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RAPID IDENTIFICATION OF BACTERIA BY INTACT CELL MATRIX-ASSISTED LASER DESORPTION/IONISATION TIME OF FLIGHT MASS SPECTROMETRY

Diane Dare[1], Helen Sutton[1], Carrina Keys[2], Haroun Shah[2], Therese McKenna[3] and Leonhard Pollack[3]

[1]Manchester Metropolitan University, Manchester, UK. [2]Public Health Laboratory, London, UK. [3]Waters Corporation, Manchester, UK

Presented at ASM 2002, Salt Lake City, Utah, USA, 19th-23rd May, 2002

ABSTRACT

Preliminary work has demonstrated that MALDI-TOF-MS spectra from intact cells of a range of different bacteria produced by different operators at three different laboratories are highly reproducible, provided appropriate protocols are followed. Since these spectra are representative of surface components present on the bacterial cell, they can be used to generate a unique pattern of peaks for each bacterium; the mass spectra fingerprint pattern. This has enabled a database to be generated, which can be used for the rapid identification of these bacteria. The database consists of 703 entries, containing a variety of species of 76 different genera. The bacteria within the database are all strains of known provenance provided by the National Collection of Type Cultures, London.

In order to validate the database, the Medical Identification Service Unit of the Central Public Health Laboratory Service, Colindale, London, together with the Manchester Metropolitan University, produced parallel sets of spectra. One set was used to produce the database and the other set to challenge the database entries. The results of these with respect to the genera *Aeromonas, Bacillus, Burkholderia, Cedecea, Corynebacterium, Moraxella, Salmonella, Staphylococcus and Yersinia* are presented, a total of 65 strains. Accurate identification was obtained for genus (n=1); species (n=10); subspecies (n=12); and strain (n=36). These results demonstrate the enormous potential for rapid identification of bacteria.

INTRODUCTION

The accurate rapid identification of bacteria is necessary for a wide range of industries including food, water, pharmaceutical and diagnostic microbiology. Techniques to increase the speed of identification after primary isolation are continually being sought to improve treatment for the patient, approval of products, withdrawal of contaminated products etc. In the majority of laboratories the working taxonomic unit is the species, but occasionally more exacting information is required and time-consuming methods have to be undertaken to sub-type further.

Characterisation of bacteria using matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF-MS) is a newly described rapid identification method. This technique uses the surface ions generated from the soft ionisation of the intact bacterial cell to generate apparent reproducible fingerprint spectra within a few minutes [1,2,3,4]. There is sufficient data within the spectrum to identify the bacterial species and further studies have shown that for some bacterial species, the generated fingerprints are sufficiently discriminatory to allow simultaneous sub-typing [5,6].

A preliminary study [7] describes the robustness of the technique where a diverse selection of bacterial species were analysed on three instruments in three different centres over a period of three months. The generated spectra were shown to be consistently reproducible provided that the appropriate protocols were followed.

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As a result of this study a substantial database of over 700 bacterial samples prepared at Manchester Metropolitan University was validated by challenging against a parallel set of spectra produced by the Medical Identification Service Unit of the Central Public Health Laboratory Service, Colindale, London.

METHOD

Bacterial cultures

The bacterial species for the preparing the database, together with those used for validating the database were supplied from The National Collection of Type Cultures, London, UK (NCTC). A list of the genera and number of entries used for the database is given in **Table 1**. Those used to challenge the database are given in **Table 2**. Two freeze dried ampoules of each bacteria were used, one ampoule was reconstituted, cultured and analysed by the Medical Identification Service Unit (MISU) of the Central Public Health Laboratory (CPHL), London, UK, whilst the other was cultured and analysed by Manchester Metropolitan University (MMU), Manchester, UK. The majority of the bacteria were reconstituted and sub-cultured onto Columbia blood agar (Oxoid, Basingstoke UK) containing 5% (v/v) horse blood (TCS Microbiology, Botolph Claydon, Bucks. UK.), as supplied by the Public Health Laboratory Service accredited laboratories in London and Chester, and incubated for 24 at 37 °C in the appropriate atmosphere. They were sub-cultured twice prior to analysis by MALDI-TOF-MS.

Genus	No entries	Genus	No entries	Genus	No entries
Acinetobacter	3	*Fusobacterium*	3	*Photobacterium*	2
Actinobacillus	6	*Gordonia*	1	*Plesiomonas*	2
Actinomadura	1	*Haemophilus*	18	*Propionibacterium*	1
Aerococcus	1	*Hafnia*	3	*Proteus*	25
Aeromonas*	12	*Helicobacter*	2	*Providencia*	13
Alcaligenes	5	*Klebsiella*	30	*Pseudomonas*	22
Arcanobacterium	1	*Kocuria*	3	*Psychrobacter*	3
Bacillus*	17	*Kurthia*	2	*Rhodococcus*	3
Bacteroides	13	*Kytococcus*	1	*Rothia*	1
Bordetella	3	*Lactobacillus*	6	***Salmonella****	19
Brevibacillus	1	*Lactococcus*	1	*Serratia*	16
Brochothrix	1	*Legionella*	7	*Shewanella*	1
Burkholderia*	8	*Listeria*	17	*Shigella*	2
pylobacter	45	*Listonella*	2	*Sphingomonas*	1
Cardiobacterium	1	*Mannheimia*	4	***Staphylococcus****	51
Cedecea*	3	*Micrococcus*	1	*Stenotrophomonas*	3
Chromobacterium	1	*Moraxella (Branhamella)*	5	*Streptococcus*	60
Chryseobacterium	3	***Moraxella (Moraxella)****	8	*Streptomyces*	3
Citrobacter	14	*Morganella*	6	*Tatumella*	1
*Coryne **bacterium****	9	*Mycobacterium*	9	*Vibrio*	16
Edwardsiella	3	*Neisseria*	36	*Virgibacillus*	1
Eikenella	1	*Nocardia*	2	***Yersinia****	10
Enterobacter	20	*Oligella*	1	CMBT & Media control	1
Enterococcus	30	*Paenibacillus*	3	Standard pep mix control	1
Erysipelothrix	1	*Pantoea*	1	a-cyano & Media control	1
Escherichia	48	*Pasteurella*	16		
Fluoribacter	3	*Pediococcus*	2	Total	703

* Genera tested

Table 1. List of genera and number of entries in the database

* Omitting Corynebacterium cystitidis NCTC 11863, Corynebacterium pilosum NCTC 11862& Moraxella nonliquefaciens NCTC 10464 as invalid results & including Corynebacterium xerosis 11861 as correct after filtering results for correct growth conditions.

Genus	Species	Subspecies	Strain	NCTC No.	Correct genus	Correct species	Correct subspecies	Correct strain
Aeromonas	*hydrophila*	*hydrophila*	Type strain	8049	✓	✓	✓	✓
Aeromonas	*salmonicida*			10402	✓	✓	X	X
Aeromonas	*sobria*			11215	✓	✓	✓	✓
Bacillus	*badius*		Type strain	10333	✓	✓	✓	✓
Bacillus	*cereus*			10320	✓	✓	✓	✓
Bacillus	*cereus*		Serotype 1	11143	✓	✓	X	X
Bacillus	*cereus*		Type strain	2599	✓	✓	✓	✓
Bacillus	*cereus*			7464	✓	✓	X	X
Bacillus	*circulans*		Type strain	2610	✓	✓	✓	✓
Bacillus	*coagulans*		Type strain	10334	✓	✓	✓	✓
Bacillus	*firmus*		Type strain	10335	✓	✓	✓	✓
Bacillus	*lentus*		Type strain	4824	✓	✓	✓	✓
Bacillus	*licheniformis*		Type strain	10341	✓	✓	✓	✓
Bacillus	*megaterium*		Type strain	10342	✓	✓	✓	✓
Bacillus	*pumilus*		Type strain	10337	✓	✓	X	X
Bacillus	*sphaericus*		Type strain	10338	✓	✓	✓	✓
Bacillus	*subtilis*	*subtilis*	Type strain	3610	✓	✓	✓	✓
Burkholderia	*cepacia*			10661	✓	✓	✓	✓
Burkholderia	*cepacia*			10734	✓	✓	X	X
Burkholderia	*cepacia*			10743	✓	✓	X	X
Burkholderia	*cepacia*		Genomavar III	13008	✓	✓	X	X
Burkholderia	*cepacia*		Genomavar III	13009	✓	✓	✓	✓
Burkholderia	*cepacia*		Type strain, Genomavar III	13010	✓	✓	✓	✓
Burkholderia	*multivorans*		Type strain	13007	X	X	X	X
Burkholderia	*stabilis*		Type strain	13011	✓	✓	✓	✓
Cedecea	*davisae*			11465	✓	✓	✓	✓
Cedecea	*lapagei*			11466	✓	✓	✓	✓
Cedecea	*neteri*		Type strain	12120	X	X	X	X
Corynebacterium	*bovis*		Type strain	3224	✓	✓	✓	✓
Corynebacterium	*cystitidis*		Type strain	11863	X	X	X	X
Corynebacterium	*jeikeium*		Type strain	11913	✓	✓	✓	✓
Corynebacterium	*kutscheri*		Type strain	11138	✓	✓	✓	✓
Corynebacterium	*pilosum*		Type strain	11862	X	X	X	X
Corynebacterium	*pseudodiphtheriticum*		Type strain	11136	✓	✓	✓	✓
Corynebacterium	*ulcerans*			7907	✓	✓	✓	✓
Corynebacterium	*xerosis*		Type strain	11861	X	X	X	X
Moraxella (Moraxella)	*bovis*		Type strain	11013	✓	✓	✓	✓
Moraxella (Moraxella)	*lacunata*		Type strain	11011	✓	✓	✓	✓
Moraxella (Moraxella)	*nonliquefaciens*			10464	X	X	X	X
Moraxella (Moraxella)	*osloensis*		Type strain	10465	✓	✓	✓	✓
Moraxella (Moraxella)	*ovis*		Type strain	11227	✓	✓	✓	✓
Salmonella	*choleraesius*	*salamae*	Serotype Dar-es-salaam, 1,9,12:l,w:e,n,x	5773	✓	✓	X	X
"Salmonella"	*"enterica"*	*"diarizonae"*	6,7:1,v :z53 Ar 27:23:25, *Salmonella* Subsp.IIIb	12417	✓	✓	✓	✓
Salmonella	*choleraesius*	*houtenae*	Type strain, 45:g,z51:-. *Salmonella* Subsp. IV	12418	✓	✓	✓	✓
"Salmonella"	*"enterica"*	*"enterica"*	Anaerogenic, Serotype Pullorum, 9,12:-:-	5776	✓	✓	✓	✓
Staphylococcus	*aureus*	*aureus*	Methicillin resistant (MRSA)	10442	✓	✓	✓	X
Staphylococcus	*aureus*	*aureus*	Disinfectant test strain	10788	✓	✓	✓	X
Staphylococcus	*aureus*	*aureus*	Beta lactamase producing control strain	11561	✓	✓	✓	X
Staphylococcus	*aureus*	*aureus*	Methicillin resistant control (MRSA)	12493	✓	✓	✓	✓
Staphylococcus	*aureus*	*aureus*		12973	✓	✓	✓	X
Staphylococcus	*aureus*	*aureus*		12981	✓	✓	✓	X
Staphylococcus	*aureus*	*aureus*	EMRSA-03	13130	✓	✓	✓	X
Staphylococcus	*aureus*	*aureus*	EMRSA-04	13131	✓	✓	✓	✓
Staphylococcus	*aureus*	*aureus*	EMRSA-05	13132	✓	✓	✓	X
Staphylococcus	*aureus*	*aureus*	EMRSA-06	13133	✓	✓	✓	X
Staphylococcus	*aureus*	*aureus*	EMRSA-07	13134	✓	✓	✓	X
Staphylococcus	*aureus*	*aureus*	EMRSA-08	13135	✓	✓	✓	X
Staphylococcus	*aureus*	*aureus*	EMRSA-09	13136	✓	✓	✓	✓
Staphylococcus	*aureus*	*aureus*		1803	✓	✓	✓	X
Staphylococcus	*aureus*	*aureus*	Disinfectant test strain	4163	✓	✓	✓	X
Staphylococcus	*aureus*	*aureus*	Heatley Oxford	6571	✓	X	X	X
Staphylococcus	*aureus*	*aureus*	Type strain, serovar 3, Serotype 3	8532	✓	✓	X	X
Yersinia	*enterocolitica*	*enterocolitica*	Bitotype 3, Serotype 1(2a,3)	10460	✓	✓	X	X
Yersinia	*intermedia*		Type strain	11469	✓	✓	✓	✓
Yersinia	*kristensenii*		Type strain	11471	✓	✓	✓	✓
Total				65	59	58	48	36
%					91%	89%	74%	55%
Corrected total*				62	60	59	49	37
Corrected %					97%	95%	79%	60%

Table 2. Results of validating the database with parallel sets of data produced by the CPHL

Preparation of the bacteria for analysis by MALDI-TOF MS

Colonies of bacteria were removed from the media plate by sweeping a sterile loop across the plate. These are applied directly on to the sample targets and allowed to dry for a least one hour. Twelve target wells were inoculated for each strain. A 1µL aliquot of either α-cyano-4-hydroxycinnamic acid (αCHCA) (Sigma-Aldrich), for Gram-negative bacteria, or 5-Chloro-2-mercaptobenzothiazole (CMBT) (Sigma-Aldrich) for Gram-positive bacteria was applied to each target well and allowed to dry for at least one hour prior to mass spectrometric analysis. The UV-absorbing matrix solutions used (αCHCA and CMBT) were freshly prepared as saturated solutions in water, methanol, and acetonitrile, 1:1:1 containing 0.1% formic acid and 0.01M 18-crown-6 ether.

Instrument operation

The MALDI-TOF-MS analysis was performed using a M@LDI-Linear time of flight mass spectrometer (Micromass UK. Ltd., Manchester, UK.). A nitrogen laser giving a 337nm output of 3ns pulse width was used. The laser fluence was set just above the threshold for ion production. The mass spectrometer was used in the positive ion detection mode using an acceleration voltage of +15kV. On loading each target plate, automatic, accurate indexing of the sample/reference wells was performed, followed by calibration of the m/z range of the instrument, using the 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).

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The acquisition mass range was from m/z 500 to 10000 Da. For maximum throughput of samples the bacterial mass fingerprints were acquired automatically using the MAXspec real-time data selection algorithm to optimise the bacterial fingerprint signal in the mass range 600 to 3000 Da. Spectra from the reference wells, for lock mass calibration, were also acquired automatically.

MicrobeLynx™ data analysis

The twelve replicate spectra produced at Manchester Metropolitan University were compared for reproducibility using the root mean square (RMS) value, **Figure 1**. This value is obtained by comparing each replicate in turn with the average of the other 11 replicates. An RMS rejection value of 3 was used to identify outliers significant at the 0.1% level. The acceptable spectra were then combined to give a representative spectral pattern for each organism, **Figure 2**, from which a database of over 700 bacterial samples was prepared. Each bacterial reference strain was generally represented in the database by twelve mass spectral fingerprints from the Manchester Metropolitan University instrument.

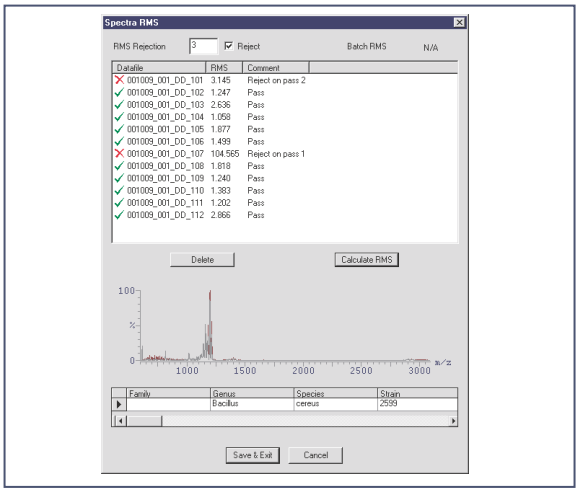

Figure 1. Comparison of the RMS values for 12 replicate spectra of Bacillus cereus NCTC 2599

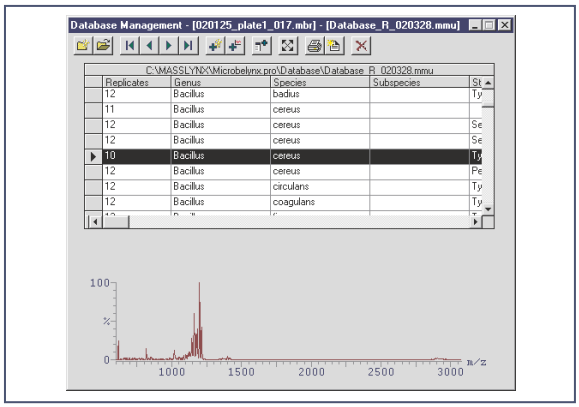

Figure 2.

Parallel sets of twelve replicate spectra were prepared for a selection of the bacteria at the Medical Identification Service Unit of the Central Public Health Laboratory. These twelve spectra were combined to produce an average spectrum, which was used to challenge the Manchester Metropolitan University database using a pattern recognition algorithm. This algorithm uses all the mass and intensity data in the mass spectrum to give the best database match with a probability score. A comparative display of the test spectrum and the differences from the best database match is produced in a browser format, **Figure 3**.

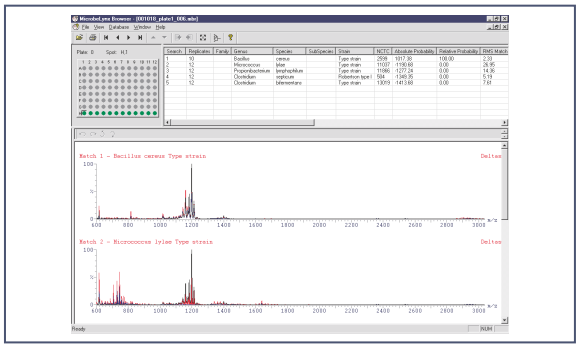

Figure 3. Browser results for CPHLS sample of Bacillus cereus 2599 against the 703 MMU database entries

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RESULTS

Table 2 shows the results of validating the database with parallel sets of data produced by the CPHL for the samples tested. The results of the best match from the database are compared with the identity of the sample tested at genus, species, subspecies and strain level.

The results show the correct matches at genus (n=1); species (n=10); subspecies (n=12); and strain level (n=36) for the 65 different strains against a database of 703 entries. Giving an overall percentage match of 91%, 89%, 74% and 55% respectively.

Details of the 6 strains, which failed to match, are given in **Table 3**. The correct match to strain level was obtained 2nd for two samples. For one of these samples, the top match could be discarded on the grounds of incorrect growth conditions. A third sample gave the correct match 4th and two further samples gave all 5 matches with incorrect growth conditions. These results were therefore considered invalid. Only one sample was considered to have produced a poor result and upon inspection of the 2 parallel spectra, showed them to be significantly different, requiring further investigation. This sample was also considered invalid.

If these observations are taken into account, the results can be corrected to genus (n=1); species (n=10); subspecies (n=12); and strain level (n=37) and the number of valid samples reduced to 62. The overall percentage matches then become 97%, 95%, 79% and 60% respectively.

Genus	Species	Subspecies	Strain	NCTC No.	Comment
Burkholderia	*multivorans*		Type strain	13007	2nd match correct to strain level.
Cedecea	*neteri*		Type strain	12120	4th match correct to strain level.
Corynebacterium	*cystilidis*		Type strain	11863	Parallel spectra very similar, top 5 matches incorrect growth conditions.
Corynebacterium	*pilosum*		Type strain	11862	Parallel spectra very similar, top 5 matches incorrect growth conditions.
Corynebacterium	*xerosis*		Type strain	11861	2nd match to strain level. Only 2nd match had correct growth conditions.
Moraxella (Moraxella)	*nonliquefaciens*			10464	Parallel spectra significantly different.

Table 3. Results of samples failing to match at genus level

CONCLUSION

- MALDI-TOF-MS analysis of intact bacteria cells provides a new rapid method of identification in minutes for a number of different genera.
- Identification can be achieved against a substantial database of 703 quality-controlled spectra.
- Identification to genus (n=1); species (n=10); subspecies (n=12); and strain level (n=36) for 65 different species has been achieved. An overall correct percentage match of 91%, 89%, 74% and 55% respectively.
- The identification can be improved to genus (n=1); species (n=10); subspecies (n=12); and strain level (n=37) by correcting the number of samples to 62 and filtering the results for appropriate growth conditions. An overall correct percentage match of 97%, 95%, 79% and 60% respectively.
- MALDI-TOF-MS analysis of intact bacteria cells has the potential to revolutionize bacterial identification.

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Author to whom all correspondence should be addressed:
Thérèse McKenna
Waters Corporation
(Micromass UK Limited)
Floats Road, Wythenshawe
Manchester, M23 9LZ
Tel: + 44 (0) 161 946 2400
Fax: + 44 (0) 161 946 2480
e-mail: therese.mckenna@micromass.co.uk

WATERS CORPORATION
34 Maple St.
Milford, MA 01757 U.S.A.
T: 508 478 2000
F: 508 872 1990
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