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Rapid identification of acetic acid bacteria using MALDI-TOF mass spectrometry fingerprinting

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ABSTRACT

Acetic acid bacteria (AAB) are widespread microorganisms characterized by their ability to transform alcohols and sugar-alcohols into their corresponding organic acids. The suitability of matrix-assisted laser desorption-time of flight mass spectrometry (MALDI-TOF MS) for the identification of cultured AAB involved in the industrial production of vinegar was evaluated on 64 reference strains from the genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter*. Analysis of MS spectra obtained from single colonies of these strains confirmed their basic classification based on comparative 16S rRNA gene sequence analysis. MALDI-TOF analyses of isolates from vinegar cross-checked by comparative sequence analysis of 16S rRNA gene fragments allowed AAB to be identified, and it was possible to differentiate them from mixed cultures and non-AAB. The results showed that MALDI-TOF MS analysis was a rapid and reliable method for the clustering and identification of AAB species.

Introduction

Acetic acid fermentation is a process whereby ethanol is transformed into acetic acid by a particular group of microorganisms known as acetic acid bacteria (AAB). These widespread microorganisms play an important role in multiple natural processes that result in high-value food and beverage products, such as vinegar, chocolate and kombucha, as well as chemicals of industrial interest, including cellulose, ascorbic acid (vitamin C) or dihydroxyacetone (DHA). Some symbiotic N_2-fixing AAB strains are currently studied in order to improve non-legume coffee, rice and sugarcane cultures. However, a subset of these bacteria has recently been described as emerging opportunistic human pathogens that are resistant to several microbicidal agents [24,25,1,8].

Over the last few years, the taxonomy of AAB has been subjected to continuous remodeling because of the availability of new molecular techniques for bacterial identification and classification [29,23,6]. Currently, AAB are classified in the family *Acetobacteraceae* and grouped within twelve genera including *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Acidomonas*, *Asaia*, *Neoasaia*, *Kozakia*, *Frauteria*, *Granulibacter*, *Saccharibacter*, *Swaminathania* and *Tanticharoenia* [38,36]. Of these, particularly those classified in the genera *Acetobacter* (*A. aceti*, *A. pasteurianus*, *A. cerevisiae*, *A. oeni*, *A. malorum*, *A. estunensis* and *A. pomorum*), *Gluconobacter* (*G. oxydans*) and *Gluconacetobacter* (*Ga. europaeus*, *Ga. hansenii*, *Ga. entanii*, *Ga. intermedius*, *Ga. oboediens*, *Ga. liquefaciens*, and *Ga. xylinus*) are involved in industrial vinegar production because of their capacity to oxidize ethanol to acetic acid and their extreme resistance to high acetic acid concentrations [9,37,18,10,33].

For the identification and typing of AAB, several DNA-based techniques have been developed, such as restriction fragment length polymorphism (RFLP), sequence analysis of several genomic targets, and fingerprinting analysis of genomic repetitive elements [19,11,7,17]. An alternative to DNA-based molecular typing methods is provided by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), a recently developed protein fingerprinting method that allows organisms to be distinguished at the species or even subspecies level [28,3].

The aim of this study was to test the suitability of MALDI-TOF MS for the rapid identification of the most important culturable acetic acid bacteria belonging to the genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* using the SARAMIS™ software package (Spectral Archive and Microbial Identification System; Anagnostec Gmbh, Germany).

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Materials and methods

Bacterial strains, media and growth conditions

The 64 reference strains used in this study were representative of 22 AAB species and were purchased from the Belgian Coordinated Collection of Microorganisms (BCCM/LMG) (Table 1A). Strains isolated in our laboratory from superficial and submerged semi-continuous acetification processes were used to evaluate the accuracy of the identification methodology supported by MALDI-TOF MS (Table 1B).

For MS analysis purposes, reference strains and isolates were grown on YPM (0.5% yeast extract, 0.3% peptone, 2.5% mannitol, 1.5% agar) and RAE 1a/2e (1% yeast extract, 4% glucose, 1% peptone, 0.338% $Na_2HPO_4{\cdot}2H_2O$, 0.15% citric acid$\cdot H_2O$, 1% acetic acid, 2% ethanol; ethanol and acetic acid were sterilized by filtration and added to sterile medium). RAE medium was poured using a double layer system: 2% agar on the top and 1% agar on the bottom. Bacterial cultures were incubated for 2–5 days at 30 °C.

Molecular identification by 16S rRNA gene sequence analysis

Isolates from vinegar were identified in our laboratory using multiple techniques in order to obtain a unique and accurate identification. To cross-reference the data and verify the accuracy of MALDI-TOF MS analysis, the re-sequencing of the 16S rRNA gene was performed for the reference strains and the isolates from vinegar, and the sequence analyses were compared with the clustering data from the MALDI-TOF MS analysis.

DNA extraction was performed using the GenElute™ Bacterial Genomic DNA Kit (Sigma–Aldrich, Buchs, Switzerland), according to the manufacturer's instructions for Gram-negative bacteria. PCR amplification of the nearly complete 16S rRNA gene was performed using the primers 16Sd (5′-GCTGGCGGCATGCTTAACACAT-3′) and 16Sr (5′-GGAGGTGATCCAGCCGCAGGT-3′), and the conditions described by [26]. Purified PCR products were sequenced at Fasteris S.A. (Geneva, Switzerland).

Sequences were compared with those from the GenBank database [4] using the BLAST program hosted by the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast), and were then deposited in the GenBank database under the accession numbers shown in Tables 1A and 1B.

Phylogenetic analyses were carried out using MEGA5 (Arizona State University) [22,32]. Sequences were aligned using the MUSCLE multiple alignment algorithm [13,14] with default parameters, and an unrooted phylogenetic tree was created based on the neighbor-joining method [27] using the Kimura 2-parameter correction model [21]. The reliability of the groups was evaluated by the bootstrap method with 1000 resamplings.

MALDI-TOF MS spectra acquisition and analysis

To establish a reproducible protocol for the routine identification of AAB, YPM agar medium was chosen for the bacterial cultures. Cells from a single bacterial colony grown on agar medium (YPM or RAE) were transferred in duplicate to a target spot on a 48-well stainless steel FLEXImass™ target plate (Shimadzu-Biotech, Kyoto, Japan) using a disposable loop, overlaid with 1 μL of matrix solution containing 30 mg mL^{-1} α-cyano-4-hydroxycinamic acid (CHCA) in acetonitrile/ethanol/water (1:1:1) supplemented with 3% trifluoroacetic acid, and air-dehydrated at room temperature. Protein mass fingerprints were obtained using a MALDI-TOF Mass Spectrometry Axima™ Confidence machine, with detection in the linear positive mode at a laser frequency of 50 Hz and within a mass range from 2000 to 20,000 Da (Shimadzu-Biotech, Kyoto, Japan). The acceleration voltage was 20 kV, and the extraction delay time was 200 ns. A minimum of 20 laser shots per sample was used to generate each ion spectrum. For each sample, 50 protein mass fingerprints were averaged and processed using the Launchpad™ v.2.8 software (Shimadzu-Biotech, Kyoto, Japan). For peak acquisition, the average smoothing method was chosen, with a smoothing filtering width of 50 channels. Peak detection was performed using the threshold-apex peak detection method, with an adaptive voltage threshold that roughly followed the signal noise level, and subtraction of the baseline was set with a baseline subtraction filter width of 500 channels. For each sample, a list of the significant spectrum peaks was generated that included the *m*/*z* values for each peak, mass deviations, and signal intensities. Calibration was conducted for each target plate using the spectra of the reference strain *Escherichia coli* K12 (GM48 genotype).

Protein mass fingerprints were imported into the SARAMIS™ software package and analyzed using the m/z spectra peaks of a mass range from 3000 to 20,000 Da and an analytical error for mass accuracy of 800 ppm. Cluster analysis using the single-link agglomerative algorithm of SARAMIS™ was performed to compare the spectra and to produce taxonomic trees.

To further evaluate the accuracy of the MS protein fingerprinting analysis, several blind tests were performed. Different sets of AAB reference strains were randomly selected, and each strain was analyzed in duplicate or quadruplicate.

Results and discussion

Comparison of 16S rRNA phylogeny and MALDI-TOF MS analysis of the reference strains

Phylogenetic analysis of the 64 reference strains of AAB used in this study reproduced the classification reported in recent publications (Fig. 1A). The taxonomy of the reference strains was confirmed in all cases, except for *Acetobacter pasteurianus* LMG 1607 and *Gluconacetobacter liquefaciens* LMG 1383. The analysis of the 16S rRNA gene sequence of strain LMG 1607 identified it as *Acetobacter indonesiensis* (sequence 100% identical to AJ419841) and not as an authentic *A. pasteurianus* strain, whereas strain LMG 1383 was identified as *Gluconobacter japonicus* (sequence 100% identical to AB178400) and not as *Ga. liquefaciens* [Note from the author: Strain LMG 1383 received in our laboratory corresponded to a batch that may not be a subculture of Asai Ac-8, the original strain identified and deposited as *Ga. liquefaciens* (Cleenwerck, personal communication)]. The reclassification of these reference strains as *A. indonesiensis* and *G. japonicus*, respectively, was consistent with the clustering observed after MALDI-TOF MS analysis (Fig. 1B). In the MALDI-TOF MS dendrogram, the reference strain LMG 1607 clustered in an independent group, at quite a distance from the main *A. pasteurianus* group, which was formed by strains LMG 1262^T and LMG 1555. Similarly, the reference strain LMG 1383 clustered in the group formed by the *Gluconobacter* strains, which were closely related to *G. cerinus* LMG 1407.

The dendrogram constructed from the MALDI-TOF MS data analysis correlated well with the phylogenetic analysis based on the 16S rRNA gene sequences. The MS clusters formed by the reference strains belonging to the genus *Gluconobacter* showed the same clustering observed in the phylogenetic tree (Fig. 1A and B). Even if the genera *Acetobacter* and *Gluconacetobacter* were not clearly separated in the MS dendrogram, the strains belonging to the different species clustered according to the main branches revealed by the phylogenic analysis (Fig. 1A and B). Four AAB species showed split clusters: *A. peroxydans*, *Ga. xylinus*, *Ga. europaeus* and *G. cerinus* (Fig. 1B). The split cluster might be the consequence of a unique