width.
- Laser fluence was set just above the threshold for ion production in the positive ion detection mode.
- Acceleration voltage of +15 kV.
- Mass calibration using average molecular weights from a standard peptide mixture (bradykinin, angiotensin I, glu-fibrinopeptide B, renin substrate tetra decapeptide, ACTH (18-39 clip) all at 1pmol/µL, bovine insulin 2pmol/µL and ubiquitin 10pmol/µL).
- The data acquisition mass range was from m/z 500 to 10000 Da.

Data analysis using Micromass MicrobeLynx™ software

- Spectra were analysed in batches of twelve replicates and compared for reproducibility using the root mean square (RMS) value obtained by comparing each replicate in turn with the average of the other 11 replicates.
- A RMS rejection value of 3 was used to identify outliers significant at the 0.1% level.
- Acceptable spectra were combined to give representative spectra for each strain.
- Dendrograms were produced by an algorithm where the proximity of one organism to another was generated using a spectral RMS calculation.
- Each node of the dendrogram was singly linked.
- Clusters of organisms were grouped together using a simple average proximity.
- The relative difference axis on the dendrogram represents a relative scale normalised between 0 and 1. A difference of 0 indicates the clusters are exactly the same. A difference of 1 indicates that the clusters are the least similar clusters in the dataset.

Results

i) Potential discrimination can be demonstrated

- **Figure 1** demonstrates that the 8 clinical isolate of *Prevotella intermedia* can be separated into two distinct clusters.
- One cluster contains four Ribotype 4 strains
- The second cluster contains Ribotype 1 & 3 strains

ii) Potential identification of clinical isolates against an NCTC type strain database

- Search results, as expected give poor matches for the clinical isolates.
- Poor results were probably due to different culture conditions. Clinical isolates used CBA, 37°C, 24 hours compared to the NCTC anaerobe database entries which used FAA, 37°C, 24 or 48 hours.

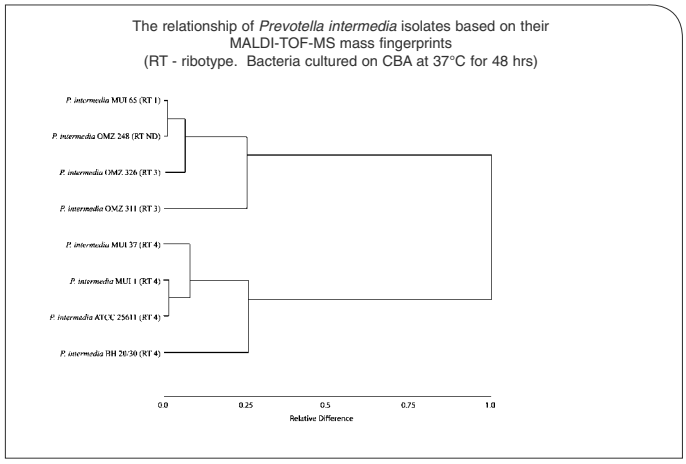

Figure 1

Conclusion

- MALDI-TOF-MS has the potential to discriminate between different Ribotypes of Prevotella intermedia grown under the same conditions.
- Database searches require culture conditions to be kept constant, to obtain meaningful comparisons.
- Further studies require the 8 clinical isolates to be cultured on FAA before comparison with the NCTC database entries.

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