Systematic and Applied Microbiology 36 (2013) 75–81

Contents lists available at SciVerse ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

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

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ARTICLE INFO

Article history:
Received 15 March 2012
Received in revised form 5 September 2012
Accepted 7 September 2012

Keywords:
MALDI-TOF MS
Whole-cell identification
Acetic acid bacteria
Vinegar

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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http://dx.doi.org/10.1016/j.syapm.2012.09.002

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

Table 1A
Reference strains related with industrial vinegar production, used to establish a reference database for MALDI-TOF MS-based species identification.

Bacterial species	Strain	Biological origin	Specificity	AC number
Acetobacter aceti	LMG 1496	–	100	JF793948
Acetobacter aceti	LMG 1504^T	Beech-wood shavings of vinegar plant	100	JF793949
Acetobacter aceti	LMG 1505	Quick vinegar	100	JF793950
Acetobacter aceti	LMG 1512	Film in fermentor of rice vinegar	100	JF793951
Acetobacter cerevisiae	LMG 1545	Film in fermentor of rice vinegar	100	JF793952
Acetobacter cerevisiae	LMG 1592	Manufacture of vinegar	100	JF793953
Acetobacter cerevisiae	LMG 1625^T	Beer (ale) in storage	100	JF793954
Acetobacter cerevisiae	LMG 1682	Beer	100	JF793955
Acetobacter estunensis	LMG 1626^T	Cider	100	JF793958
Acetobacter estunensis	LMG 1572	Cider	100	JF793959
Acetobacter estunensis	LMG 1580	Beer	100	JF793960
Acetobacter malorum	LMG 1746^T	Rotting apple	90	JF793956
Acetobacter oeni	LMG 21952^T	Spoiled red wine	100	JF793961
Acetobacter orleanensis	LMG 1583^T	Beer	100	JF793962
Acetobacter pasteurianus	LMG 1262^T	Beer	100	JF793964
Acetobacter pasteurianus	LMG 1513	Film in fermentor of rice vinegar	100	JF793965
Acetobacter pasteurianus	LMG 1555	–	100	JF793966
Acetobacter pasteurianus[a]	LMG 1607	Vinegar	91.7	JF793967
Acetobacter peroxydans	LMG 1633	Ditch water	100	JF793968
Acetobacter peroxydans	LMG 1635^T	Ditch water	87.5	JF793969
Acetobacter pomorum	LMG 18848^T	Industrial cider vinegar fermentation	100	JF793970
Gluconacetobacter azotocaptans	LMG 21311^T	*Coffea arabica* L., rhizosphere	100	JF793972
Gluconacetobacter azotocaptans	LMG 23156	*Coffea arabica* L., rhizosphere	100	JF793974
Gluconacetobacter azotocaptans	LMG 23157	*Coffea arabica* L., rhizosphere	100	JF793975
Gluconacetobacter diazotrophicus	LMG 7971	*Saccharum officinarum*, stem	100	JF793978
Gluconacetobacter diazotrophicus	LMG 8065	*Saccharum officinarum*, stem	100	JF793979
Gluconacetobacter diazotrophicus	LMG 22174	*Oryza sativa*, root tissue	100	JF793980
Gluconacetobacter europaeus	LMG 1510	Vinegar	100	JF793981
Gluconacetobacter europaeus	LMG 1521	From mixed culture NCTC 6716 isolated from vinegar brew	100	JF793982
Gluconacetobacter europaeus	LMG 18494	Red wine vinegar produced in submerged bioreactor	100	JF793983
Gluconacetobacter europaeus	LMG 18890^T	Submerged culture vinegar generator	100	JF793984
Gluconacetobacter hansenii	LMG 1524	Vinegar	100	JF793985
Gluconacetobacter hansenii	LMG 1527^T	Celluloseless mutant 1 derived from NCIB 8745	100	JF793986
Gluconacetobacter hansenii	LMG 1528	Celluloseless mutant 2 derived from NCIB 8745	100	JF793988
Gluconacetobacter hansenii	LMG 23726	Kombucha tea ferment	100	JF793989
Gluconacetobacter intermedius	LMG 18909^T	Commercially available tea fungus beverage (Kombucha)	100	JF793990
Gluconacetobacter johannae	LMG 23153	*Coffea arabica* L., rhizosphere	100	JF793993
Gluconacetobacter johannae	LMG 23154	*Coffea arabica* L., rhizoplane	100	JF793994
Gluconacetobacter liquefaciens	LMG 1348	Fruit	100	JF793995
Gluconacetobacter liquefaciens	LMG 1381^T	*Diospyros* sp., dried fruit	100	JF793996
Gluconacetobacter liquefaciens[b]	LMG 1383	Fruit	100	JF793998
Gluconacetobacter liquefaciens	LMG 1503	–	100	JF793999
Gluconacetobacter oboediens	LMG 1517	–	100	JF794001
Gluconacetobacter oboediens	LMG 1688	–	100	JF794002
Gluconacetobacter oboediens	LMG 1689	–	100	JF794003
Gluconacetobacter oboediens	LMG 18849^T	Red wine vinegar fermentation	90	JF794004
Gluconacetobacter sacchari	LMG 19747^T	Sugar cane, leaf sheath	100	JF794007
Gluconacetobacter sacchari	LMG 19748	Mealy bug	100	JF794009
Gluconacetobacter xylinus	LMG 25	Mountain ash berries	100	JF794012
Gluconacetobacter xylinus	LMG 1515^T	Mountain ash berries	75	JF794013
Gluconacetobacter xylinus	LMG 1518	–	100	JF794014
Gluconacetobacter xylinus	LMG 1523	Vinegar	100	JF794015
Gluconobacter cerinus	LMG 1368^T	*Prunus* sp. (cherry)	100	JF794016
Gluconobacter cerinus	LMG 1390	*Rheum rhabarbarum*, flower	100	JF794017
Gluconobacter cerinus	LMG 1407	Bakers' yeast	100	JF794018
Gluconobacter frateurii	LMG 1357.1	*Dahlia* sp., flower	100	JF794019
Gluconobacter frateurii	LMG 1365^T	*Fragaria ananassa*	75	JF794020
Gluconobacter frateurii	LMG 1366	*Fragaria ananassa*	100	JF794021
Gluconobacter frateurii	LMG 1369.1	*Prunus* sp. (cherry)	100	JF794022
Gluconobacter oxydans	LMG 1394	–	80	JF794023
Gluconobacter oxydans	LMG 1403*	Derived from ATCC 621	100	JF794024
Gluconobacter oxydans	LMG 1408^T	Beer	100	JF794026
Gluconobacter oxydans	LMG 1411	–	100	JF794028
Gluconobacter oxydans	LMG 1516	–	100	JF794029

[a] Identified as *Acetobacter indonesiensis*.
[b] Identified as *Gluconobacter japonicus*.
^{T} Type strain.
* Synonym strains.

characteristic of the individual strains, which was translated in some specific and unique mass peaks. For example, *Ga. europaeus* LMG 18890^T showed the specific characteristic of requiring acetic acid for growth [30]. Nevertheless, this might respond to a previous misclassification of the AAB strains concerned. For example, *Ga. europaeus* LMG 25, which clustered independently and at quite a distance from the group formed by the other three *Ga. europaeus* strains, has been reclassified recently as *Ga. swingsii* (Cleenwerck, personal communication). The strain *G. cerinus* LMG 1407 clustered with *Ga. liquefaciens* LMG 1383 (identified as *G. japonicus*) in a

Table 1B
Submerged running vinegar isolates analyzed.

Isolate	Vinegar origin	Acidity (%AcH)	Isolation media	AC number
COR1	White wine surface vinegar (2008)	6.5	YPM	JQ513819
COR2		6.5	YPM	JQ513820
COR5		11.6	YPM	JQ513821
COR6		11.6	YPM	JQ513822
COR7		11.6	YPM	JQ513823
M50.2	Red wine submerged vinegar (2007)	5.4	YPM	JQ513824
M50.3		5.4	YPM	JQ513825
M100		5.4	YPM	JQ513826
M100.3		5.4	YPM	JQ513827
CAV69	Balsamic submerged vinegar (2007)	6.6	GYC	JQ513828
CAV75		7.2	GYC	JQ513829
CAV82		8.7	GYC	JQ513830
TP1-23	Red wine submerged vinegar (2010)	5.8	RAE	JQ513831
TP1-25		5.8	RAE	JQ513832
TP1-30		6.3	RAE	JQ513833
TP1-40		8.6	RAE	JQ513834
TP1-52		6.0	RAE	JQ513835
TP1-63		7.2	RAE	JQ513836
TP1-90		6.0	RAE	JQ513837
TP1-105		8.1	RAE	JQ513838
RD1	Cider submerged vinegar (2011)	5.1	RAE	JQ513839
RD2		5.1	RAE	JQ513840
RD4		5.1	RAE	JQ513841
RD5		5.1	RAE	JQ513842
RD6		5.1	RAE	JQ513843
RD11		5.1	RAE	JQ513844
RD12		5.1	RAE	JQ513845
EV1	Red wine submerged vinegar (2011)	8.0	RAE	JQ513846
EV3		8.0	RAE	JQ513847
EV8		8.0	RAE	JQ513848
EV9		8.0	RAE	JQ513849
EV11		8.0	RAE	JQ513850
EV12		8.0	RAE	JQ513851
EVB7	White wine submerged vinegar (2011)	10.7	RAE	JQ513852
EVB8		10.7	RAE	JQ513853
EVB9		10.7	RAE	JQ513854
La5	Spirit submerged vinegar (2010)	5.2	RAE	JQ513855
La29		6.3	RAE	JQ513856
170B	Spirit submerged vinegar (2008)	5.6	RAE	JQ513857
174B		5.6	RAE	JQ513858
BalC	Running tap water[a] (2007)	–	YPM	JQ513859
BalJ		–	YPM	JQ513860
3Pe4	Red wine submerged vinegar (2006)	7.0	YPM	JQ513861
4Pe2		10.2	YPM	JQ513862
5Pe3		6.5	YPM	JQ513863
PlA	Spirit submerged vinegar (2011)	8.8	YPM	JQ513864

[a] Non-AAB strains isolated from running tap water, used as negative control for the evaluation of MALDI-TOF MS analysis.

different cluster to the one formed by the other *G. cerinus* strains. The nature of this strain, which was closer to *G. japonicus* than to the other *G. cerinus* studied, was confirmed by the analysis of the 16S rRNA gene.

The results of MALDI-TOF MS analysis suggested that the classification of strains LMG 1407 as *G. cerinus*, LMG 1523.1 as *Ga. xylinus*, and possibly LMG 1510 and LMG 1521 as *Ga. europaeus* should be verified.

The topology of the phylogenetic tree constructed by using the neighbor-joining method was consistent with data found in the literature [6,36]. The genera *Gluconobacter* and *Acetobacter* were first divided based on the capability of *Gluconobacter* to oxidize glucose to gluconic acid rather than ethanol to acetic acid, and its incapability to overoxidize acetic acid to CO_2 and H_2O because of the lack of a functional tricarboxylic acid (TCA) cycle [2]. The genus *Gluconacetobacter* was later proposed to be independent from *Acetobacter* due to their different ubiquinone systems Q-10 and Q-9, respectively [35,37].

The consistency of the clustering observed in the dendrogram shown in Fig. 1B was very high, and the clustering specificity for each strain is shown in Table 1A. A total of 48 strains isolated from vinegar were used to verify the suitability of MALDI-TOF MS analysis for the identification of AAB. It was shown that 40 of the 48 isolates clustered within specific AAB species groups in the reference dendrogram (Fig. 2). The other eight isolates clustered independently according to their molecular identification as non-AAB. The analysis of the 16S rRNA gene sequences identified these isolates as environmental bacteria from the genera *Acinetobacter*, *Burkholderia*, *Paenibacillus*, *Sphingopyxis* and *Sphingomonas*. The presence of these environmental species in the vinegar samples could be explained by the non-sterile practices that are typically observed in the vinegar industries, which can lead to

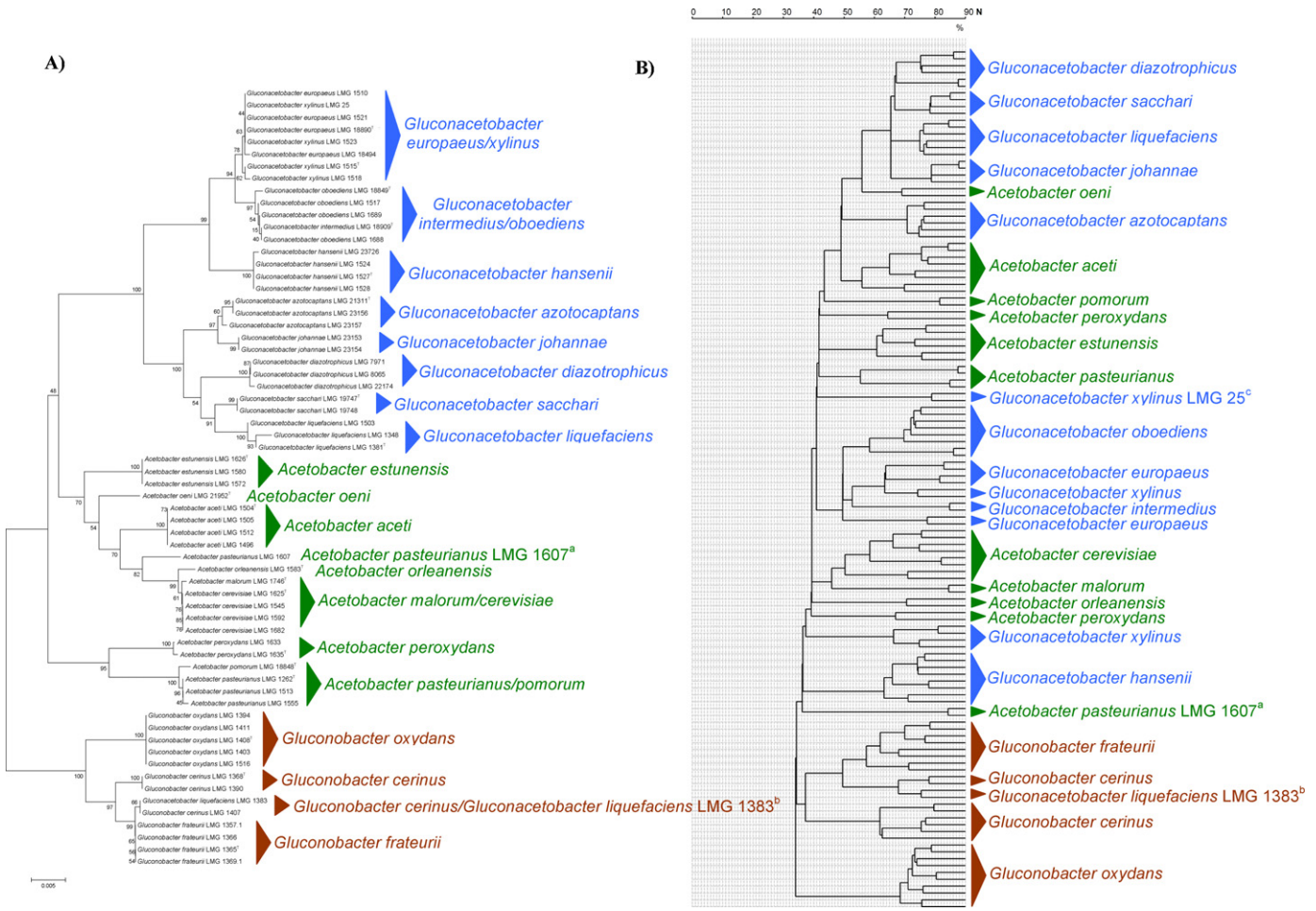

Fig. 1. (A) Unrooted neighbor-joining phylogenetic tree, generated using nearly complete 16S rRNA gene sequences (1366 bp in the final dataset) of 64 reference strains of AAB. The optimal tree with the sum of branch length = 0.24 is shown. The evolutionary distances were computed using the Kimura 2-parameter correction. Values at nodes indicate bootstrap percentages for 1000 replicates. All ambiguous positions were removed for each sequence pair. (B) Single-linkage clustering analysis of MALDI-TOF mass spectra (error 0.08%, *m*/*z* mass range from 3000 to 20,000) for the 64 reference strains. [a]Strain identified as *A. indonesiensis*. [b]Strain identified as *G. japonicus*. [c]Strain recently reclassified as *Ga. swingsii* (Cleenwerck, personal communication).

contamination of the samples prior to their arrival at the laboratory. Among the 40 isolates considered to be AAB species, the position of 37 in the MS dendrogram was consistent with their preliminary identification by 16S rRNA gene sequencing analysis. CAV75, which was identified as *Burkholderia fungorum*, was the only non-AAB isolate that clustered in the *Ga. xylinus* group (Fig. 2).

The 16S rRNA gene sequence obtained for isolate COR5 from vinegar showed a maximum identity of 90% with several sequences of *A. pasteurianus* and *A. pomorum* (FJ715624, AB680052, AB569643). On the other hand, MALDI-TOF MS analysis clustered this isolate independently of any AAB group. The comparative sequence analysis did not provide the resolution for species/genus assignment [12], so the taxonomic position of the isolate could not be verified by MALDI-TOF, though it was very likely different from AAB (Fig. 2). Four other isolates, CAV69, CAV75, CAV82 and 3Pe4, clustered together to form a branch within the *Ga. xylinus* group (Fig. 2). CAV69, CAV82 and 3Pe4 were previously identified within the *Ga. xylinus* group (99–100% identical to *Ga. xylinus*, *Ga. europaeus* and *Ga. swingsii* sequences: AB645736, AB680040, Y15289, FJ716523). In contrast, CAV75 could not be identified at the species/genus level by sequence analysis, because the 16S rRNA gene sequence obtained showed a maximum identity of 91% with sequences of *Burkholderia fungorum* (AF215706). This AAB phylogenetic group showed a high degree of heterogeneity in its MS protein profiles, which explained why the former group of four isolates from vinegar could not be clustered within a defined AAB group of species. The evaluation of a higher number of *Ga. xylinus*, *Ga. europaeus* and other species belonging to the *Ga. xylinus* group, in order to construct a reference framework, should help to distinguish these heterogenic bacterial strains.

The molecular identification of bacteria using 16S rRNA gene analysis as the only phylogenetic marker fails to discriminate between closely related species. This phenomenon was observed for most of the isolates from vinegar, which were identified as belonging to a group of two or more AAB: *A. pasteurianus*/*A. pomorum*, *Ga. xylinus*/*Ga. europaeus*/*Ga. swingsii*/*Ga. nataicola*, or *Ga. intermedius*/*Ga. oboediens*. In contrast, MALDI-TOF MS whole-cell protein fingerprinting succeeded in identifying the following isolates as unique AAB species: *A. pasteurianus* (COR2, COR7, M50.2, M50.3, M100, M100.3, RD4), *Ga. intermedius* (RD1, RD2, RD5, RD11, RD129) or *Ga. oboediens* (170B, 174B) (Fig. 2).

Prospectives

In this study, it was shown that MALDI-TOF MS is a suitable fingerprinting technique for the rapid identification of the most common acetic acid bacteria associated with vinegar production. Nevertheless, to apply mass spectrometry for the unequivocal identification of unknown isolates, it will be necessary to create SuperSpectra for the species most frequently found during

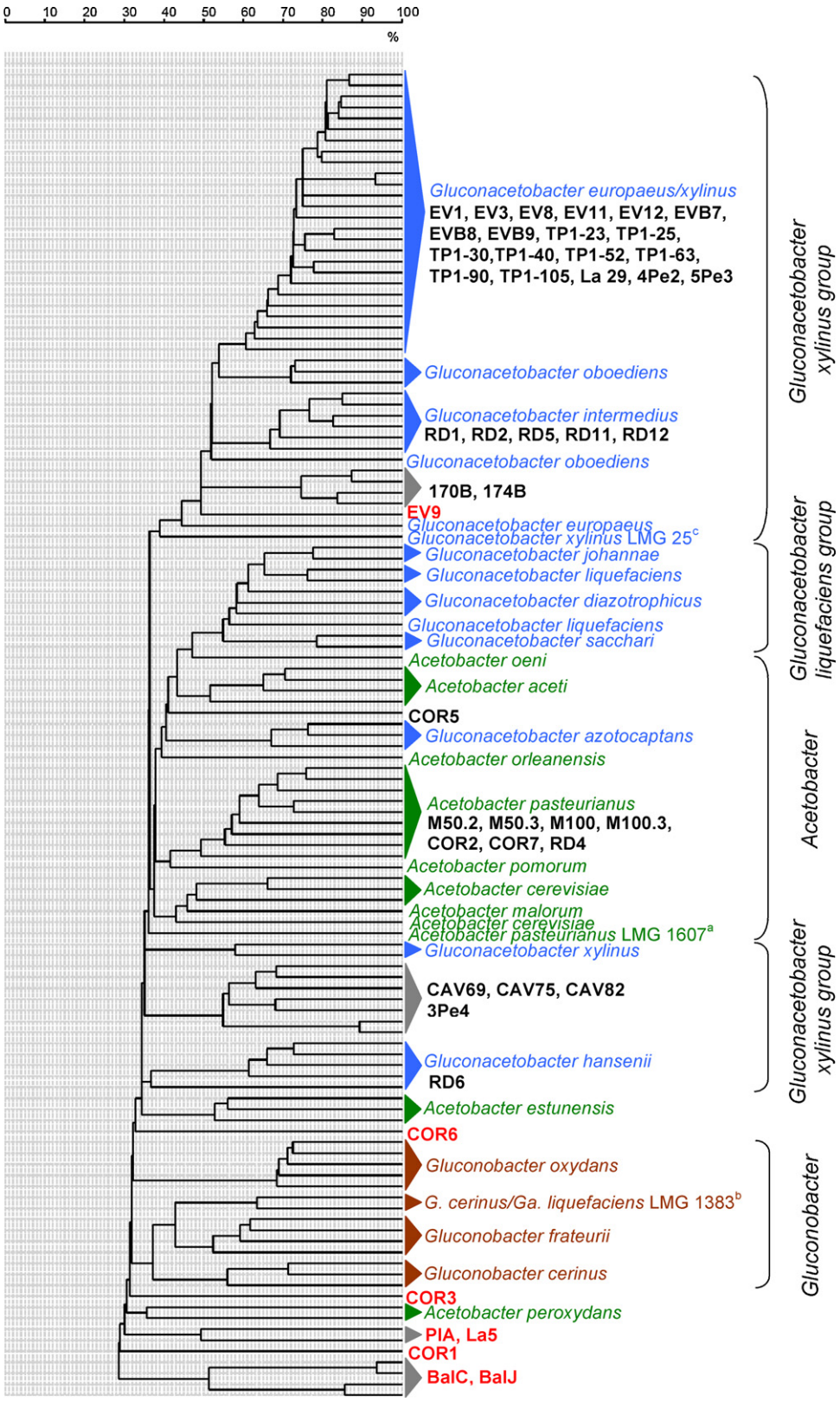

Fig. 2. Single-linkage clustering analysis of MALDI-TOF mass spectra (error 0.08%, *m*/*z* mass range from 3000 to 20,000) for reference strains and isolates from vinegar. [a]Strain identified as *A. indonesiensis*. [b]Strain identified as *G. japonicus*. [c]Strain recently reclassified as *Ga. swingsii* (Cleenwerck, personal communication).

vinegar production and manufacturing. SuperSpectra are created on the basis of the species-specific masses selected following the analysis of the mass spectra for multiple strains of the same species. The integration of these SuperSpectra into the SARAMIS^{TM} database will allow the rapid and automated identification of new isolates by species-specific MS spectra comparison [20].

The application of MALDI-TOF MS analysis for simultaneous detection of bacterial strains without previous isolation has already

been established [5]. However, future studies should center on the application of MALDI-TOF mass SuperSpectra database searches for the analysis of samples directly harvested from high-acid vinegars. High-acid vinegars (which are vinegars showing a high concentration of acetic acid) have the significant drawback of being problematic for culturing in solid or liquid medium. Nevertheless, some attempts have been made to culture spirit vinegar with > 10% acidity [31,34,15,16]. Although researchers have had relative success with culturing high-acid vinegars by applying different enrichment protocols, the cultivation of these types of vinegars is not yet a routine practice. Direct analysis of the vinegar samples will avoid the time-consuming step of *in vitro* cultivation for bacterial isolation, and it will also prevent the biased results that can be obtained because of the loss of non-culturable bacterial strains. Direct identification by using MALDI-TOF MS will be the final challenge for ecological and population dynamics studies during acetic acid fermentation.

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