Lipid fingerprinting of *Bacillus* spp. using online MALDI-TOF mass spectrometry†

Xi Shu, Miao Liang, Bo Yang, Yueyan Li, Changgeng Liu, Youfeng Wang and Jinian Shu*

Received 5th June 2012, Accepted 9th September 2012

DOI: 10.1039/c2ay25579k

Rapid identification of *Bacillus* spores has attracted great interest in the medical and forensic communities due to the highly pathogenic nature of certain *Bacillus* species and its potential application in bioweapons. In this study, we evaluated the feasibility of rapid and reliable identification of several *Bacillus* spp. (*B. subtilis*, *B. cereus*, *B. megaterium*, *B. pumilus*, and *B. sphaericus*) using online MALDI-TOF mass spectrometry combined with lipid profiling. The bacterial cultures in liquid medium were extracted with methanol and chloroform, and the extracts were aerosolized into droplets and immediately analyzed with an online MALDI-TOF mass spectrometer. Results showed that phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diglycosyldiacylglycerol (DGDG) were present in the mass spectra, as alkali metal adducts. The unique lipid profiles obtained from these *Bacillus* spp. allowed us to differentiate between them. The reliability and reproducibility of the rapid identification method for *Bacillus* were tested under different experimental conditions. An alternative online chemotaxonomic method for bacterial identification is demonstrated.

Introduction

Bacillus spp. are ubiquitous in natural environments such as fresh and saline water, soils, plants, animals and air. There are currently 65 valid *Bacillus* spp. with new species continually being described.¹ Known *Bacillus* spp. include industrial, pathogenic and non-pathogenic bacteria. For example, *B. anthracis* is a causative agent of anthrax. *B. cereus* is occasionally associated with food poisoning. *B. subtilis* and *B. pumilus* are capable of producing γ -polyglutamic acid (PGA), and have been used in the processing of fermented soybean foods.² It is important to establish a rapid and simple method for identification of *Bacillus* spp. to assist in diagnosing diseases, anticipating public health hazards, recognizing warfare threats, and for monitoring possible contamination in stored foods and grains.

A variety of traditional methods, such as nucleic acid hybridization,³ polymerase chain reaction (PCR),⁴ enzyme-linked

immunosorbent assays (ELISA),^{5,6} immunofluorescence assays (IFAs),⁷ dielectrophoretic impedance analysis,⁸ and several other methods,⁹ have been used for collecting, isolating and identifying pathogens from different specimens and samples. Such traditional methods are able to reliably differentiate bacteria at the species and sub-species levels, but their usefulness is hindered by the prolonged time (hours to days) and their expensive cost.¹⁰ Thus, a rapid detection method to detect airborne environmental bacterial particles could mitigate the spread of disease.

Matrix assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a straightforward method for rapid identification and taxonomy of microorganisms.^{11–16} In several recent studies, MALDI-TOF MS has been shown capable of identifying >84% of bacterial species and >95% of bacterial genera in large sets of clinical isolates, with overall analysis costs reduced to 22–32% of that required by conventional bacterial identification methods.^{14,17,18} Previously, the identification of *Bacillus* spp. using MALDI-TOF MS was mainly based on a comparison of mass spectra of proteins,^{19–26} lipopeptides,^{27–29} and peptides.^{30,31} The MALDI-TOF MS method relies on reproducible detection of microbial protein patterns, thus providing complementary information to genotypic or phenotypic test methods.

Lipids are important cellular components,³² which feature variable composition in cell membranes of different bacteria and provide important information for differentiation of bacterial species.³³ MALDI-TOF MS has also been applied to obtain abundant, accurate mass ion signals of the phospholipids in bacteria.^{34–39} Therefore, the characterization of lipids in bacteria and other microorganisms plays a crucial role for taxonomic identification, differentiation of pathogenic species, and understanding of metabolic processes under normal or stressed growth conditions. However, there are few reports on the application of online MALDI-TOF MS coupling with lipid profiling for identification of bacterial species.

In this study, an online MALDI-TOF mass spectrometer was used to differentiate *Bacillus* spp. based on lipid profiling. Five strains of *Bacillus* spp. (*B. subtilis*, *B. cereus*, *B. megaterium*, *B. pumilus*, and *B. sphaericus*) were selected to verify the feasibility of the combined MALDI-TOF MS lipid profiling method. The influence of experimental conditions and reproducibility were examined. MALDI-TOF mass spectra obtained by online MALDI-TOF MS were compared to those obtained using MALDI-TOF MS (Autoflex III, Bruker Daltonics, Germany).

Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. E-mail: jshu@rcees.ac.cn; Fax: +86 010 6292 3563; Tel: +86 010 6284 9508

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2ay25579k

Materials and methods

Chemicals and microbial strains

2,4-Dihydroxybenzoic acid (2,4-DHB), 2,5-dihydroxybenzoic acid (2,5-DHB), 2',4',6'-trihydroxyacetophenone monohydrate (THAP), Sinapic acid (SA), and 2-(4'-hydroxybenzeneazo)benzoic acid (HABA) were obtained from Sigma (Beijing, China); α -cyano-4-hydroxycinnamic acid (CHCA) was purchased from ACROS Organics (Beijing, China); methanol, chloroform, ethanol, acetonitrile, isopropanol, sodium chloride (NaCl), potassium chloride (KCl), peptone, and beef extract were purchased from Beijing Shiji (Beijing, China); deionized water was supplied by the RCEES Supporting Laboratory. All chemicals were of analytical grade.

The strains of *B. subtilis* (CGMCCC1.1389), *B. cereus* (CGMCCC1.0126), *B. megaterium* (CGMCCC1.0151), *B. pumilus* (CGMCCC1.0271), and *B. sphaericus* (CGMCCC1.1371) were obtained from the China General Microbiological Culture Collection Center (CGMCC). After resuscitation, the strains were grown aerobically on a liquid nutrient medium at 37 °C for 24 h. The liquid nutrient medium was prepared by dissolving 3 g of beef extract, 5 g of NaCl, and 10 g of peptone in 1 L of deionized water (final pH 7.0).

Sample preparation

After incubation, the bacterial cells were harvested by centrifugation at 9000 rpm for 3 min at room temperature. The cell pellet was washed and centrifuged three times with deionized water for desalting, re-suspended in 3 mL of water, and inactivated by adding 7 mL of ethanol. The samples were stored at 4 °C prior to further analysis. Preliminary results indicated that the extracts of the bacterial cells were stable at 4 °C for several weeks. The number of cells per volume was counted under a light microscope. Unless otherwise specified, the corresponding cell density in the final extract was approximately 1.4×10^7 cells per mL.

Lipids from the bacterial pellets were extracted with chloroform/methanol (2 mL/1 mL) via 5 min ultrasonication at room temperature. The solid materials in the mixtures were removed by centrifugation at 9000 rpm for 3 min. A 0.5 mL aliquot of the extract was added to 4.5 mL of matrix solution (20 mM) for MALDI analysis. The matrixes (2,4-DHB, 2,5-DHB, THAP, SA, HABA, and CHCA) were dissolved in the mixed solution (acetonitrile–water–isopropanol, 1 : 1 : 2 v/v). Then, the analyte/matrix solution was pneumatically nebulized into suspended droplets and immediately analyzed by online MALDI-TOF MS. Liquid consumption of nebulization was set at 0.2 mL min⁻¹.

MALDI-TOF MS analysis

The detailed design of the laboratory-built online MALDI-TOF MS and the experimental procedures are described elsewhere.^{40,41} Briefly, suspended droplets were introduced into the instrument by an aerodynamic lens assembly tilted at 40° from the horizontal plane. The droplets were deposited continuously on a tungsten target plate which also served as an ion repeller for the reflectron mass spectrometer. The deposition spot was ca. 0.5 mm in diameter. A 10 Hz pulsed 266 nm laser (frequency quadrupling of the Nd:YAG laser output, New Wave Research, Polaris III) was applied to desorb and ionize the sample with an incident angle of 50°. The laser energy was 0.45–1.1 mJ per pulse. The ions generated by the MALDI process

were detected with a reflectron mass spectrometer. Each mass spectrum was acquired by averaging the ions generated from 128 laser shots. The mass spectra were calibrated using the ions of α -cyclodextrin, β -cyclodextrin, and polyethylene glycol (PEG) 1000 cationized by Na⁺. The calibration errors of mass spectra were within ± 0.3 Da. 2,4-DHB was used as the matrix as it is presently the most characterized MALDI matrix in the lipid field. It has a high absorption efficiency at 266 nm.⁴² Each sample was analyzed at least in triplicate.

The bacterial extracts were also analyzed using Autoflex III (Bruker Daltonics, Germany) MALDI-TOF MS in the positive ionization reflection mode for lipid analysis. Dried-droplet samples were prepared by spotting 1 μ L of the mixture on the AnchorChip™ target plate. A 337 nm nitrogen laser with a frequency of 100 Hz was used, and the laser power was adjusted to 60–80% to obtain the best spectra. Each mass spectrum was typically summed with 500 laser shots. External calibration of MALDI-TOF mass spectra was carried out using a mixture of polypropylene glycol. The mass spectra were analyzed by Flex Analysis software provided by Bruker Daltonics Corp, Germany.

Results and discussion

Cationization of bacterial lipids

The formation of alkali metal ion adducts could not completely be excluded in the positive ion mode mass analysis of bacterial cell lipids. In order to assist the assignment of the mass spectra obtained, we conducted MALDI analyses on the extracts doped with alkali salts. Results showed that the MALDI-TOF mass spectra of *B. megaterium* substantially changed after the extracts were doped with salts (Fig. 1). The mass spectra of *B. megaterium* were mainly composed of two bunches of mass peaks located around m/z 755 and 916, respectively. After doping with alkali salts, several homologous series of mass peaks with a space of 14 Da (Series II to VII) appeared (Fig. 1B and C). The mass peaks of Series V to VII (Fig. 1C) had a shift of 32 Da to Series II to IV (Fig. 1B) respectively. Therefore, the mass peaks of Series II to IV (Fig. 1B) and Series V to VII (Fig. 1C) were likely from the di-sodium adduct species and di-potassium adduct species, respectively. It is reported that

Fig. 1 MALDI-TOF mass spectra of *B. megaterium* lipids from raw extracts (A), and those doped with 10 mM NaCl (B) and KCl (C).

phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are the major phospholipids in *B. megaterium*.⁴³ Based on the LIPID MAPS database, mass peaks at m/z 694.3, 708.4, and 722.4 peaks of Series II (Fig. 1B) were assigned to PE(29:0) + 2Na–H, PE(30:0) + 2Na–H, and PE(31:0) + 2Na–H, respectively. The space between these mass peaks (14 Da) corresponds to a methylene group. By analogy, the mass peaks of Series III m/z 739.6, 753.6, and 767.6 are assigned to PG(30:0) + 2Na–H, PG(31:0) + 2Na–H, and PG(32:0) + 2Na–H, respectively, and those of Series IV (Fig. 1B) are assigned to PE(43:2) + 2Na–H, PE(44:2) + 2Na–H, PE(45:2) + 2Na–H, and PE(46:2) + 2Na–H, respectively. The mass peaks at m/z 724.5, 755.6, 769.5, 916.5, 930.5, and 944.5 (Fig. 1A) are suppressed and disappear (Fig. 1B and C). However, several new mass peaks with 16 Da shift to these peaks were observed (Fig. 1B and C) suggesting that the suppressed mass peaks were sodium–potassium adducts.

Similar experiments were conducted with the other bacteria (Fig. S1–S4†). The mass spectra obtained indicated that there was sufficient sodium and potassium ions in the bacterial samples to facilitate ionization. Nonetheless, doping added salt simplified the mass spectrum by suppressing other cationic adducts and helped to ascertain the form of salt adducts. Our data showed that the addition of salt to the raw extracts significantly influenced the mass spectra. However, the subtotal intensities of each phospholipid class barely changed. In all mass spectra, the mass peaks of lipids were consistently in the form of alkali metal adducts. The binding mode of adducts depended on salt concentration in the extract and intrinsic property of the lipid. The phospholipids such as PE and PG preferred to bind two cations, while glycolipids such as DGDG tended to couple with one cation.

Discrimination of the genus *Bacillus* spp.

Based on previous studies,^{29,44–47} the lipid database, and salt doping experiments mentioned above, typical MALDI-TOF mass spectra of lipids extracted from *B. subtilis*, *B. cereus*, *B. megaterium*, *B. pumilus*, and *B. sphaericus* (Fig. 2) were assigned (Table 1). Values for the mass peaks represent the relative contribution (%) to the overall lipid profile. The mean values and standard deviation were calculated from the mass spectra obtained in triplicate experiments.

Fig. 2 The representative MALDI-TOF mass spectra of five different *Bacillus* species obtained by online MALDI-TOF MS: *B. subtilis* (A), *B. sphaericus* (B), *B. pumilus* (C), *B. megaterium* (D), and *B. cereus* (E).

The mass peaks of the *B. cereus* mass spectrum (Fig. 2A) mainly occur between m/z 650 and 800, contributed from PGs and PEs with 28–32 carbons in the two acyl groups. The mass spectrum of *B. megaterium* (Fig. 2B) shares the mass peaks of PGs and PEs with 30–32 carbons with *B. cereus*. However, *B. megaterium* possesses the mass peaks of PEs with 43–46 carbons while lacking the PEs with less than 30 carbons found in *B. cereus*. In addition, the total relative intensity of PGs in *B. megaterium* is approximately twice that of *B. cereus* PGs. Similarly, the mass spectrum of *B. pumilus* (Fig. 2C) contained PGs with 30–32 carbons (Table 1). However, PEs are absent in the mass spectrum, and total relative intensity of these PGs is over four times that of *B. cereus*. *B. pumilus* showed bunched mass peaks around m/z 900, suggesting the presence of DGDGs rather than the PEs observed in *B. megaterium*. The mass spectrum of *B. sphaericus* (Fig. 2D) is characterized by two dominating mass peaks at m/z 724.4 (26.2%) and 740.3 (50.8%). *B. subtilis* (Fig. 2E) shares the same DGDGs with *B. pumilus*, but the relative intensity is significantly different. Additionally, *B. subtilis* has two strong unidentified mass peaks at m/z 1058.7 and 1107.6, both of which have similar adducting behaviors to other lipids (Fig. S4†). Together these results indicate that each *Bacillus* species has a characteristic lipid profile and that these five *Bacillus* species can be discriminated by their lipid profiles.

Similar experiments were also performed with Autoflex III (Bruker Daltonics, Germany) MALDI-TOF MS. Representative MALDI-TOF mass spectra of five bacterial extracts obtained with Autoflex III MALDI-TOF MS are shown in Fig. S5†. The mass spectra shown in Fig. S5† represent the overall profiles similar to those shown in Fig. 2. However, there are small differences between the mass spectra obtained by the two different MALDI-TOF MS. Fig. 3 shows the detailed mass spectra of *B. cereus* lipid extracts obtained with Autoflex III MALDI-TOF MS (A) and online MALDI-TOF MS (B), respectively. The mass spectrum obtained with online MALDI-TOF MS is dominated by sodium adducts while those obtained with Autoflex III MALDI-TOF MS consists of more potassium adducts. Similar phenomena are also observed in the other bacterial mass spectra. The discrepancy may be caused by the different laser wavelength (266 nm to 337 nm) or sample preparation.

Effects of experimental conditions on lipid profiling

It is known that the sample preparation technique strongly affects the reproducibility and informativeness of spectrum obtained by MALDI-TOF MS.⁴⁸ In this study, the sample preparation method differed from traditional MALDI and the aerosol MALDI. The droplets were deposited continuously on a stationary place and the accumulated particles were ionized *in situ* by laser. In addition, the laser wavelength of 266 nm rather than 337 nm or 355 nm was used. The effects of matrix species, matrix to analyte ratio, and analyte concentration on the mass spectra were also assessed.

The influences of the matrices were examined in the experiment. Fig. 4 shows the MALDI-TOF mass spectra of *B. cereus* lipid extracts added with 20 mM 2,4-DHB (A), 2,5-DHB (B), SA (C), THAP (D), CHCA (E), and HABA (F), respectively. The results indicated that the different matrix had no significant influence on the lipid profiles except for relative intensity. The mass spectra obtained with 2,5-DHB, and CHCA exhibited a lower signal to noise ratio and signal intensity. This may be caused by the lower absorption coefficient of the matrices at 266 nm laser wavelength. HABA gave rise to

Table 1 The probable assignments for MALDI-TOF mass spectra of *B. subtilis*, *B. cereus*, *B. megaterium*, *B. pumilus*, and *B. sphaericus*. Relative peak intensities and standard deviations are listed. The relative peak intensities are the percentages to the total lipid intensity detected in each mass spectrum^a

<i>m/z</i>	<i>B. cereus</i>		<i>B. megaterium</i>		<i>B. pumilus</i>		<i>B. sphaericus</i>		<i>B. subtilis</i>	
	Assignment	RI ($\pm$ SD)	Assignment	RI ($\pm$ SD)	Assignment	RI ($\pm$ SD)	Assignment	RI ($\pm$ SD)	Assignment	RI ($\pm$ SD)
672.2	PE(29:0) + Na	1.6 $\pm$ 0.7								
680.2	PE(28:0) + 2Na-H	4.5 $\pm$ 0.8								
686.2	PE(30:0) + Na	3.2 $\pm$ 1.8								
692.2	PE(29:1) + 2Na-H	2.3 $\pm$ 0.2								
694.2	PE(29:0) + 2Na-H	8.0 $\pm$ 1.2								
696.2	PE(28:0) + Na + K-H	2.1 $\pm$ 0.2								
706.2	PE(30:1) + 2Na-H	1.6 $\pm$ 0.1								
708.2	PE(30:0) + 2Na-H	16.8 $\pm$ 2.5	PE(30:0) + 2Na-H	6.2 $\pm$ 1.5			PE(30:0) + 2Na-H	1.7 $\pm$ 0.6		
710.2	PE(29:0) + Na + K-H	3.1 $\pm$ 0.5								
720.2	PE(31:1) + 2Na-H	3.7 $\pm$ 0.4								
722.2	PE(31:0) + 2Na-H	7.5 $\pm$ 0.5	PE(31:0) + 2Na-H	1.3 $\pm$ 0.4						
724.2	PE(30:0) + Na + K-H	8.5 $\pm$ 1.2	PE(30:0) + Na + K-H	7.8 $\pm$ 0.9			PE(30:0) + Na + K-H	26.2 $\pm$ 4.3		
725.2			PG(29:0) + 2Na-H	2.1 $\pm$ 0.1						
726.2							PE(29:0) + 2K-H	3.4 $\pm$ 1.5		
734.3	PE(32:1) + 2Na-H	1.1 $\pm$ 0.4								
736.3	PE(32:0) + 2Na-H	10.7 $\pm$ 0.6	PE(32:0) + 2Na-H	1.2 $\pm$ 0.3						
738.3	PE(31:0) + Na + K-H	2.7 $\pm$ 0.6	PE(31:0) + Na + K-H	1.5 $\pm$ 0.1			PE(31:0) + Na + K-H	1.1 $\pm$ 0.7		
739.3	PG(30:0) + 2Na-H	3.1 $\pm$ 0.6	PG(30:0) + 2Na-H	11.0 $\pm$ 2.5						
740.3			PE(30:0) + 2K-H	4.1 $\pm$ 0.1			PE(30:0) + 2K-H	50.8 $\pm$ 4.9		
752.3	PE(32:0) + Na + K-H	7.4 $\pm$ 0.8	PE(32:0) + Na + K-H	0.9 $\pm$ 0.2			PE(32:0) + Na + K-H	3.0 $\pm$ 0.3		
753.3	PG(31:0) + 2Na-H	4.9 $\pm$ 0.4	PG(31:0) + 2Na-H	1.4 $\pm$ 0.2						
754.3							PE(31:0) + 2K-H	2.3 $\pm$ 0.8		
755.3	PG(30:0) + Na + K-H	1.6 $\pm$ 0.5	PG(30:0) + Na + K-H	14.6 $\pm$ 0.7	PG(30:0) + Na + K-H	30.6 $\pm$ 1.1	PG(30:0) + Na + K-H	3.6 $\pm$ 0.9		
767.3	PG(32:0) + 2Na-H	3.0 $\pm$ 0.6	PG(32:0) + 2Na-H	0.7 $\pm$ 0.2						
769.3	PG(31:0) + Na + K-H	1.2 $\pm$ 0.5	PG(31:0) + Na + K-H	1.0 $\pm$ 0.4	PG(31:0) + Na + K-H	4.8 $\pm$ 0.3				
771.3			PG(30:0) + 2K-H	1.8 $\pm$ 0.6	PG(30:0) + 2K-H	25.9 $\pm$ 0.6	PG(30:0) + 2K-H	7.9 $\pm$ 2.0		
783.3	PG(32:0) + Na + K-H	1.7 $\pm$ 0.5	PG(32:0) + Na + K-H	0.8 $\pm$ 0.3	PG(32:0) + Na + K-H	7.6 $\pm$ 0.3				
785.3					PG(31:0) + 2K-H	2.9 $\pm$ 0.4				
799.3					PG(32:0) + 2K-H	4.6 $\pm$ 0.2				
886.3			PE(43:2) + 2Na-H	1.0 $\pm$ 0.4						
887.4					DGDG(30:0) + Na	8.5 $\pm$ 0.2			DGDG(30:0) + Na	15.7 $\pm$ 1.5
900.3			PE(44:2) + 2Na-H	14.7 $\pm$ 0.7						
901.4					DGDG(31:0) + Na	1.9 $\pm$ 0.2			DGDG(31:0) + Na	15.6 $\pm$ 3.1
903.4					DGDG(30:0) + K	6.8 $\pm$ 0.9				

Table 1 (Contd.)

<i>m/z</i>	<i>B. cereus</i>		<i>B. megaterium</i>		<i>B. pumilus</i>		<i>B. sphaericus</i>		<i>B. subtilis</i>	
	Assignment	RI ($\pm$ SD)	Assignment	RI ($\pm$ SD)	Assignment	RI ($\pm$ SD)	Assignment	RI ($\pm$ SD)	Assignment	RI ($\pm$ SD)
914.3			PE(45:2) + 2Na-H	3.3 $\pm$ 0.5						
915.4					DGDG(32:0) + Na	2.8 $\pm$ 0.3			DGDG(32:0) + Na	64.1 $\pm$ 4.4
916.3			PE(44:2) + Na + K-H	16.5 $\pm$ 1.3						
917.4					DGDG(31:0) + K	1.4 $\pm$ 0.4				
928.3			PE(46:2) + 2Na-H	1.3 $\pm$ 0.3						
930.3			PE(45:2) + Na + K-H	3.7 $\pm$ 1.0						
931.4					DGDG(32:0) + K	2.1 $\pm$ 0.2			DGDG(32:0) + K	4.7 $\pm$ 1.9
932.3			PE(44:2) + 2K-H	1.3 $\pm$ 0.6						
944.3			PE(46:2) + Na + K-H	1.6 $\pm$ 0.6						

^a PE indicates phosphatidylethanolamine, PG phosphatidylglycerol, and DGDG diglycosyldiacylglycerol. Numbers in parenthesis indicate the number of carbon atoms and double bonds in the fatty acid side chains, respectively. RI and SD represent the relative intensity and the standard deviation, respectively. RI and SD are calculated from the peak intensities of the mass spectra obtained in triplicate experiments.

Fig. 3 Mass spectra of *B. cereus* lipid extracts obtained with Autoflex III MALDI-TOF MS (A) and online MALDI-TOF MS (B), respectively.

Fig. 4 MALDI-TOF mass spectra of *B. cereus* lipid extracts added with 20 mM 2,4-DHB (A), 2,5-DHB (B), SA (C), THAP (D), CHCA (E), and HABA (F), respectively.

more matrix cluster ions with mass peaks over 900 *m/z*.⁴⁹ These cluster ions may overlap the mass peaks of lipids. Hence, the HABA was not suited to ionize the lipid either. A similar phenomenon of matrix effect was observed in the other bacterial mass spectra (Fig. S6†). Therefore, 2,4-DHB, SA, and THAP are better matrices to use for lipid analysis at 266 nm laser wavelength and in positive mode.

The ratio of matrix to analyte had a significant impact on the lipid profiles (Fig. 5). Fig. 5A was dominated by sodium and di-sodium adducts, while in Fig. 5B and C, the minor shoulder peaks contribution from unsaturated lipid adducts (Series IV) and sodium-potassium adducts (Series V) were increased. In addition, the mass peaks of the matrix clusters observed in the mass range of 560–650 Da were substantially higher in Fig. 5A than those in Fig. 5B and C. These DHB matrix clusters were similar to those reported in a

previous study.⁵⁰ Fig. 5C shows that the lipid profile was poor when the matrix concentration was <2 mM. These results indicated that the matrix concentration is critical for lipid profiling. The optimal matrix concentration is *ca.* 10 mM.

The MALDI-TOF mass spectra of lipids extracted from *B. cereus* cells (Fig. 6) showed that the analyte concentration did not significantly influence the lipid profile. With the increase in cell density, the single sodium PE adducts (Series I) disappeared while the sodium-potassium PE adducts (Series III) increased. In addition, the PG adducts (Series IV) almost disappeared in Fig. 6C. These changes were likely resulted from the increase in cell-associated potassium ions in the extracts. A higher analyte concentration was not tested due to potential contamination of the MALDI-TOF MS system and interference with next sample detection. Fig. 6C shows that the increased analyte concentration suppressed the matrix clusters.

Fig. 5 MALDI-TOF mass spectra of *B. cereus* lipid extracts added with 50 mM (A), 10 mM (B), and 2 mM (C) DHB. Cell density was 1.4×10^7 cells per mL.

Fig. 6 MALDI-TOF mass spectra of lipids extracted from *B. cereus* cells of 2.8×10^6 cells per mL (A), 1.4×10^7 cells per mL (B), 7.0×10^7 cells per mL (C). The matrix concentration was 20 mM.

The optimal bacterial densities for MALDI-TOF MS have previously been reported as 4 mg cells per 30 μ L⁵¹ or 10^6 and 10^7 cells per mL.^{48,52} In the present study, we found the optimal bacterial density as ca. 10^7 cells per mL, indicating that, to some extent, the cell density is flexible for MALDI lipid profiling.

Conclusion

In this study, lipid profiles were obtained from five *Bacillus* species (*B. subtilis*, *B. cereus*, *B. megaterium*, *B. pumilus*, and *B. sphaericus*) with online MALDI-TOF MS. The lipid profiles were similar to the results obtained with the traditional MALDI-TOF MS, indicating the reliability of the online MALDI-TOF MS. Mass spectra obtained from bacterial extracts doped with alkali salts clearly showed a homologous series of lipid mass peaks, which assisted assignment of the mass spectra. Major components of bacterial lipids such as PEs and PGs were detected in the mass spectra, and the lipids constantly occurred as di-cationic adducts. The experimental results show that the matrices had no significant influence on lipid profiling except for signal intensity. 2,4-DHB, SA, and THAP were better matrices to use for lipid analysis at 266 nm laser wavelength and positive mode.

The dependence of signal intensities to matrix concentration and bacterial cell density was investigated, and the optimal matrix and bacterial concentration were 10 mM and 10^7 cells per mL, respectively. Our work demonstrates the potential of online MALDI-TOF MS for identification of *Bacillus* at species level. The method is rapid, reliable and provides an alternative online chemotaxonomic method for bacteria identification.

Acknowledgements

This study was funded by the National High Technology Research and Development Program of China (863 Program, 2009AA06Z401) and the National Natural Science Foundation of China (20777082).

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