Effects of Growth Medium on Matrix-Assisted Laser Desorption–Ionization Time of Flight Mass Spectra: a Case Study of Acetic Acid Bacteria

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The effect of the growth medium used on the matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectra generated and its consequences for species and strain level differentiation of acetic acid bacteria (AAB) were determined by using a set of 25 strains. The strains were grown on five different culture media that yielded a total of more than 600 mass spectra, including technical and biological replicates. The results demonstrate that the culture medium can have a profound effect on the mass spectra of AAB as observed in the presence and varying signal intensities of peak classes, in particular when culture media do not sustain optimal growth. The observed growth medium effects do not disturb species level differentiation but strongly affect the potential for strain level differentiation. The data prove that a well-constructed and robust MALDI-TOF mass spectrometry identification database should comprise mass spectra of multiple reference strains per species grown on different culture media to facilitate species and strain level differentiation.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been applied in medical (1), environmental (2–4), and food-related (5–8) studies as an excellent cost- and time-effective tool for the identification of microorganisms. Different types of identification approaches were recently reviewed (9). These include linking of protein biomarkers derived from the mass spectra to a corresponding experimental database or comparing mass spectra with *in silico*-generated spectra predicted by whole-genome sequence analyses (10, 11). Yet, the most frequently used approach is the comparison of mass spectra of unknowns with those of well-characterized reference strains stored in a profile database. These comparisons are conducted by using either a peak- or a curve-based algorithm (9, 12). With a peak-based algorithm, the presence of specific biomarker peaks in the unknown isolate's mass spectrum is verified (6, 13). A curve-based method considers the complete spectrum, i.e., not only the presence of certain peaks but also the variation in peak signal intensity. The major drawback of these pattern recognition approaches is that they can be prone to experimental variations (14).

Previous studies revealed that MALDI-TOF mass spectra of bacteria consist of signals derived mostly from ribosomal and other abundant proteins (15–18). For proteins that are of ribosomal origin (19), the effect of growth conditions on the mass spectra and thus on the identification result is expected to be minimal (20). However, growth conditions can influence the expression pattern of other proteins (21) and hence alter the mass spectrum (16, 22). Additionally, growth medium compounds can interfere with the ionization of the bacterial biomolecules, especially when the bacterial cells have a tendency to adhere to the culture medium surface (15). Nevertheless, several studies found that variations related to the growth medium had no impact on the species level identification of unknowns (16, 23–26).

We are currently investigating the use of MALDI-TOF MS as an identification tool for bacterial isolates originating from spoiled beer and brewery samples and are constructing an identification database including spectra of strains originating from

spoiled beer and brewery samples but also from other niches where the same species occur in order to encompass the species' phenotypic diversity wherever possible. Beer spoilage bacteria are taxonomically diverse and include several acetic acid bacteria (AAB) that can be grown on different culture media.

The present study investigated the effect of the growth medium on the generated mass spectra by using a set of 25 AAB grown on five different culture media and its consequences for species and strain level differentiation.

MATERIALS AND METHODS

Strains and growth media. Twenty-five strains of AAB (see Table S1 in the supplemental material) were obtained from the BCCM/LMG bacterial collection (Ghent University, Ghent, Belgium) (<http://www.belspo.be/bccm/>) and cultured according to the provider's instructions and on additional selective and nonselective growth media. *Acetobacter* strains were grown on acetic acid medium (AAM) (1.0% D-glucose, 1.5% bacteriological peptone, 0.8% yeast extract, 0.3% acetic acid, 0.5% ethanol, 1.5% agar, pH 3.5) (27), deoxycholate-mannitol-sorbitol (DMS) agar (0.1% D-glucose, 0.1% D-mannitol, 0.1% sorbitol, 1.0% bacteriological peptone, 0.3% yeast extract, 1.5% calcium lactate, 0.1% potassium phosphate, 0.01% sodium deoxycholate, 0.002% magnesium sulfate, 0.003% bromocresol, 1.5% agar, pH 4.5) (28), GY agar (5% D-glucose, 1% yeast extract, 1.5% agar), GYAE agar (GY agar supplemented with 1% acetic acid and 2% ethanol), and YPM agar (0.5% yeast extract, 0.3% bacteriological peptone, 2.5% D-mannitol, 1.5% agar). *Gluconacetobacter* and *Gluconobacter* strains were cultured on the same growth media, with the exception of GYAE agar, which was replaced with reinforced AE (RAE)

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agar (4% D-glucose, 1% yeast extract, 1% bacteriological peptone, 0.338% $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.15% citric acid, 1% acetic acid, 2% ethanol; a layer of this medium containing 1% agar is topped with a layer containing 2% agar) (29). AAB are strictly aerobic bacteria, and cultures were always incubated at 28°C. The incubation time on each medium was kept constant per strain but varied between 24 and 72 h among strains in order to obtain sufficient growth; yet some strains grew so poorly on, at most, one growth medium that mass spectra of acceptable quality (see below) could not be obtained.

MALDI-TOF MS sample preparation and data acquisition. Resuscitated lyophilized cultures were subcultured twice on each culture medium, and cell extracts were prepared from the subsequent third, fourth, and the fifth generations. Hence, variation induced by resuscitation and adaptation to the growth medium was excluded and three generations, i.e., three biological replicates, were included. Five milligrams of wet cells taken from a single colony was suspended in 300 μl Milli-Q water, after which 900 μl pure ethanol was added. After centrifugation (3 min, $20,817 \times g$, 4°C), 50 μl 70% formic acid and 50 μl acetonitrile were added to the bacterial cell pellet. After vigorous shaking and centrifugation (3 min, $20,817 \times g$, 4°C), 1 μl of the supernatant (= the cell extract) was spotted in duplicate onto a MALDI-TOF MS stainless steel target plate to obtain two technical replicates. Immediately after drying, the spots were overlaid with 1 μl matrix solution, which consisted of 5 mg α -cyano-4-hydroxycinnamic acid dissolved in 1 ml 50:2:48 acetonitrile–trifluoroacetic acid–Milli-Q water solvent. Prior to analysis, the mass spectrometer was externally calibrated with a peptide mixture of adrenocorticotrophic hormone (fragment 18-39; Sigma-Aldrich), insulin (Sigma-Aldrich), ubiquitin (Sigma-Aldrich), cytochrome *c* (Sigma-Aldrich), and myoglobin (Sigma-Aldrich). A 4800 Plus MALDI TOF/TOF Analyzer (AB Sciex) was used in the linear mode and covered a mass range of 2 to 20 kDa. The mass spectrometer used a 200-Hz frequency-tripled Nd:YAG laser operating at a wavelength of 355 nm. Generated ions were accelerated at 20 kV through a grid at 19.2 kV into a 1.5-m, linear, field-free drift region toward the detector. For each spot, 40 subspectra resulting from 50 laser shots from randomized positions within the spot were collected and presented as one spectrum (a total of 2,000 laser shots). The laser intensity was set between 4,200 and 5,700 procedure defined units (pdu). With every set of measurements comprising 384 spots, the Bacterial Test Standard (Bruker Daltonics) was included as a positive control.

MALDI-TOF MS data analysis. The mass spectra were retrieved as t2d files from the 4800 Plus MALDI TOF/TOF Analyzer via the 4000 Series Explorer software (AB Sciex). Data Explorer 4.0 software (AB Sciex) was used to convert the t2d files to text files that were subsequently used as input files for the BioNumerics 7.1 software package (Applied Maths, Sint-Martens-Latem, Belgium). A spectrum was considered to be of acceptable quality if the absolute signal intensity of the highest peak was >500 counts, if more than five peaks with a signal-to-noise (S/N) ratio of >20 were detected in the 3- to 20-kDa range, and if there were no repetitive signals in the 2.1- to 3-kDa range. The spectral data were imported by using an optimized preprocessing template in the BioNumerics 7.1 software package (30). After the data were imported, the preprocessing involved consecutive continuous wavelet transform noise estimation, Savitsky-Golay filter smoothing, and baseline subtraction with the rolling-disk algorithm. Each peak with a S/N ratio of at least 5 and an absolute intensity of at least 6 counts was annotated. For each strain grown on each culture medium, both technical and biological replicates were combined into a single summary spectral profile (SSP). A peak-matching analysis was conducted with constant and linearly varying tolerance values of 1 m/z and 800 ppm, respectively (30). The minimum peak detection rate was set at 100%, meaning that each summary peak occurred in each individual spectrum of the technical and biological replicates. The use of the 100% peak detection rate during this summarizing procedure excluded any technical or biological variation from the analysis. The signal intensity for each data point in the SSP was calculated by averaging the respective signal intensities in the technical and biological replicates.

The peak-based data analysis matches all of the peaks in the SSP to a peak class with constant and linearly varying tolerance values of 2 m/z and 800 ppm, respectively (30). The data set obtained was converted into a binary character set. The latter data set was then used to compare the SSPs by using the binary Dice coefficient. The curve-based data analysis of SSPs was conducted by using the Pearson product-moment correlation coefficient. In order to visualize the variation between the SSPs, multidimensional scaling (MDS) plots were created as described by De Bruyne et al. (24). In brief, the MDS algorithm starts with the similarity matrix generated after either peak- or curve-based analysis and then assigns a location to each data point in the n -dimensional space by using a nonlinear least-squares fit, minimizing the distances between the data points (24). The resulting data positions can be displayed by three-dimensional visualization. The degree of variation among the five SSPs of each strain was described by the minimal similarity value (MSV), which was defined as the lowest average similarity level among the five SSPs.

RESULTS AND DISCUSSION

Media that do not sustain optimal growth can influence the SSP strongly. A total of 17 AAB could be cultured on all five media as described above (see Table S1 in the supplemental material). The MDS plots obtained after peak-based numerical analysis of the five SSPs of each of these strains revealed that the diversity among the SSPs was variable, suggesting a strain-dependent effect of the growth medium on the mass spectra (Fig. 1).

The MSVs among SSPs of the 11 *Acetobacter* strains ranged from 50.5% (*Acetobacter fabarum* LMG 24630) to 81.0% (*A. fabarum* LMG 1701), with an average of $66.1\% \pm 9.4\%$ (Fig. 1A). The SSPs of strain LMG 1701 had 31 (52%) out of 60 peak classes in common. In contrast, only 12 (23%) out of 53 peak classes were common to the SSPs of strain LMG 24630. Nine additional shared peak classes could be detected in the SSPs obtained from the latter strain grown on four culture media but not YPM agar. Strain LMG 24630 did not grow well on the latter medium, and longer incubation times were required to obtain sufficient cell material for MALDI-TOF MS analysis. It is unclear what caused the aberrant SSP, but these slow-growing subcultures may have been sampled in a different physiological state, which may affect MALDI-TOF mass spectra (31). A similar decrease in the number of shared peak classes and concomitantly in the MSV was observed for *Acetobacter aceti* strain LMG 1504^T when it was grown on this culture medium. A total of 52 peak classes were detected for this particular strain, 19 of which were common to all five SSPs, and again 7 additional peak classes were present in the SSPs generated after growth on four media but not on YPM agar (Fig. 2). This was again associated with poor growth on YPM agar. Other strains like *A. aceti* LMG 5 (MSV of 66.9%) and *A. fabarum* LMG 1701 (MSV of 81.0%) grew well on YPM agar, which resulted in SSPs that were more comparable to the strain-specific SSPs generated from growth on the other four culture media (Fig. 1A).

The five SSPs of *Acetobacter pasteurianus* LMG 1590 had 10 (19%) out of 53 peak classes in common, yet the signal intensities of these common peak classes varied with the growth medium used (Fig. 3). The most intense peak observed after cultivation of LMG 1590 on GY, GYAE, and AAM agar was detected in the peak class at m/z 5,268.97 $\pm$ 6.22; each of these growth media comprises D-glucose as the sole carbon source. On the other hand, peak class m/z 6,790.66 $\pm$ 7.43 had the highest signal intensity in the SSPs generated after growth on the other two culture media, which comprised D-glucose (DMS agar), D-mannitol (YPM and DMS agar), and sorbitol (DMS agar) as C sources. Similar results were

FIG 1 MDS plots obtained after peak-based numerical analysis of the SSPs of 11 *Acetobacter* strains (A) and 1 *Gluconacetobacter* (*Ga.*) and 5 *Gluconobacter* (*G.*) strains (B) grown on five culture media. For each strain, single-color dots represent the SSPs derived from the five different culture media. The boxes display the MSVs among the respective strain-specific SSPs. The higher the level of similarity between two SSPs, the more closely located the dots are to each other.

obtained with three other *Acetobacter* strains grown on multiple media, suggesting a link between certain medium compounds, i.e., the carbon source, and the peptides detected. Remarkably, seven peak classes were unique to the DMS agar-derived SSP (Fig. 3). These seven peak classes were not present in SSPs of other strains grown on DMS agar, indicating that these are strain-specific rather than DMS agar-specific peak classes. A detailed comparison of SSPs generated on each of the five growth media revealed similar observations for the other *Acetobacter* strains where no growth medium-specific peak classes were found. This contrasts with results obtained by Dieckmann et al. (16), who detected two medium-related peak classes in the mass spectra of isolates grown on a blood-containing culture medium. However, they smeared the bacterial cells directly onto the MALDI-TOF MS target plate, an approach that increases the potential for contamination with medium-derived peaks (32).

The MSVs among SSPs of the *Gluconacetobacter* and *Gluconobacter* strains ranged from 58.9 to 78.5%, with an average of $70.5\% \pm 8.7\%$ (Fig. 1B). The lowest MSVs were observed for *Gluconobacter cerinus* LMG 1678 (58.9%, 15 out of 52 peak classes were shared) and *Gluconacetobacter liquefaciens* LMG 1381^T (61.6%, 19 out of 53 peak classes were shared), and for both strains, this was related to the RAE agar-derived SSPs, as both strains grew poorly on this culture medium. Omission of the RAE agar data improved the MSVs to 80.5 and 83.4% for strains LMG 1678 and LMG 1381^T, respectively. The highest MSV (78.5%) was observed for strain *Gluconobacter oxydans* LMG 1406, which grew well on all of the media, including RAE agar, and whose SSPs had 25 (48%) out of 52 peak classes in common (see Fig. S2 in the supplemental material).

The culture medium used affects strain level differentiation. The SSPs of eight *G. oxydans* strains (see Table S1 in the supplemental material) grown on AAM were compared by using the binary Dice

coefficient, as strain level differentiation is best accomplished by the presence-or-absence analysis of specific peak classes (13, 26, 33, 34). An MSV of 54.1% was obtained, and only 11 (13%) out of 85 peak classes were shared (Fig. 4). Although these 11 shared peak classes had the same *m/z* value, a strain-dependent variation in peak signal intensity was observed (Fig. 4). When the same eight strains were grown on YPM and GY agar, only 8/88 (9%, MSV of 59.5%) and 12/79 (15%, MSV of 49.3%) peak classes, respectively, were shared (Table 1). An analysis of all of the SSPs showed that only 7 (7%) out of 105 peak classes were consistently present in these *G. oxydans* SSPs, irrespective of the growth medium used (Table 1). However, the same analysis also revealed many strain-specific peak classes in SSPs obtained after growth on each of the three culture media (Table 2) (to identify strain-specific peak classes, the SSPs of all of the strains obtained after growth on the same culture medium were considered). The number of strain-specific peak classes varied with the growth medium used (Table 2). For instance, the LMG 1674 SSPs were characterized by four and two strain-specific peak classes when that strain was grown on AAM and GY agar, respectively; however, no strain-specific peak classes were found when LMG 1674 was grown on YPM agar. Similarly, the LMG 1683 SSPs were characterized by 3, 4, and 0 strain-specific peak classes when that strain was grown on AAM, YPM, and GY agar, respectively. It should also be noted that some of these strain-specific peak classes are present in SSPs obtained after growth on multiple culture media. For example, the peak class characterized by an *m/z* value of about 5,591 was present in each of the strain LMG 1676 SSPs, while for strains LMG 1683 and LMG 1398, each strain-specific peak class was observed after growth on a single growth medium only (Table 2).

Likewise, five *Acetobacter cerevisiae* strains (see Table S1 in the supplemental material) were cultured on AAM, DMS, YPM, and GY agar and their SSPs were compared by using the binary Dice coefficient. Eight (6%) out of 142 peak classes were common to all

FIG 2 Mass spectra derived from *A. aceti* strain LMG 1504^T grown on the five culture media indicated in the black boxes on the right. All of the shared peak classes (19 out of 52) of the five SSPs are indicated by asterisks, and the corresponding *m/z* values are represented in the black boxes at the top. The seven additional peak classes after omission of the YPM-derived SSP are indicated by squares within the spectra, and the corresponding *m/z* values are shown in the gray boxes at the top. r. int., relative intensity.

of the SSPs generated after growth on all four culture media (see Table S3 in the supplemental material). The same analysis also revealed strain-specific peak classes (see Table S4 in the supplemental material), and again the number of strain-specific peak classes varied with the growth medium used. For example, the LMG 1545 SSPs were characterized by two, eight, five, and five strain-specific peak classes when that strain was grown on AAM, DMS, YPM, and GY agar, respectively. Moreover, some of these strain-specific peak classes were common to SSPs obtained after growth on multiple media. For instance, eight strain-specific peak classes (*m/z* values of about 4,300,

5,335, 6,366, 6,399, 6,574, 7,889, 10,676, and 12,737) were present in each SSP of strain LMG 1699 grown on the four different culture media. Similarly, two strain-specific peak classes characterized by *m/z* values of about 6,560 and 7,126 were shared in all of the LMG 1599 SSPs. In contrast, the four SSPs of strain LMG 1545 had no strain-specific peak classes that were present in each of the SSPs, but still several peak classes were present in two or three SSPs (see Table S4 in the supplemental material).

These results confirm that the number of strain-specific peak classes is culture medium dependent and demonstrate that the

FIG 3 Mass spectra derived from *A. pasteurianus* strain LMG 1590 grown on five different culture media. The shared peak classes (10 out of 53) are indicated by asterisks, and their m/z values are shown in the black boxes at the top. The seven unique peak classes detected after culturing on DMS agar are indicated with gray squares, and the corresponding m/z values are shown in the gray boxes at the top. The two most abundant peak classes of these seven DMS-specific peak classes are inside the rectangle. r. int., relative intensity.

selected culture medium affects the potential for strain level differentiation.

A growth medium-dependent core set of peak classes decreases with an increasing number of strains. During the analysis of strain-specific peak classes, the number of shared peak classes appeared to decrease when more strains were examined. To investigate this in more detail, the SSPs of the eight *G. oxydans* strains grown on GY, AAM, and YPM agar were reanalyzed. Per growth medium, the effect of the sequential inclusion of an SSP of an additional strain was analyzed in all of the possible combinations.

Subsequently, the number of shared peak classes was determined for all of the possible combinations and plotted as a function of the number of strain SSPs sequentially added to the data set. The plots represented in Fig. 5 visualize this decrease in shared peak classes with increasing numbers of strain SSPs. The lowest set of shared peak classes (8 [9%] out of 88) was found when the eight strains were grown on YPM agar rather than on AAM (11 [13%] out of 85) or GY agar (12 [15%] out of 79) (Table 1). Seven out of 105 peak classes were common to the SSPs of the strains grown on the three media (Table 1). The same study was performed with the five

FIG 4 Mass spectra generated for eight *G. oxydans* strains grown on AAM agar. The shared and strain-specific peak classes are indicated by asterisks and squares, respectively. Annotations of the peak classes are shown in black or gray boxes for, respectively, the common or strain-specific peaks. r. int., relative intensity.

TABLE 1 Peak classes common to SSPs derived from eight *G. oxydans* strains grown on AAM, YPM, or GY agar^a

<i>m/z</i> value	Peak class		
	AAM ^b	YPM ^c	GY ^d
2,460.83 ± 3.97	X		
3,090.78 ± 4.55			X
3,188.40 ± 4.55	X		X
4,178.58 ± 5.34	X	X	X
4,514.83 ± 5.61			X
4,926.60 ± 5.94	X	X	X
4,968.53 ± 5.97	X	X	X
5,211.47 ± 6.17		X	
5,627.75 ± 6.50	X		X
6,186.62 ± 6.95	X	X	X
6,382.37 ± 7.11	X	X	X
7,431.76 ± 7.95	X		X
8,362.86 ± 8.69	X	X	X
9,942.86 ± 9.95	X	X	X

^a Only the peak classes marked with an X are present in the respective medium, and the peak classes common to all of the SSPs are in bold (7/105 [7%]).
^b Eleven (13%) of 85 peak classes shared.
^c Eight (9%) of 88 peak classes shared.
^d Twelve (15%) of 79 peak classes shared.

A. cerevisiae strains grown on four culture media. Although only five strains were examined, a similar decrease in shared peak classes was observed after growth on AAM, DMS, YPM, and GY agar (see Fig. S5 in the supplemental material).

These data suggest that there is a core set of peak classes that is consistently present when multiple strains of a species are examined and that this core is culture medium dependent. The data confirm that multiple reference strains grown on multiple growth media should always be analyzed to cover the intraspecies diversity when constructing a MALDI-TOF MS identification database (35). It also demonstrates that the decrease in shared peak classes can be used as a measure of taxonomic intraspecies diversity.

Species level differentiation is growth medium independent and is not biased by the similarity coefficient used. The MDS plots derived from the peak-based numerical analysis as visualized in Fig. 1 demonstrated that most of the AAB species were clearly distinguishable, irrespective of the growth medium used. One exception, however, is *A. malorum* strain LMG 1746^T, which could not be distinguished from the *A. cerevisiae* strains (Fig. 1A). Similar results were obtained in the study by Andrés-Barrao et al. (5). It is well known that these two species are very closely related and can only be distinguished by minimal differences revealed primarily by sequence analysis of the 16S-23S rRNA gene internal transcribed spacer region (36, 37). In addition, two strains, i.e., *A. pasteurianus* LMG 1590 and *G. cerinus* LMG 1678, did not group with the other strains of these respective species (Fig. 1). *G. cerinus* LMG 1678 had only 2 out of 96 peak classes in common with other *G. cerinus* strain SSPs, suggesting that it may be erroneously assigned to this species. The latter was confirmed by sequence analysis of the housekeeping genes *rpoB* and *dnaK* as described by Cleenwerck et al. (38), and strain LMG 1678 should therefore be considered misclassified (A. D. Wieme, unpublished data). Similarly, *A. pasteurianus* strain LMG 1590 also grouped separately and had only 5 out of 109 *A. pasteurianus* SSP peak classes in common, which confirms the established taxonomic diversity of this species (39).

In addition to the peak-based analysis with the binary Dice coefficient, the same data set was also analyzed by using the Pear-

TABLE 2 Strain-specific peak classes found in SSPs derived from eight *G. oxydans* strains cultured on three growth media^a

Strain	<i>m/z</i> value for indicated peak class		
	AAM	YPM	GY
LMG 1398	3,867.46 ± 5.09		4,616.35 ± 5.69
		5,318.36 ± 6.25 10,644.00 ± 10.52	
LMG 1406	3,728.15 ± 4.98	4,616.79 ± 5.69	
	5,036.67 ± 6.09		5,406.82 ± 6.33
		6,341.08 ± 7.07 7,158.83 ± 7.73 7,224.86 ± 7.78	
	10,822.60 ± 10.66	10,821.40 ± 10.66	9,971.87 ± 9.98
LMG 1408 ^T	2,627.62 ± 4.10 3,910.06 ± 5.13	2,627.42 ± 4.10	2,627.51 ± 4.10 3,910.36 ± 5.13 3,944.98 ± 5.16 4,356.70 ± 5.49 5,318.39 ± 6.25 6,819.21 ± 7.46
	7,852.49 ± 8.28	7,852.91 ± 8.28	7,922.93 ± 8.34 8,719.65 ± 8.98
	10,643.90 ± 10.52		
LMG 1519	4,895.25 ± 5.92	5,035.04 ± 6.03 5,395.70 ± 6.32 5,934.74 ± 6.75	4,894.64 ± 5.92 9,795.83 ± 9.84
LMG 1581		5,267.04 ± 6.21	6,608.63 ± 7.29 7,254.69 ± 7.80
	7,369.34 ± 7.90		7,741.57 ± 8.19
	7,829.83 ± 9.86	9,827.70 ± 9.86	9,828.18 ± 9.86 10,770.30 ± 10.62
	10,770.50 ± 10.62		
LMG 1674	5,394.77 ± 6.32 7,676.08 ± 8.14 9,967.32 ± 9.97 10,797.20 ± 10.64		2,911.32 ± 4.33 7,675.78 ± 8.14
LMG 1676		3,681.46 ± 4.95	3,728.02 ± 4.98 3,894.66 ± 5.12
	5,591.13 ± 5.47 5,954.07 ± 6.76 6,239.26 ± 6.99	4,082.31 ± 5.27 5,591.62 ± 6.47 6,239.71 ± 6.99 6,652.23 ± 7.32	5,591.79 ± 6.47 5,954.59 ± 6.76 6,239.69 ± 6.99
	7,255.28 ± 7.80		6,904.59 ± 7.52
	7,796.98 ± 8.24 7,867.55 ± 8.29 7,938.71 ± 8.35	7,395.64 ± 7.92 7,796.61 ± 8.24 7,867.55 ± 8.29	7,796.76 ± 8.24 7,867.43 ± 8.29
			8,170.35 ± 8.54 9,812.64 ± 9.85 12,488.40 ± 11.99
LMG 1683	6,956.09 ± 7.56 7,905.84 ± 8.32 14,902.00 ± 13.92	3,309.27 ± 4.65 3,970.36 ± 5.18 5,334.88 ± 6.27 6,679.51 ± 7.34	

^a The strain-specific peak classes common to the strain-specific SSPs of bacteria grown on two or three different culture media are in bold.

FIG 5 The decrease in shared peak classes among SSPs of eight *G. oxydans* strains grown on GY (A), AAM (B), or YPM agar (C). The number of shared peak classes is plotted as a function of the number of strain-specific SSPs sequentially added. Squares represent the average of shared peak classes per sequential addition of a strain-specific SSP.

son product-moment correlation coefficient, taking into account the complete molecular mass range (2 to 20 kDa) and the averaged signal intensities. In general, the MDS plots were similar to those based on the comparison of SSPs by means of the binary Dice coefficient and thus facilitate species differentiation with the same exceptions discussed above (Fig. 6). The MSVs obtained through the curve-based numerical analysis of the 11 *Acetobacter* strain SSPs ranged from 44.7 to 92.0%, with an average of $68.4\% \pm 13.6\%$. For the SSPs of the *Gluconacetobacter* and *Gluconobacter* strains, the MSVs ranged from 65.0 to 94.4%, with an average of $81.9\% \pm 11.3\%$.

In general, the curve-based analysis yielded more variability between strain-specific SSPs obtained after growth on different culture media than did peak-based analysis. However, curve-based analysis of some strains, like *A. pasteurianus* LMG 1262^T,

yielded MSV values among SSPs that were higher than those obtained by peak-based analysis (MSV of 89.3% compared to 65.2%), and this was related to errors or problems during peak class assignment. For instance, in *A. pasteurianus* LMG 1262^T SSPs, the peak characterized by m/z $7,211.49 \pm 7.77$, which was present in the YPM-derived SSP, was not assigned to the m/z $7,197.90 \pm 7.76$ peak class (Fig. 7; see Fig. S6 in the supplemental material). Also, a double peak that was present in the *G. oxydans* strain LMG 1406 SSPs (see Fig. S2 in the supplemental material) was not consistently annotated during preprocessing because of the limited resolution achieved by MALDI-TOF MS analysis. This double peak was therefore not detected during summarization and had no influence on the peak-based numerical analysis (MSV of 78.5%), while it was accounted for in the curve-based analysis (MSV of 94.4%).

FIG 6 MDS plots obtained after curve-based numerical analysis of the generated SSPs of 11 *Acetobacter* strains (A) and 1 *Gluconacetobacter* (*Ga.*) and 5 *Gluconobacter* (*G.*) strains (B) grown on five different culture media. For each strain, single-color dots represent the SSPs derived from growth on five different culture media. The MSVs among each specific strain's SSPs are shown within the respective boxes.

FIG 7 Mass spectra of *A. pasteurianus* strain LMG 1262^T grown on five different culture media. The shared peak classes are indicated by asterisks and annotated in the black boxes at the top. The rectangle highlights the peak that shifted in the mass spectrum derived from the YPM SSP and that was erroneously missed by the peak detection algorithm. r. int., relative intensity.

In conclusion, the present study demonstrates that the culture medium can have a profound effect on the mass spectra of AAB, as observed in the presence and various signal intensities of peak classes. Growth media that do not sustain optimal growth can influence the SSP strongly. The growth medium effects do not disturb species level differentiation but strongly affect the potential for strain level differentiation. A decrease in the number of shared peak classes was observed with an increasing number of strains examined and may be indicative of the degree of intraspecies diversity. Altogether, our data demonstrate that a well-constructed and robust MALDI-TOF MS identification database ide-

ally comprises mass spectra of multiple reference strains per species grown on different culture media.

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