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Optimization of mass spectral features in MALDI-TOF MS profiling of *Acinetobacter* species

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ABSTRACT

The influence of the matrix solution, sample form and deposition technique on the quality MALDI-TOF mass spectra was examined and assessed with the aim to improve MALDI-TOF MS performance for the identification of microorganisms and to enable automatic spectra acquisition. It was observed that the use of matrix compounds ferulic and sinapinic acid may result in improved mass spectral features, in terms of signal resolution and S/N ratio, as compared to alpha-cyano-4-hydroxycinnamic acid, which was, on the other hand, found to be the only matrix compound that enabled fully automatic mass spectra acquisition. The robustness of the whole sample preparation procedure was then assessed on a set of 25 strains of four *Acinetobacter* species. Results showed reproducible detection of subtle mass spectral differences between strains belonging to the same species, although they do not confirm the possibility of reliable strain typing.

Introduction

During the past 15 years, Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) has been progressively increasing its influence in the field of bacterial taxonomy. Currently, the method is being introduced as a rapid tool for routine bacterial identification in clinical laboratories. Recent studies demonstrated its ability to identify greater than 84% of bacterial species and greater than 95% of bacterial genera in studies of large sets of clinical isolates, with overall analysis costs reduced to 22–32%, as compared to conventional bacteria identification methods [2,4,12]. The ability of MALDI-TOF MS to provide reproducible and informative spectra was found to be highly dependent on the sample preparation technique employed [13]. Different laboratories usually develop their own sample preparation procedures for particular bacterial groups of interest, but the applicability of MALDI-TOF MS profiling in practice will be strongly dependent on the universality and robustness of the sample preparation protocol.

Sample preparation protocols have been optimized to develop a universal technique for identification of both Gram-positive and Gram-negative bacteria [6,7,14]. Similar bacterial sample preparation protocols are recommended by producers of mass spectrometry equipment, who also provide bioinformatics tools and MALDI-TOF MS profile databases for identification. A method based on bacterial sterilization, using 75% ethanol, protein extraction with a solvent mixture consisting of acetonitrile/formic acid/water (50:35:15, v/v), combined with dried extract overlain with a saturated solution of alpha-cyano-4-hydroxycinnamic acid in acetonitrile/trifluoroacetic acid/water (50:2.5:47.5, v/v), actually appears as a prospective standard protocol for MALDI-TOF MS bacteria profiling [5,8].

The genus *Acinetobacter* includes Gram-negative, strictly aerobic, oxidase-negative, non-motile coccobacilli bacteria that are widely distributed in nature. The genus currently comprises 33 genomic species, of which the names of 22 have been validly published [3,9]. Some *Acinetobacter* species, including *Acinetobacter baumannii* in particular, have been implicated in opportunistic infections in individuals with reduced resistance, mostly in patients in intensive hospital care. Despite the recent developments of molecular identification methods, identification of *Acinetobacter* at the species level is problematic in most diagnostic laboratories. Therefore, a method providing accurate, quick and cost-effective identification of clinical acinetobacter strains is urgently needed. The MALDI-TOF MS analysis of selected representative *Acinetobacter* spp. has been accomplished, without deeper insight into the discriminatory abilities of the method [1,10].

Abbreviations: MALDI-TOF MS, Matrix-Assisted Laser Desorption Ionization-Time-Of-Flight Mass Spectrometry; CHCA, alpha-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid; SA, sinapinic acid; FerA, ferulic acid; TFA, trifluoroacetic acid; a.u., arbitrary units.

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The aim of our work was to find ways to improve of the sample preparation protocol [5], to enhance MALDI-TOF mass spectral features. These experiments were carried out on a set of strains of *Acinetobacter* species, although, the conclusions drawn are relevant for other bacterial taxa. Well defined strains of *Acinetobacter* spp. were used to assess the robustness and discriminatory abilities of the methods.

Experimental

Chemicals

Sinapinic acid (SA), alpha-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were obtained from Bruker Daltonik (Leipzig, Germany). Ferulic acid (FerA) and ethanol were from Sigma–Aldrich (Steinheim, Germany). Trifluoroacetic acid and acetonitrile were from Merck (Darmstadt, Germany), formic acid from Riedel de Haën (Seelse, Germany). Water was prepared on a Milli-Q plus 185 apparatus (Millipore, Bilerica, MA, USA). All chemicals were of analytical grade.

The strains

The strains of *Acinetobacter bereziniae* and *Acinetobacter guillouiae* were described previously [9] and *Acinetobacter haemolyticus* NIPH 510^T, *Acinetobacter beijerinckii* NIPH 838 and *A. beijerinckii* NIPH 2111 were obtained from the National Institute of Public Health, Prague (A. Nemec). The strains were cultivated on Nutrient agar (Oxoid) for 20 h at 30 °C. Strains of the same species were epidemiologically unrelated and genotypically distinct, as determined by sequence analysis of RNA polymerase-β-subunit (*rpoB*) gene [9].

Sample preparation

Approximately 5 mg (wet weight, one full 1 μl sterile loop) of the cultivated biomass was sterilized with 1.2 ml of 75% EtOH. After centrifugation and removal of the supernatant, sterilized cells were extracted with 50 μl of a acetonitrile/formic acid/water (50:35:15, v/v) mixture by vortexing at 2000 rpm for 1 min. The supernatant after centrifugation was deposited on three positions (wells) of the sample plate at a volume of 0.3 μl and, after drying at room temperature, overlain with 0.3 μl of the saturated CHCA matrix solution in acetonitrile:water:TFA (50:47.5:2.5, v/v) mixture ("Overlayer" method). Examined variations of the protocol are described in separate paragraphs of Results and discussion section.

MALDI-TOF MS analysis

MALDI-TOF mass spectra measurements were carried out using an Ultraflex III instrument (Bruker Daltonik, Bremen, Germany) operated in linear positive mode under FlexControl 3.0 software. The spectra were exportable in a format compatible with BioNumerics software. External calibration of the mass spectra was performed using *Escherichia coli* DH5 alpha standard peaks (4346.3, 5095.8, 5380.4, 6254.4, 7273.5 and 10,299.1 Da). Laser power was set to 120% of the threshold laser power for a particular type of the sample. Five independent spectra comprising 1000 laser shots each were acquired from each of the wells. Within an individual well, the laser was directed according to a pre-defined lattice raster, where minimum 200 and maximum 400 shots were obtained from one raster position. Mass spectra were processed using Flex Analysis (version 3.0; Bruker Daltonik) and BioTyper software (version 1.1; Bruker Daltonik). Signals present in at least 11 of 15 spectra obtained from each sample were taken into account for cluster analysis. The MALDI-TOF mass spectra-based dendrogram was generated, using the correlation distance measure with the average linkage algorithm settings of the software.

Results and discussion

To achieve particular improvement of the features of *Acinetobacter* MALDI-TOF MS profiles, three parameters of the sample preparation procedure were examined, namely the matrix choice, sample treatment method and sample deposition on the MALDI target.

Matrix choice

Matrix compounds FerA, DHB and SA were dissolved in the same solvent and deposited in the same way as CHCA, described in the protocol [5]. The concentration of the matrix compounds was 10 mg ml^{-1}. For the comparison of matrix performance, two mass spectral parameters were selected: signal resolution ($R = M/\Delta M$; ΔM at 50% peak intensity) and signal-to-noise ratio at various m/z ranges, specifically for peaks with m/z = 5175, 6930 and 10,857 of *A. haemolyticus* strain and 5175, 6930 and 10,513 of *A. beijerinckii* strains. These intense peaks were observed in all replicate analyses and were always well separated from the rest of the signals. Another factor was taken into account, which is not commonly considered in studies aimed at MALDI-TOF MS profiling methodology development, i.e., the possibility of automatic spectra acquisition.

Each sample was spotted onto 18 wells of the same MALDI sample plate and the laser 'shots' were directed, using a pre-defined lattice raster. After accumulating 200 laser shots, only those spectra accumulations with signal intensities exceeding 600 a.u. and mass resolutions over 500 were accepted. When 10 raster positions were unsuccessfully examined, the well was classified as unusable. From the examined matrix solutions, it was possible to obtain mass spectra successfully from samples treated with 100% CHCA, 78% FerA, 44% SA and 0% DHB, which was in close relation to the homogeneity of the dried sample. CHCA and FerA formed small crystals covering the entire sample well, while SA created larger, needle-shaped crystals. To obtain equal or similar numbers of mass signals per sample for each matrix solution, in order to enable reliable comparisons of their performance, additional analyses had to be carried out for FerA and SA. The main reason for unsuccessful automatic spectra acquisition with DHB was due to crystal formation only near the well border, out of the path pre-defined for the laser shot positions. Even with manual laser setting, it was difficult to find positions with protein signals and pre-defined paths directing laser pulses on the well border did not provide successful spectra acquisition. For this reason, DHB was excluded from the following experiments. CHCA was determined to be the only matrix compound that guarantees full success in automatic mass spectra acquisition. This is not just important for the throughput of the analyses, but also for the reproducibility, as operator-dependent mass spectra can be obtained when laser directing is carried out manually [11].

The influence of particular matrix on signal resolution and signal-to-noise ratio is displayed in Table 1. The values of signal-to-noise ratio were obtained with relatively large confidence limits and did not imply improvement over the entire mass range for particular matrix solutions, only SA in the high mass range showed increases in the S/N values. Lower reproducibility of the S/N ratio was observed for SA, as compared to the other matrix solutions, indicating the presence of sporadic "sweet spots" (matrix-sample co-crystals with improved MALDI ionization properties). On the other hand, signal resolution appeared to be clearly dependent on the chosen matrix compound, showing that FerA and SA yielded protein signals with better resolution for all selected peaks. The sig-