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MALDI—TOF MASS SPECTROMETRY AS A TOOL FOR THE RAPID & SPECIFIC IDENTIFICATION OF PATHOGENS LINKED WITH HOMELAND DEFENCE

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OVERVIEW

Introduction

Rapid identification of pathogens associated with homeland defence is currently required due to the potential release of biological agents. In particular *B. anthracis*, since this pathogen has already been the subject of recent investigations. However the genus *Bacillus* is immensely complex and is presently undergoing major taxonomical revisions based upon 16S rDNA sequence analysis. For many isolates identification to the sub-genus level is difficult due to the high degree of homogeneity within this region of the gene. The UK Health Protection Agency now compliments 16S rDNA analysis with the analysis of other genes.

This study examines the potential use of Matrix Assisted Laser Desorption/Ionisation Time of Flight (MALDI-TOF) mass spectrometry as an alternative more rapid identification technique for this genus.

Methods & Instrumentation

Intact *Bacillus* cells are overlaid with the MALDI matrix 5-Chloro-2-mercaptobenzothiazole (CMBT). The co-crystallised sample is then irradiated with a N_2 laser and the resulting plume of positive ions separated using time of flight mass spectrometry. This produces a characteristic mass spectral fingerprint pattern, which forms the basis of identification against a database containing representative spectra of the genus.

In this study data for ~ 50 NCTC strains are used to challenge a database of ~3500 spectral entries representing over 400 different bacterial species. Approximately 90 strains of *Bacilli* covering the species *B. badius, B. cereus, B. circulans, B. coagulans, B. firmus, B. lentus, B. licheniformis, B. megaterium, B. mycoides, B. pumulis, B. sphaericus and B. subtilis* were included.

Preliminary data

Accurate identification to the genus level was achieved for at least 90% of the isolates tested while > 80% were also successfully speciated. This suggests that speciation of this problematic genus can be achieved without the need for the more complex and time-consuming DNA extraction techniques using one or more genes. These results therefore suggest MALDI-TOF mass spectrometry has the potential to rapidly identify pathogens linked with homeland defence.

INTRODUCTION

The genus *Bacillus* is immensely complex and many species have been reclassified as detailed in the International Journal of Systematic Bacteriology (IJSB) / International Journal of Systematic and Evolutionary Microbiology (IJSEM), either as an original article or in the "Validation List". Some examples of these are *B. atrophaeus* NCTC 10073 originally *"B. globigii"* and *B. subtilis; B. coagulans* NCTC 3991, 3992 & 3993 originally *"B thermoacidurans"; B. licheniformis* NCTC 10452[1], 6346 & 5399 originally *B. subtilis; B. pumulis* NCTC 7198 & 8241 originally *B. subtilis; B. sphaericus* NCTC 7585, 5896 & 2668 originally *"B. fusiformis"* and *Virgibacillus pantothenticus* NCTC 8162, 8124, 8123 & 8122 originally *B. pantothenticus*.

Many of these reclassifications result from modification of the methods used for microbial characterisation. Traditionally, identification and characterisation was based upon morphology and physiological properties. These were superseded by chemotaxonomic analysis in the 1970-80's, which extended the range of phenotypic characters of bacterial cells. The current approach now tends towards nuclei acid analysis, in particular 16S rDNA sequence analysis. However *Bacillus anthracis, Bacillus thuringiensis* and *Bacillus cereus* which manifest very different diseases, share 100% sequence homology in the 16S rDNA gene, making discrimination difficult[2].

This study examines the potential use of Matrix Assisted Laser Desorption/Ionisation Time of Flight (MALDI-TOF) mass spectrometry as a rapid identification technique for this genus without the need for the more complex and time-consuming DNA extraction techniques[1,3,4,5].

METHOD

Bacterial Culture Conditions

MMU Database

- Bacterial strains supplied as freeze dried ampoules from the National Collection of Type Cultures (NCTC) used to produce the database.
- Ampoules were reconstituted in accordance with NCTC guidelines, 'Opening of Ampoules' followed generally by a 24 hour incubation period at 37°C on Columbia Blood agar (CBA, Oxoid, Basingstoke, UK) containing 5% (v/v) horse blood (TCS Microbiology, Botolph Claydon, Bucks. UK.), in an aerobic atmosphere.
- This process was followed by two further sub-cultures under the same conditions prior to MALDI-TOF MS analysis.
- A significant number of the database entries covering bacteria associated with urinary tract infections were also incubated for 16 hours at 35°C on Cysteine Lactose Electrolyte Deficient agar (CLED, Oxoid, Basingstoke, UK) in an aerobic atmosphere, followed by two further subcultures under the same conditions.
- A number of organisms required other culture conditions to ensure growth e.g. strict anaerobes were grown anaerobically, some required more than 24 hours incubation, temperatures other than 37°C or other media types.
- In general the anaerobes were cultured on CBA and Fastidious Anaerobic agar (FAA, IDG/LabM code Lab90, LabM Ltd.Topley House, 52 Walsh Road, Bury) with 5% (v/v) horse blood (TCS Microbiology, Botolph Claydon, Bucks, UK).
- All media was supplied as prepared plates by the Media Services Unit of the Public Health Laboratory Service accredited laboratories (Chester or London, UK).

HPA Test strains of *Bacillus* species

- The test strains of *Bacillus* species are listed in Table 1.
- These were prepared from ampoules as for the database in **parallel experiments** carried out by the Molecular Identification Services Unit (MISU) of the Health Protection Agency (HPA), London, UK, using:
  - Different Waters Micromass M@LDI-Linear instrument.
  - Different operator.
  - Different ampoule.
  - Same culture conditions.
- All the strains tested were cultured for 24 hours at 37°C on CBA in an aerobic atmosphere.
- The bacteria were inoculated onto MALDI target plates and co-crystallised with 5-chloro-2-mercaptobenzothiazole as detailed below.
- Followed by MALDI-TOF MS analysis.
- The data collected was searched against the MMU database.
- The 8 closest spectral matches were identified.

Preparation for MALDI-TOF MS analysis of NCTC database and test strains

- Bacterial colonies from the agar plate were applied to 12 MALDI target plate wells, using a 1µL culture loop (i.e. twelve wells per bacterial strain).
- Samples were air-dried for at least 1 hour.
- Overlayed with a 1µL aliquot of MALDI matrix solvent (Acetonitrile: Methanol: Water (1:1:1) with 0.1% (v/v) formic acid and 0.01M 18-crown-6-ether.) containing either 3 mg/mL of 5-chloro-2-mercapto-benzothiazole (Sigma-Aldrich Chemical Company) for gram-positive bacteria or 14mg/mL of α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemical Company) for gram-negative bacteria.
- Allowed to air dry before MALDI–TOF MS analysis.

Identification no.	Genus	Species	No in MMU DB	No HPA spectra tested
1	*Bacillus*	*atrophaeus*	1	1
2	*Bacillus*	*badius*	1	1
3	*Bacillus*	*cereus*	13	12
4	*Bacillus*	*circulans*	5	5
5	*Bacillus*	*coagulans*	5	5
6	*Bacillus*	*firmus*	1	1
7	*Bacillus*	*lentus*	1	1
8	*Bacillus*	*licheniformis*	17	16
9	*Bacillus*	*megatarium*	8	7
10	*Bacillus*	*mycoides*	5	1
11	*Bacillus*	*pumulis*	8	7
12	*Bacillus*	*sphaericus*	9	0
13	*Bacillus*	*subtilis*	11	9
			85	**66**

Table 1. Number of Bacillus species tested and number contained within the database.

MALDI–TOF analysis

Analysis was performed using a Waters Micromass M@LDI-Linear time of flight mass spectrometer (Waters Corporation, Manchester, UK) fitted with:

- A nitrogen laser producing a 337nm output of 3ns pulse width.
- A laser fluence set just above the threshold for ion production in the positive ion detection mode.
- An acceleration voltage of +15kV.
- Automatic and accurate indexing of the sample/reference wells.
- Automatic mass calibration using the average molecular weights from a (1:1) standard peptide mixture containing bradykinin, angiotensin I, glu-fibrinopeptide B, rennin substrate tetra decapeptide, ATCH (18-39 clip) all at 1pmol/µL, bovine insulin 2pmol/µL and ubiquitin 10pmol/µL with MALDI matrix solvent containing 14mg/ML of α-cyano-4-hydroxycinnamic acid.
- Automatic collection of bacterial mass fingerprints over the mass range from m/z 500 to 10,000 Da. together with spectra from reference wells for lock mass correction.

MMU Database construction

- MMU database of **3424** spectral entries:
  - Covering **158** different genera.
  - **508** different species.
  - **2349** different NCTC strains.
- Each database entry consisted of a representative average spectrum derived from combining up to 12 replicate spectra for each bacterial strain.
- Significant outliers were eliminated using a root mean square (RMS) rejection value of 3, Figure 1.

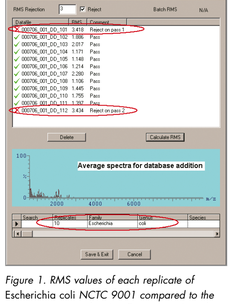

Figure 1. RMS values of each replicate of Escherichia coli *NCTC 9001 compared to the average spectra, showing 2 spectra rejected.*

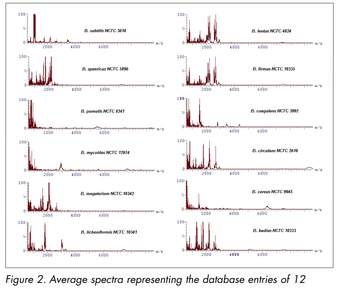

Figure 2. Average spectra representing the database entries of 12 species of the genus Bacillus.

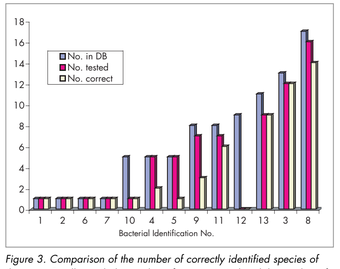

Figure 3. Comparison of the number of correctly identified species of the genus Bacillus *with the number of species tested and the number of species contained within the database.*

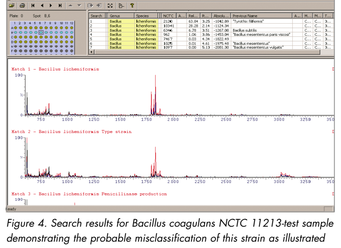

Figure 4. Search results for Bacillus coagulans NCTC 11213-test sample demonstrating the probable misclassification of this strain as illustrated by the 7 top matches.

RESULTS

- Figure 2 demonstrates the similarity between species of the *Bacillus* genus.
- The results of testing 66 of the 85 database strains of Bacillus by parallel data against a total database of ~3500 spectral entries are presented graphically in Figure 3 and demonstrate:
  - *B. atrophaeus, B. badius, B. firmus, B lentus, B. mycoides* and *B. subtilis* were all identified correctly to species.
  - All *B. cereus* strains tested were identified correctly to species with filtering for the correct culture conditions.
  - The majority of *B. licheniformis* were identified correctly to species. The remaining 2 correctly identified to genus.
  - 6 of the 7 *B. pumulis* samples tested were correctly identified.
  - Results for *B. circulans, B. coagulans* and *B. megatarium* were generally correct to genus with the correct species appearing among the top 5 matches.
    - For *B. coagulans* NCTC 11213 reclassification as *B. licheniformis* is suggested, Figure 4.
    - Noisy data obtained for *B. megatarium* NCTC 5637.
    - *Bacillus* coagulans NCTC 3993 & *Bacillus megaterium* NCTC 5637 demonstrated no top matches to the *Bacillus* genus.
- Typical results for *B. cereus, B. pumulis & B. licheniformis, B subtilis* are demonstrated for *B. licheniformis*, Figure 5 which show the majority of the top database matches suggesting the same species*.

  **B. subtilis* NCTC 10452 reclassified as *B. licheniformis*[1]

CONCLUSION

- 92% successful identification to genus.
- 82% successful identification to species (including NCTC 11213 & excluding NCTC 5637).
- 88% successful identification to species in top 5.
- Conclusive ID for *B. cereus, B. pumulis & B. licheniformis, B subtilis* was generally achieved.
- MALDI-TOF MS is an appropriate tool for the rapid & specific identification of pathogens linked with Homeland Defence.

Search	Genus	Species	NCTC
1	Bacillus	licheniformis	9932
2	Bacillus	subtilis	10452
3	Bacillus	licheniformis	1025
4	Bacillus	licheniformis	962
5	Bacillus	licheniformis	6346
6	Bacillus	licheniformis	2120
7	Bacillus	licheniformis	7589
8	Bacillus	licheniformis	10341

Figure 5. Typical search results for test samples of Bacillus licheniformis *demonstrate the confidence of the correct species match. These results also illustrate the misclassification of B. subtilis NCTC 10452 strain, confirmed by 16S rDNA seqence analysis as* B. licheniformis[1].

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