Evaluation of Matrix-Assisted laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) in Comparison to 16S rRNA Gene Sequencing for Species Identification of Nonfermenting Bacteria

A. Mellmann[1*], J. Cloud[2], Th. Maier[3], U. Keckevoet[1], I. Ramminger[1], P. Iwen[4], J. Dunn[5], G. Hall[6], D. Wilson[6], P. LaSala[7], M. Kostrzewa[3], D. Harmsen[8]

[1]Institute for Hygiene, University Hospital Muenster, D-48149 Muenster, GERMANY; [2]ARUP Institute for Clinical and Experimental Pathology®, Salt Lake City, Utah, USA; [3]Bruker Daltonik GmbH, Leipzig, GERMANY; [4]University of Nebraska Medical Center, Department of Pathology and Microbiology, Omaha, Nebraska, USA; [5]Cook Children's Medical Center, Fort Worth, Texas, USA; [6]Cleveland Clinic Foundation, Microbiology, Cleveland, Ohio, USA; [7]University of Texas Medical Branch, Dept. of Pathology, Clinical Microbiology, Galveston, Texas, USA; [8]Department for Periodontology, University Hospital Muenster, D-48149 Muenster, GERMANY

*Corresponding author:
Dr. med. Alexander Mellmann, MD
Institut fuer Hygiene, Universitätsklinikum Muenster
Robert-Koch-Str. 41
48149 Muenster, Germany
Phone: +49 251 8352316
Fax: +49 251 8355688
Email: mellmann@uni-muenster.de

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Abstract

Nonfermenting bacteria are ubiquitous environmental opportunists causing infection especially in compromised patients. Due to their limited biochemical reactivity and different morphotypes, misidentification by classical phenotypic means occurs frequently. Therefore, we evaluated matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for species identification. Using 248 nonfermenting culture collection strains composed of 37 genera most relevant for human infections, a reference database was established for MALDI-TOF MS-based species identification following manufacturer's recommendations for microflex measurement and MALDI BioTyper™ software (Bruker Daltonik GmbH, Leipzig, Germany), i.e. using a mass range of 2,000 to 20,000 Dalton and a new pattern matching algorithm. To evaluate the database, 80 blind-coded clinical nonfermenting bacterial strains were analyzed. As a reference method for species designation, partial 16S rRNA gene sequencing was applied. Using 16S rRNA gene sequencing, 57 of the 80 isolates produced unique species identification (≥ 99% sequence similarity); 11 further isolates gave ambiguous results at this threshold and were rated as identified at genus level only. 10 isolates were identified to genus level (≥ 97% similarity) and 2 isolates were below this threshold and counted as not identified and excluded from further analysis. MALDI-TOF MS identified 67 of the included 78 isolates (85.9%) in agreement with the reference method, 9 were misidentified and 2 unidentified. Identification of 10 randomly selected strains was 100% correct using 3 different mass spectrometers and 4 different cultivation media. Thus, MALDI-TOF MS-based species identification of nonfermenting bacteria provided accurate and reproducible results within 10 minutes without any substantial costs for consumables.

INTRODUCTION

The genera *Pseudomonas*, *Burkholderia*, *Stenotrophomonas* and others belong to the large group of nonfermenting bacteria that are unable to ferment sugars. Nonfermenting bacteria are ubiquitous environmental opportunists and some species can cause severe infections especially in immuno-compromised patients (30). Especially in the group of cystic fibrosis patients, nonfermenting bacteria are a main cause of morbidity and mortality (18). Furthermore, antibiotic resistance of some nonfermenting bacterial species often complicates therapy (25, 30). An accurate species diagnosis is therefore critical not only because the prognosis of an infection differs significantly depending on the species identification (33) but also because in some cases, e.g., infections with *Burkholderia cepacia* genomovar III (5), strict infection control measures have to be established for cystic fibrosis patients (5, 31).

In a routine clinical laboratory, species identification of cultured isolates usually relies on phenotypic methods such as panels of biochemical reactions, antibiotics, and fatty acid patterns (28). However, due to their limited biochemical reactivity and variable morphology, nonfermenters are frequently misidentified by classical methods (21, 26). Moreover, isolates from chronic infections often loose their characteristic phenotypes (12, 26). To overcome these drawbacks, genotypic identification methods have become widely used, most of them based on the polymorphism of the 16S rRNA genes. Species-specific PCRs, restriction patterns, or more recently partial DNA sequencing are used for species identification. Nowadays, sequencing of the 16S rRNA gene is accepted as reference method for species identification, and several studies have shown its superiority to phenotypic methods for various groups, including for nonfermenting bacteria, e.g. (2, 3, 8, 9, 14, 15, 27). However, a prerequisite for valid identification results is the use of an extensive and comprehensive quality-controlled database (6, 16).

47 Based on analysis of the protein composition of a bacterial cell, matrix-assisted laser
48 desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a new
49 technology for species identification. By measuring the exact size of peptides and small proteins,
50 which are assumed to be characteristic for each bacterial species, it is possible to determine the
51 species within few minutes starting from whole cells, cell lysates, or crude bacterial extracts (13,
52 17, 22). However, due to difficulties in its reproducibility of results using different cultivation
53 conditions and the limited availability of reference data sets, MALDI-TOF MS has not yet been
54 widely used for species identification.

55 In this study, we therefore established a reference database for MALDI-TOF MS-based
56 nonfermenter identification, analyzed the reproducibility using different cultivation conditions
57 and mass spectrometer instruments, and evaluated the methodology using 80 blind-coded clinical
58 nonfermenter strains that were analyzed by partial 16S rRNA gene sequencing as the reference
59 method.

MATERIAL AND METHODS

Bacterial strains. To establish a reference database for MALDI-TOF MS based species identification, 248 nonfermenter culture collection strains composed of 37 genera most relevant for human infections were used (Table 1).

The 80 clinical nonfermenting strains that were used to evaluate the MALDI-TOF MS reference database were recovered from clinical specimens received by the Cleveland Clinic Foundation (Cleveland, OH), University of Texas Medical Branch (Galveston, TX), Cook Children's Medical Center (Fort Worth, TX), and the University of Nebraska Medical Center (Omaha, NE) during the 2004 calendar year.

16S rRNA gene sequencing and sequence analysis. DNA isolation, amplification, and cycle sequencing of the clinical isolates were performed at the ARUP Institute for Clinical and Experimental Pathology® (Salt Lake City, UT). DNA was extracted as previously described (27). Four microliters of the extract were used in each PCR reaction. PCR was performed in a total volume of 40 µl containing 1x FastStart DNA Master Plus SYBR Green (Roche Diagnostics Corp., Indianapolis, IN) and 500 nM of each primer 16S-27f and 16S-519r (27) at 4 mM Mg^{2+} concentration. Thermal cycling reactions were performed using the RotorGene 3000 real-time PCR instrument (Corbett Research, Sydney, Australia) and consisted of an initial denaturation (10 min at 95°C) followed by 35 cycles of denaturation (30 s at 95°C), annealing (20 s at 55°C), and extension (30 s at 72°C), with a single final extension (2 min at 72°C). Negative controls, containing water instead of template DNA, were run in parallel in each run. The PCR product was purified by an enzymatic method with exonuclease I (New England Biolabs GmbH, Frankfurt-Hoechst, Germany) and shrimp alkaline phosphatase (Amersham Pharmacia Biotech, Freiburg, Germany) modified from Dugan et al. (11). Briefly, 5 µl of the PCR product was

84 incubated with 1 U of each enzyme at 37°C for 30 minutes. The enzymes were then inactivated at
85 80°C for 15 minutes and the PCR products stored at 4°C. The amplicons were sequenced using
86 the ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied
87 Biosystems, Foster City, CA). The sequencing reaction required 0.5 µl of premix from the kit, 1.8
88 µl TrisHCl/$MgCl_2$ buffer (400mM Tris-HCl; 10mM $MgCl_2$), 10 pmol of sequencing primer
89 (same as PCR primers), and 2 µl of the cleaned PCR product in a total volume of 10 µl.
90 For sequencing chemistry mixes, the same primers as those used in the PCR reaction
91 (16S-27f or 16S-519r) were used to obtain forward and reverse sequence data for partial 5'-16S
92 rRNA gene sequencing. All sequencing reactions were performed using a standard thermocycler
93 to complete 25 cycles of denaturation (10 s at 96°C), annealing (5 s at 53°C), and extension (4
94 min at 60°C). The sequencing products were purified using the Centri-Sep Spin Columns
95 (Princeton Separations, Adelphia, NJ), followed by preparation for running on the ABI Prism 310
96 or 3100 Avant Genetic Analyzer in accordance with the instructions of the manufacturer (Applied
97 Biosystems). The double-stranded sequences corresponding to *E. coli* 16S rRNA gene positions
98 54 to 510 were analyzed in accordance to the RIDOM procedure (16).
99
100 **MALDI-TOF MS sample preparation, spectra generation, and data analysis.** A
101 colony of a fresh overnight culture was used for the sample preparation before measurement. The
102 material was thoroughly suspended in 300 µl doubly distilled water and 900 µl ethanol were
103 added and mixed well. Prior to shipment to the place of measurement, the samples in
104 ethanol/water were centrifuged, the supernatant was removed and the pellets were dried. For
105 sample extraction, 50 µl of formic acid (70% in water) and, after thorough mixing, 50 µl
106 acetonitrile were added to the bacterial pellet. After a centrifugation for 2 minutes at 13,000x g,
107 one µl of the supernatant containing the bacterial extract was transferred to a sample position of a

ground steel MALDI target plate and allowed to dry at room temperature. Subsequently, the sample was overlaid with 2 µl of MALDI matrix (saturated solution of HCCA in 50% acetonitrile/2.5% trifluor acetic acid) and dried again.

For database construction and validation, measurements were performed using a microflex LT (Bruker Daltonik GmbH, Leipzig, Germany) bench-top mass spectrometer equipped with a 20 Hz Nitrogen laser (parameter setting: IS1 20 kV, IS2 18.5 kV, lens 8.5 kV, detector gain 2650 V, no gating). Spectra were recorded in the positive linear mode for the mass range of 2,000 to 20,000 Dalton at maximum laser frequency. The database references (main spectra) for the newly investigated bacteria were constructed using the automated functionality of the MALDI BioTyper™ 1.1 software package (Bruker Daltonik GmbH). Briefly, for each database entry 20 individually measured mass spectra were imported into the software. After smoothing, baseline correction, and peak-picking the resulting peak lists were used by the program to calculate and to store a main spectrum containing the information about average peak masses, average peak intensities, and frequency.

For microorganism identification, raw spectra of the unknown bacteria were imported into the MALDI BioTyper™ software and analyzed by standard pattern matching (default parameter settings) against the BioTyper™ database (an integrated part of the BioTyper™ software) containing main spectra of 2506 microorganisms as reference data. The reference database consisted of the 248 newly created main spectra for the investigated nonfermenting bacteria and other clinical, veterinary, or environmental bacterial strains. Sample preparation of the 248 nonfermenter reference strains was performed at the Institute for Hygiene, University Hospital Muenster (Muenster, Germany). The clinical nonfermenting strains for evaluation were prepared at the ARUP Institute for Clinical and Experimental Pathology® (Salt Lake City, UT).

Preparation of the ground steel plate, mass spectrometry of 96 samples, and database similarity search for species identification took about three hours for the microflex instrument. A single sample could be identified in approx. 10 minutes.

MALDI-TOF MS reproducibility testing. Complementary to the already determined intra-species (4, 36) and inter-laboratory (37) reproducibility of MALDI-TOF MS-based species identification, spectra of 10 randomly chosen nonfermenter strains (*Brevundimonas aurantiaca* DSM 4731, *B. intermedia* DSM 4732, *B. andropogonis* DSM 9511, *B. caribensis* DSM 13236, *Flavobacterium johnsoniae* DSM 2064, *F. mizutaii* DSM 11724, *Pseudomonas aeruginosa* DSM 50071, *P. beteli* LMG 978, *P. boreopolis* LMG 979, *P. extremorietalis* DSM 15824) were determined under different conditions. First, the testing was done by parallel measurement on three different MALDI-TOF MS instruments (microflex LT, autoflex II TOF/TOF [Bruker Daltonik GmbH] with a 50 Hz nitrogen laser, ultraflex III TOF/TOF [Bruker Daltonik GmbH] with a 200 Hz smartbeam^{TM} laser) to test the instrument comparability. The parameter settings for the additional instruments were as follows: IS1 20 kV, IS2 18.7 kV, lens 8.0 kV, detector gain 1756 V, gating max. 1500 Da (autoflex) and IS1 25 kV, IS2 23.45 kV, lens 6.0 kV, detector gain 1650 V, gating max. 1500 Da (ultraflex). Second, the influence of different cultivation conditions was characterized by cultivation of the 10 strains on 4 different media (Columbia blood agar, chocolate agar, Muller-Hinton agar, and tryptic soy agar; Heipha, Eppelheim, Germany) at 30°C under aerobic conditions for 48 h. Finally, the influence of the age of bacterial cultures was investigated by analyzing three strains (*B. aurantiaca* DSM 4731, *B. caribensis* DSM 13236, and *P. aeruginosa* DSM 50071) that were cultivated for 48 h at 30°C on Columbia blood agar and subsequently stored for 2, 5, and 7 days at room temperature. All samples for reproducibility testing were blind-coded for MALDI-TOF MS analysis.

155

156 **Evaluation of MALDI-TOF MS based species identification using clinical isolates.** To
157 evaluate the MALDI-TOF MS reference database, 80 blind-coded clinical nonfermenters were
158 analyzed. As the reference method for species designation, partial 16S rRNA gene sequencing
159 was used. The reference partial 16S rRNA gene sequence database (*E. coli* 16S rRNA gene
160 positions 54 to 510) included all 248 nonfermenter culture collection strains used for MALDI-
161 TOF reference (Table 1). Sequence data were stored and analyzed using the RIDOM framework
162 (27). Sequence similarities of ≥ 99% were used for identification at species level and ≥ 97% for
163 identification at the genus level, respectively (3). A further differentiation was made at species
164 level between unique and ambiguous similarity search results (multiple top scoring results), the
165 latter were rated as identification at genus level only. Sequence similarities below 97% were rated
166 as not identifiable. MALDI-TOF MS results based on log(score) values calculated by the
167 BioTyper™ software were compared to the 16S rRNA gene sequence similarity search results.
168 BioTyper™ software requires log(score) values ≥ 2.0 for identification at species level and
169 values of between < 2 and ≥ 1.7 for identification at genus level, respectively. Results based on
170 log(score) values < 1.7 were rated as not identifiable by the software. These BioTyper™
171 thresholds were empirically determined based on an in-house database comprising more than
172 2,800 bacterial strains that were either culture collection strains or well characterized clinical
173 strains.

174 To determine the discriminatory ability of 16S rRNA gene sequencing and MALDI-TOF
175 MS within certain groups of strains, the pairwise distances were calculated and displayed in a
176 UPGMA-tree using the MEGA software version 4.0 (37).

177 **RESULTS**

178 From all 248 culture collection strains representing the majority of all clinical relevant
179 nonfermenter species, the cell extracts gave sufficient spectra and were included into the
180 MALDI-TOF MS reference database (database version 1.0).

181 To investigate the reproducibility of spectra generated with different mass spectrometers,
182 10 culture collection strains were randomly chosen to be analyzed with three different mass
183 spectrometers. In all 10 strains, the log(score) results gave the same species identification result
184 irrespective of the mass spectrometer with all log(score) values above 2.0. Figure 1 shows a
185 representative result of the strain Galv12 measured with the three different spectrometers. To
186 determine the influence of different cultivation media on the quality of the spectra, the 10
187 cultured strains were also analyzed after cultivation on four different media. Furthermore, three
188 strains were analyzed after up to seven days storage on Columbia blood agar at room
189 temperature. In all cases, mass spectrometry resulted in identical, correct identification results
190 relative to the reference database.

191 All 80 nonfermenting strains from the clinical specimens gave sufficient spectra for
192 species identification. In parallel to MALDI-TOF MS identification, all strains were analyzed by
193 partial 16S rRNA gene sequencing as reference method. By sequence comparison analysis, 57
194 isolates were unambiguously identified at species level (≥ 99% sequence similarity) and 10
195 isolates at genus level (≥ 97%) by partial 16S rRNA gene sequencing and sequence similarity
196 search against the RIDOM database. Eleven isolates (W35, W12, NF18, W29, W23, W26, W14,
197 W10, W06, Galv17, Galv23) gave multiple species as a result and these were therefore treated as
198 identified at genus level only. The remaining two isolates (NF08, Neb38) were below the
199 sequence threshold of 97% similarity and were therefore excluded from further analysis.

200 The identification results obtained by MALDI-TOF MS using 16S rRNA gene sequencing
201 as the reference method are shown in Table 2 together with the achievable levels of
202 identification. In total, MALDI-TOF MS identified 67 of the 78 isolates (85.9%) concordant with
203 the sequencing results; of these, 47 of 57 (82.5%) were identified at species level (log(score) ≥
204 2.0) and 20 of 21 (95.2%) at genus level (log(score) of between < 2.0 and ≥ 1.7), respectively
205 (Table 3). Of the remaining 11 isolates, four isolates (Neb14, Neb20, W15, W18) with a
206 log(score) ≥ 2.0 were correctly identified at genus level but with a discordant species designation
207 in comparison to the reference method. In four isolates (W03, W08, W22, W28), the correct
208 genus was determined by the MALDI BioTyper™ software with log(score) values between 1.7
209 and 2.0, whereas 16S rRNA gene sequencing gave a species identification. In sample NF18, the
210 genus determination was discordant to 16S rRNA gene sequencing. Finally, the two samples with
211 log(score) values < 1.7 (Neb37 and W16) were rated as non-identifiable by MALDI-TOF MS
212 (Table 3).

213 To investigate the discriminatory ability of closely related species using MALDI-TOF MS
214 in comparison to 16S rRNA gene sequencing, nine species of the former *Burkholderia cepacia*-
215 complex were investigated in detail. By partial 16S rRNA gene sequencing, the three strains of *B.*
216 *cepacia* DSM 7288 (*B. cepacia* type strain), *B. cepacia* LMG 2161 (the former reference strain
217 for *B. cepacia*-complex genomovar I), and *B. vietnamiensis* LMG 10929 (formerly genomovar V)
218 were indistinguishable (Fig. 2A). Likewise, the three strains of *B. stabilis* LMG 14294 (formerly
219 genomovar IV), *B. pyrrocinia* LMG 14191 (formerly genomovar IX), and *B. ambifaria* LMG
220 11351 (formerly genomovar VII) were also indistinguishable. In contrast, the MALDI-TOF MS
221 was able to differentiate among all species of the former *B. cepacia*-complex (Fig. 2B).

DISCUSSION

The correct species identification of nonfermenting bacteria from clinical specimens and from samples of the patient's environment is of major importance for optimal patient management and to establish effective infection control measures. To overcome the problems related to classical phenotypic species identification methods, this study evaluated the capability of MALDI-TOF MS to identify these species. As a reference method for comparison, partial 16S rRNA gene sequencing was chosen. This method has been shown to be a reliable and universal technique for species identification in clinical microbiology (6, 35). A reference database of MALDI-TOF MS spectra comprising well characterized culture collection strains only was established and evaluated using blind-coded nonfermenter isolates from clinical specimens (Table 2). With 82.5% correctly identified isolates at species and 95.2% at genus level, respectively (Table 3), MALDI-TOF MS-based identification showed a high concordance to the reference method. In contrast, the use of two current phenotypic identification methods (API 20 NE, VITEK 2) resulted in correct identification rates of only 61% and 54% in a different study for the nonfermenters (3).

The identification of species belonging to the former *B. cepacia*-complex, which was reclassified based on *recA* polymorphisms due to too small variations within the rRNA operon (10, 23), is especially problematic and has often led to misidentifications in the past (21, 26). In this study, one clinical strain was identified as a member of the former *B. cepacia*-complex (W35). Since the reference method (16S rRNA gene sequencing) was shown to be unable to differentiate among strains of the former *B. cepacia*-complex (Fig. 2A), identification was only achieved to the level of the *B. cepacia*-complex. However, MALDI-TOF MS identified this strain as *B. multivorans* with a high log(score) of 2.379 showing the ability of this method as a possible means to differentiate species within this complex. Due to the clinical relevance of these species,

246 a detailed MALDI-TOF MS analysis of the former *B. cepacia*-complex reference strains was
247 performed (Fig. 2B). This analysis corroborated the higher discriminatory ability of MALDI-
248 TOF MS in comparison to 16S rRNA gene sequencing (36) and enabled a valid identification of
249 all members of the former *B. cepacia*-complex.

250 The proof-of-principle of the utilization of MALDI-TOF MS for bacterial species
251 determination was already shown a decade ago (7, 17, 22). However, MALDI-TOF MS has not
252 been widely used in clinical microbiology due to difficulties in the reproducibility among
253 different MALDI-TOF mass spectrometer instruments, the variation in cultivation conditions,
254 and the limited availability of reference data sets. To address these challenges, we analyzed the
255 spectra generated by our method with three different mass spectrometers and four different
256 cultivation media from 10 randomly chosen strains. Furthermore, the influence of the storage of
257 cultures at room temperature before MALDI-TOF MS processing was also investigated. Under
258 all conditions using a defined culture set, the spectra showed a high uniformity (Fig. 1) and a
259 reference database search gave the correct identification with log(score) values $\geq$ 2.0 in all
260 samples tested. We conclude that using the established MALDI BioTyper™ database the
261 reproducibility is high and independent from the mass spectrometer instruments used and the
262 conditions tested.

263 In contrast to earlier work (7), which applied a mass range of 550 to 2,200 Dalton, a range
264 of 2,000 to 20,000 Daltons was used in this study. This mass range represents predominantly
265 ribosomal proteins obtained from whole bacteria or crude bacterial extracts (24, 32). These
266 proteins are abundant in the cell and positively charged, which favors their measurement in
267 MALDI-TOF mass spectrometry and results in a relative robustness against different culture
268 conditions. Another reason for the improved reproducibility is the use of a dedicated algorithm of
269 the MALDI BioTyper™ software based on pattern comparisons. Different approaches for

bacterial identification based on MADLI-TOF MS fingerprint spectra have been described (1, 19, 20, 29). The MALDI BioTyper™ software applies an adopted pattern matching to compare unknown mass spectra with reference data stored in a database. In a first step, the software extracts a peak list of the mass spectrum after smoothing and baseline subtraction. This peak list is compared with each entry of the reference database and thereby the unknown peak list is aligned with each main spectrum by a dedicated recalibration algorithm. Therefore, even suboptimal mass accuracies of measurements are sufficient for a successful analysis. In addition, the correlation of intensities of matching peaks is determined. Based on the peak matches and intensity correlation, a score and the logarithm of this score is calculated (log[score]), with a value between 0 and 3 (0 to 100% pattern match). After comparison of an unknown spectrum with all main spectra of the database, all log(score)s are ranked. Finally, MALDI-TOF MS-based species identification was hampered in the past due to the lack of a comprehensive reference database built on well characterized strains, e.g. culture collection strains, which is a prerequisite to reflect at least partially the natural diversity of bacteria. In this study, a spectra database of 248 reference nonfermenting bacteria was initially established and subsequently evaluated using blind-coded clinical isolates.

One advantage to the current study is that the same reference strains were used in both the 16S rRNA gene database and in the MALDI-TOF MS database. Comparison of databases with different strains would have prevented an accurate comparison between the methods. One limitation of the present study is the exclusive use of 16S rRNA gene sequencing as a reference identification. This method was not able to identify all clinical isolates at the species level (≥ 99% sequence similarity). Ideally, a polyphasic approach in identification with the combined use of phenotypic and genotypic characteristics should be considered. However, due to the diversity within species of nonfermenting bacteria and in the current changes in bacterial nomenclature

294 within this group, there is no clear defined strategy to identify the nonfermenting bacteria.
295 Moreover, 16S rRNA gene sequencing has already been evaluated as superior to phenotypic
296 identification techniques for the nonfermenting bacteria (3, 14). It is noteworthy that in 12 of the
297 21 isolates in this study identified at the genus level only by 16S rRNA gene sequencing,
298 MALDI-TOF MS gave species results (with a log(score) value $\geq$ 2.0). Although difficult to
299 prove, MALDI-TOF MS most likely outperformed the reference method in these cases.

300 For nonfermenting bacteria, the MALDI-TOF MS method achieved high reproducibility
301 by measurements in a mass range including characteristic signals, which are little influenced by
302 culture conditions, development of a new algorithm for pattern matching, and creation of a high
303 quality reference database. A more stringent control of cultivation conditions might however be
304 necessary for other bacterial strains that accumulate storage products (36), sporulate (e.g. for
305 *Bacillus* species), or show autolysis during long-time storage (e.g. *Streptococcus* species).

306 In summary, a MALDI-TOF MS method was described that provided accurate and fast
307 species identification in routine diagnostics of nonfermenting bacteria. These results also showed
308 the MALDI-TOF MS was more accurate than partial 16S rRNA gene sequencing for species
309 identification of the *B. cepacia*-complex. Further expansion of the MALDI-TOF MS database
310 with other bacterial groups of clinical importance will help enhance the utility of this
311 methodology to identify unknown bacterial pathogens. In the future, a polyphasic approach with
312 the combined use of 16S rRNA gene sequencing and the higher discriminatory ability of
313 MALDI-TOF MS might be an attractive alternative for those bacterial species that are hard to
314 identify.

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Competing Interests

M. Kostrzewa and Th. Maier have declared a potential conflict of interest. They are both employees of Bruker Daltonik GmhH, the company that produces the MALDI-TOF MS instruments and the software mentioned in the manuscript. All other authors have declared that no competing interests exist.

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439 **Figure legends**

440 Fig. 1. Comparison of mass spectra for one exemplary nonfermenting strain (Galv12) generated
441 on three different MALDI-TOF MS instruments (autoflex, microflex, ultraflex). Exemplary
442 masses (in Dalton) are depicted.

443

444 Fig. 2. Comparison of discriminatory ability of partial 16S rRNA gene sequencing and MALDI-
445 TOF MS within the former *Burkholderia cepacia*-complex. Nine reference strains of the former
446 *B. cepacia*-complex, the *B. cepacia* type strain (DSM 7288), and *B. gladioli* as the outgroup
447 (DSM 4285) are shown using a rooted UPGMA-tree. For tree construction, the MEGA software
448 version 4.0 was used (34). In (A), the tree is based on partial (453 bp, *E. coli* gene position 54 to
449 510) 16S rRNA gene sequences and the sequence distances are given in percent difference. In
450 (B), the tree is based on MALDI-TOF MS results and the log(score) derived distances are also
451 given in percentage. Abbreviations: LMG, culture collection of the Laboratorium voor
452 Microbiologie, Universiteit Gent, Gent, Belgium; DSM, Deutsche Sammlung von
453 Mikroorganismen und Zellkulturen, Braunschweig, Germany; T, type strain.

454 **Tables**

455 **TABLE 1.** List of 248 nonfermenter culture collection strains used to establish the reference
456 database for MALDI-TOF MS based species identification.

Genus	Strain
Achromobacter	*A. denitrificans* DSM 30026, *A. insolitus* LMG 6003, *A. piechaudii* DSM 10342, *A. ruhlandii* DSM 653, *A. spanios* LMG 5911, *A. xylosoxidans* subsp. *xylosoxidans* DSM 2402
Acidovorax	*A. avenae* subsp. *avenae* DSM 7227, *A. avenae* subsp. *citrulli* LMG 5376, *A. defluvii* DSM 12644, *A. delafieldii* DSM 64, *A. facilis* DSM 649, *A. konjaci* DSM 7481, *A. temperans* DSM 7270
Acinetobacter	*A. baumannii* DSM 30007, *A. baumannii* LMG 994, *A. baylyi* DSM 14961, *A. bouvetii* DSM 14964, *A. calcoaceticus* DSM 30006, *A. gerneri* DSM 14967, *A. grimontii* DSM 14968, *A. haemolyticus* DSM 6962, *A. haemolyticus* LMG 1033, *A. johnsonii* DSM 6963, *A. johnsonii* LMG 10584, *A. junii* DSM 6964, *A. lwoffii* DSM 2403, *A. lwoffii* LMG 1138, *A. lwoffii* LMG 1154, *A. lwoffii* LMG 1300, *A. parvus* DSM 16617, *A. radioresistens* DSM 6976, *A. radioresistens* LMG 10614, *A. schindleri* DSM 16038, *A. tandoii* DSM 14970, *A. tjernbergiae* DSM 14971, *A. towneri* DSM 14962, *A. ursingii* DSM 16037
Alcaligenes	*A. faecalis* subsp. *faecalis* DSM 30030, *A. faecalis* subsp. *parafaecalis* DSM 13975
Alishewanella	*A. fetalis* DSM 16032
Arsenophonus	*A. nasoniae* DSM 15247
Arthrobacter	*A. monumenti* DSM 16405
Balneatrix	*B. alpica* CIP 103589
Bergeyella	*B. zoohelcum* LMG 8351
Blastomonas	*B. natatoria* DSM 3183, *B. ursincola* DSM 9006
Brevundimonas	*B. aurantiaca* DSM 4731, *B. diminuta* DSM 7234, *B. intermedia* DSM 4732, *B. nasdae* DSM 14572, *B. subvibrioides* DSM 4735, *B. vesicularis* DSM 7226
Burkholderia	*B. ambifaria* LMG 11351, *B. andropogonis* DSM 9511, *B. anthina* LMG 16670, *B. caledonica* LMG 19076, *B. caribensis* DSM 13236, *B. cenocepacia* LMG 12614, *B. cepacia* DSM 7288, *B. cepacia* LMG 2161, *B. dolosa* DSM 16088, *B. fungorum* LMG 20227, *B. gladioli* DSM 4285, *B. glathei* DSM 50014, *B. glumae* DSM 9512, *B. multivorans* LMG 14293, *B. phenazinium* DSM 10684, *B. phymatum* LMG 21445, *B. plantarii* DSM 9509, *B. pyrrocinia* LMG 14191, *B. sacchari* LMG 19450, *B. stabilis* LMG 14294, *B. terricola* LMG 20594, *B. thailandensis* DSM 13276, *B. tropica* DSM 15359, *B. tuberum* LMG 21444, *B. vietnamiensis* LMG 10929, *B. xenovorans* LMG 21463

Chryseobacterium	*C. joostei* LMG 18212, *C. scophthalmum* LMG 13028
Comamonas	*C. aquatica* LMG 2370, *C. kerstersii* DSM 16026, *C. nitrativorans* DSM 13191, *C. terrigena* DSM 7099, *C. testosteroni* DSM 50244
Delftia	*D. acidovorans* DSM 39
Elizabethkingia	*E. meningoseptica* DSM 2800, *E. miricola* DSM 14571
Empedobacter	*E. brevis* LMG 4011
Flavobacterium	*F. flevense* DSM 1076, *F. gelidilacus* DSM 15343, *F. hibernum* DSM 12611, *F. hydatis* DSM 2063, *F. johnsoniae* DSM 2064, *F. pectinovorum* DSM 6368, *F. resinovorum* DSM 7478, *F. saccharophilum* DSM 1811
Inquilinus	*I. limosus* DSM 16000
Malikia	*M. spinosa* DSM 15801
Microbulbifer	*M. elongatus* DSM 6810
Myroides	*M. odoratimimus* LMG 4029, *M. odoratus* DSM 2811
Novosphingobium	*N. aromaticivorans* DSM 12444, *N. rosa* DSM 7285, *N. subarcticum* DSM 10700, *N. subterraneum* DSM 12447
Ochrobactrum	*O. anthropi* DSM 6882, *O. gallinifaecis* DSM 15295, *O. grignonense* DSM 13338, *O. intermedium* LMG 3301, *O. tritici* DSM 13340
Pandoraea	*P. apista* LMG 16407, *P. norimbergensis* DSM 11628, *P. pnomenusa* LMG 18817, *P. pulmonicola* LMG 18106
Pannonibacter	*P. phragmitetus* LMG 5414, *P. phragmitetus* LMG 5430
Pseudomonas	*P. abietaniphila* CIP 106708, *P. aeruginosa* DSM 50071, *P. agarici* DSM 11810, *P. alcaligenes* DSM 50342, *P. amygdali* DSM 7298, *P. anguilliseptica* DSM 12111, *P. antarctica* DSM 15318, *P. asplenii* LMG 2137, *P. aurantiaca* CIP 106718, *P. avellanae* DSM 11809, *P. azotoformans* DSM 106744, *P. balearica* DSM 6083, *P. beteli* LMG 978, *P. boreopolis* LMG 979, *P. brassicacearum* DSM 13227, *P. brenneri* DSM 106646, *P. caricapapayae* LMG 2152, *P. cedrina* DSM 105541, *P. chloritidismutans* DSM 13592, *P. chlororaphis* DSM 50083, *P. cichorii* DSM 50259, *P. citronellolis* DSM 50332, *P. congelans* DSM 14939, *P. corrugata* DSM 7228, *P. extremorietalis* DSM 15824, *P. flavescens* DSM 12071, *P. fluorescens* DSM 50090, *P. fragi* DSM 3456, *P. frederiksbergensis* DSM 13022, *P. fulva* LMG 11722, *P. fuscovaginae* DSM 7231, *P. geniculata* LMG 2195, *P. gessardii* CIP 105469, *P. graminis* DSM 11363, *P. grimontii* DSM 106645, *P. hibiscicola* LMG 980, *P. huttiensis* DSM 10281, *P. indica* DSM 14015, *P. jessenii* CIP 105274, *P. jinjuensis* LMG 21316, *P. kilonensis* DSM 13647, *P. koreensis* LMG 21318, *P. libanensis* CIP 105460, *P. lundensis* DSM 6252, *P. lutea* LMG 21974, *P. luteola* DSM 6975, *P. mandelii* CIP 105273, *P. marginalis* DSM 13124, *P. mendocina* DSM 50017, *P. mephitica* CIP 106720, *P. migulae* CIP 105470, *P. monteilii* DSM 14164, *P. mosselii* CIP 105259, *P. mucidolens* LMG 2223, *P. multiresinivorans* LMG 20221, *P. nitroreducens* DSM 14399, *P. oleovorans* DSM 1045, *P. orientalis* CIP 105540, *P. oryzihabitans* DSM 6835, *P. pertucinogena* LMG 1874, *P. pictorum* LMG 981, *P.*

	plecoglossicida DSM 15088, *P. poae* DSM 14936, *P. proteolytica* DSM 15321, *P. pseudoalcaligenes* DSM 50188, *P. putida* DSM 291, *P. putida* DSM 50198, *P. resinovorans* LMG 2274, *P. rhizosphaerae* LMG 21640, *P. rhodesiae* DSM 14020, *P. savastanoi* LMG 2209, *P. savastanoi* subsp. *savastanoi* LMG 5011, *P. straminea* CIP 106745, *P. stutzeri* DSM 5190, *P. synxantha* LMG 2190, *P. syringae* DSM 6693, *P. syringae* subsp. *syringae* LMG 1247, *P. taetrolens* LMG 2336, *P. thermotolerans* DSM*Z* 14292, *P. thivervalensis* DSM 13194, *P. tolaasii* LMG 2342, *P. trivialis* DSM 14937, *P. umsongensis* LMG 21317, *P. vancouverensis* CIP 106707, *P. veronii* DSM 11331, *P. viridiflava* DSM 11124
Ralstonia	*R. eutropha* DSM*Z* 531, *R. mannitolilytica* LMG 6866, *R. pickettii* DSM 6297, *R. syzygii* DSM 7385
Rhizobium	*R. radiobacter* DSM 30147, *R. rubi* DSM 6772, *R. tropici* DSM 11418
Shewanella	*S. algae* DSM*Z* 9167, *S. baltica* DSM 9439, *S. fidelis* LMG 20552, *S. frigidimarina* DSM 12253, *S. profunda* DSM 15900, *S. putrefaciens* DSM 6067
Sphingobacterium	*S. faecium* DSM 11690, *S. mizutaii* DSM 11724, *S. multivorum* DSM 11691, *S. spiritivorum* DSM 11722, *S. thalpophilum* DSM 11723
Sphingobium	*S. chlorophenolicum* DSM 7098, *S. herbicidovorans* DSM 11019, *S. xenophagum* DSM 6383
Sphingomonas	*S. adhaesiva* DSM 7418, *S. aerolata* DSM 14746, *S. aquatilis* DSM 15581, *S. aurantiaca* DSM 14748, *S. cloacae* DSM 14926, *S. faeni* DSM 14747, *S. koreensis* DSM 15582, *S. melonis* DSM 14444, *S. parapaucimobilis* DSM 7463, *S. paucimobilis* DSM 1098, *S. pituitosa* DSM 13101, *S. trueperi* DSM 7225, *S. wittichii* DSM 6014, *S. yabuuchiae* DSM 14562
Sphingopyxis	*S. macrogoltabida* DSM 8826, *S. terrae* DSM 8831
Stenotrophomonas	*S. acidaminiphila* DSM 13117, *S.africana* CIP 104854, *S. maltophilia* DSM 50170, *S. nitritireducens* DSM 12575, *S. rhizophila* DSM 14405
Terrimonas	*T. ferruginea* DSM 30193
Weeksella	*W. virosa* LMG 12995
Wolinella	*W. succinogenes* DSM 1740

Abbreviations: subsp., subspecies; CIP, Collection de l'Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; LMG, culture collection of the Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium.

460 **TABLE 2.** Identification results of the 80 clinical nonfermenter isolates using MALDI-TOF MS

461 in comparison to partial 16S rRNA gene sequence-based species identification.

Isolate No.	16S rRNA gene sequencing[a]		MALDI-TOF MS	
	Species ID	Level of ID[b]	Species ID	Log(score)[c]
Concordant ID				
Galv01	*S. maltophilia*	Species	*S. maltophilia*	2.481
Galv02	*P. aeruginosa*	Species	*P. aeruginosa*	2.418
Galv03	*P. aeruginosa*	Species	*P. aeruginosa*	2.505
Galv04	*P. aeruginosa*	Species	*P. aeruginosa*	2.477
Galv05	*P. aeruginosa*	Species	*P. aeruginosa*	2.565
Galv07	*Achromobacter xylosoxidans* subsp. *xylosoxidans*	Species	*Achromobacter xylosoxidans*	2.415
Galv08	*P. aeruginosa*	Species	*P. aeruginosa*	2.445
Galv10	*P. oleovorans*	Species	*P. oleovorans*	2.188
Galv12	*S. maltophilia*	Species	*S. maltophilia*	2.364
Galv14	*S. maltophilia*	Species	*S. maltophilia*	2.422
Galv20	*S. maltophilia*	Species	*S. maltophilia*	2.516
Galv24	*S. maltophilia*	Species	*S. maltophilia*	2.438
Galv25	*S. maltophilia*	Species	*S. maltophilia*	2.335
Neb06	*P. aeruginosa*	Species	*P. aeruginosa*	2.502
Neb07	*P. aeruginosa*	Species	*P. aeruginosa*	2.510
Neb15	*P. aeruginosa*	Species	*P. aeruginosa*	2.434
Neb16	*P. aeruginosa*	Species	*P. aeruginosa*	2.516
Neb17	*S. maltophilia*	Species	*S. maltophilia*	2.328
Neb22	*S. maltophilia*	Species	*S. maltophilia*	2.501
Neb23	*P. aeruginosa*	Species	*P. aeruginosa*	2.456
Neb27	*S. maltophilia*	Species	*S. maltophilia*	2.494
Neb32	*S. maltophilia*	Species	*S. maltophilia*	2.418
Neb33	*Alcaligenes faecalis* subsp. *faecalis*	Species	*Alcaligenes faecalis* subsp. *faecalis*	2.299
Neb34	*Alcaligenes faecalis* subsp. *faecalis*	Species	*Alcaligenes faecalis* subsp. *faecalis*	2.373
Neb40	*Achromobacter xylosoxidans* subsp. *xylosoxidans*	Species	*Achromobacter xylosoxidans*	2.328
NF02	*S. maltophilia*	Species	*S. maltophilia*	2.489
NF03	*P. aeruginosa*	Species	*P. aeruginosa*	2.587
NF05	*S. maltophilia*	Species	*S. maltophilia*	2.529
NF07	*P. aeruginosa*	Species	*P. aeruginosa*	2.318
NF09	*P. aeruginosa*	Species	*P. aeruginosa*	2.444
NF11	*S. maltophilia*	Species	*S. maltophilia*	2.296
NF16	*P. aeruginosa*	Species	*P. aeruginosa*	2.545
NF17	*P. aeruginosa*	Species	*P. aeruginosa*	2.524
NF19	*S. maltophilia*	Species	*S. maltophilia*	2.519
NF21	*S. maltophilia*	Species	*S. maltophilia*	2.386
NF23	*S. maltophilia*	Species	*S. maltophilia*	2.450
NF24	*Alcaligenes faecalis* subsp. *faecalis*	Species	*Alcaligenes faecalis* subsp. *faecalis*	2.417
W01	*P. koreensis*	Species	*P. koreensis*	2.300
W05	*P. stutzeri*	Species	*P. stutzeri*	2.198
W07	*P. putida*	Species	*P. putida*	2.014

W09	*P. stutzeri*	Species	*P. stutzeri*	2.460
W11	*P. stutzeri*	Species	*P. stutzeri*	2.372
W13	*Delftia acidovorans*	Species	*Delftia acidovorans*	2.253
W17	*P. aeruginosa*	Species	*P. aeruginosa*	2.613
W19	*P. citronellolis*	Species	*P. citronellolis*	2.434
W20	*Brevundimonas diminuta*	Species	*Brevundimonas diminuta*	2.292
W30	*P. fulva*	Species	*P. fulva*	2.024
Galv13	*Pseudomonas* spp.	Genus	*P. savastanoi*	1.782
Galv19	*Stenotrophomonas* spp.	Genus	*S. maltophilia*	2.303
Galv21	*Pseudomonas* spp.	Genus	*P. monteilii*	2.034
Galv22	*Stenotrophomonas* spp.	Genus	*S. maltophilia*	2.350
Neb26	*Pseudomonas* spp.	Genus	*P. savastanoi*	1.797
Neb28	*Stenotrophomonas* spp.	Genus	*S. maltophilia*	1.792
Neb36	*Pseudomonas* spp.	Genus	*P. putida*	2.149
NF04	*Pseudomonas* spp.	Genus	*P. putida*	1.832
NF10	*Pseudomonas* spp.	Genus	*P. putida*	1.852
W02	*Pseudomonas* spp.	Genus	*P. putida*	2.446
W35	*B. cepacia*-complex	*B. cepacia*-complex[b]	*B. multivorans*	2.379
W12	*P. synxantha*/*mucidolens*/*libanesis*/*gessardii*	4 different species possible[b]	*P. tolaasii*	2.148
Galv17	*P. monteilii*/*putida*	2 different species possible[b]	*P. putida*	2.477
Galv23	*P. psychrotolerans*/*oryzohabitans*	2 different species possible[b]	*P. oryzihabitans*	1.704
W06	*P. monteilii*/*putida*	2 different species possible[b]	*P. putida*	2.127
W10	*P. monteilii*/*putida*	2 different species possible[b]	*P. monteilii*	2.011
W14	*P. monteilii*/*putida*	2 different species possible[b]	*P. monteilii*	1.941
W23	*P. monteilii*/*putida*	2 different species possible[b]	*P. putida*	2.048
W26	*Achromobacter xylosoxidans* subsp. *xylosoxidans*/*ruhlandii*	2 different species possible[b]	*Achromobacter ruhlandii*	2.395
W29	*P. monteilii*/*putida*	2 different species possible[b]	*P. putida*	2.124
Discrepant ID				
Neb14	*Elizabethkingia meningoseptica*	Species	*Elizabethkingia miricola*	2.159
Neb20	*Sphingomonas sanguinis*	Species	*Sphingomonas paucimobilis*	2.182
W03	*P. plecoglossicida*	Species	*P. putida*	1.873
W08	*Achromobacter spanius*	Species	*Achromobacter denitrificans*	1.845
W15	*P. putida*	Species	*P. fluorescens*	2.142
W18	*Achromobacter xylosoxidans* subsp. *xylosoxidans*	Species	*Achromobacter ruhlandii*	2.333
W22	*P. plecoglossicida*	Species	*P. putida*	1.770
W28	*P. plecoglossicida*	Species	*P. putida*	1.854
Neb37	*Chryseobacterium indologenes*	Species	no ID	1.547
W16	*Ralstonia insidiosa*	Species	no ID	1.678
NF18	*Brevundimonas nasdae*/*intermedia*/*vesicularis*	3 different species possible[b]	*Arthrobacter castelli*	1.800
No ID				
Neb38	no ID	no identification possible	*Acinetobacter* spp. DSM 30009	2.283

NF08	no ID	no identification possible	*Acinetobacter* spp. DSM 30009	1.922

Abbreviations: *S.*, *Stenotrophomonas*; *P.*, *Pseudomonas*; *B.*, *Burkholderia*; subsp., subspecies; spp., species; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ID, identification.

[a]Sequence comparison analyzed according to RIDOM procedure (16).

[b]The achievable level of identification derived from 16S rRNA gene sequencing as the reference method is given. Sequence similarities of ≥ 99% were used for identification at species level and ≥ 97% for identification at the genus level, respectively. A further differentiation was made at species level between unique and ambiguous similarity search results (multiple top scoring results), latter were rated as identification at genus level only. Sequence similarities below 97% were rated as not identifiable.

[c]Log(score) values ≥ 2.0 were required for the identification at the species level and values ≥ 1.7 for the identification at the genus level, respectively. Log(score) values below 1.7 were rated as not identifiable.

475 **TABLE 3.** Aggregated identification results of 78 clinical nonfermenter isolates using MALDI-
476 TOF MS in comparison to partial 16S rRNA gene sequencing as the reference method.

16S rRNA gene level of identification (Number)	MALDI-TOF MS Identification (%)[a]		
	Concordant ID	Discrepant ID	no ID
Species (57)	47 (82.5%)	8 (14.0%)	2 (3.5%)
Genus (21)	20 (95.2%)	1 (4.8%)	-
Total (78)	67 (85.9%)	9 (11.5%)	2 (2.6%)

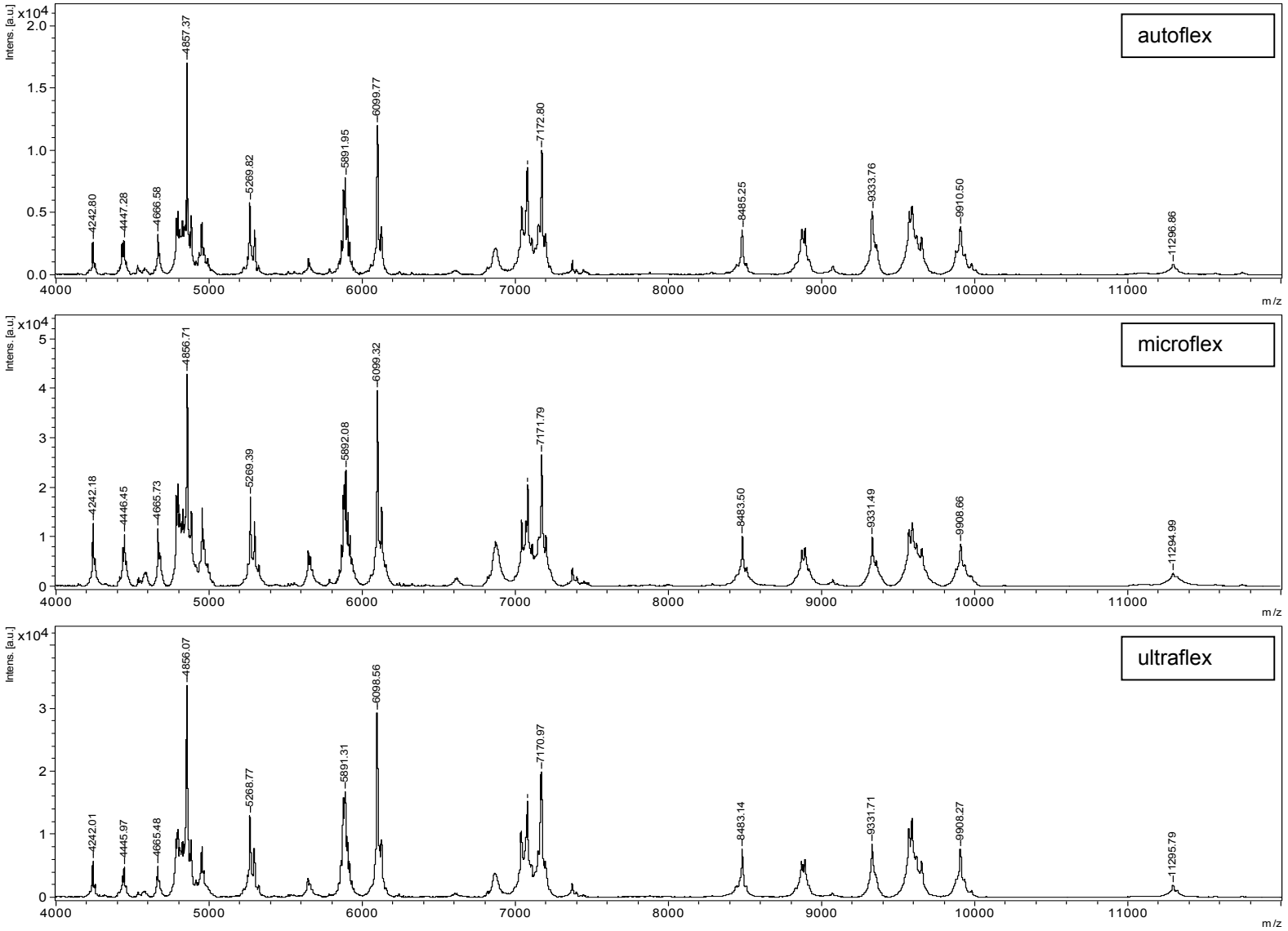
477 Abbreviation: ID, identification

478 [a]MALDI-TOF MS identification was based on resulting log(score) values after similarity search
479 against the MALDI-TOF reference database. Log(score) values ≥ 2.0 were required for the
480 identification at the species level and values ≥ 1.7 for the identification at the genus level,
481 respectively. Log(score) values < 1.7 were rated as not identifiable.

482

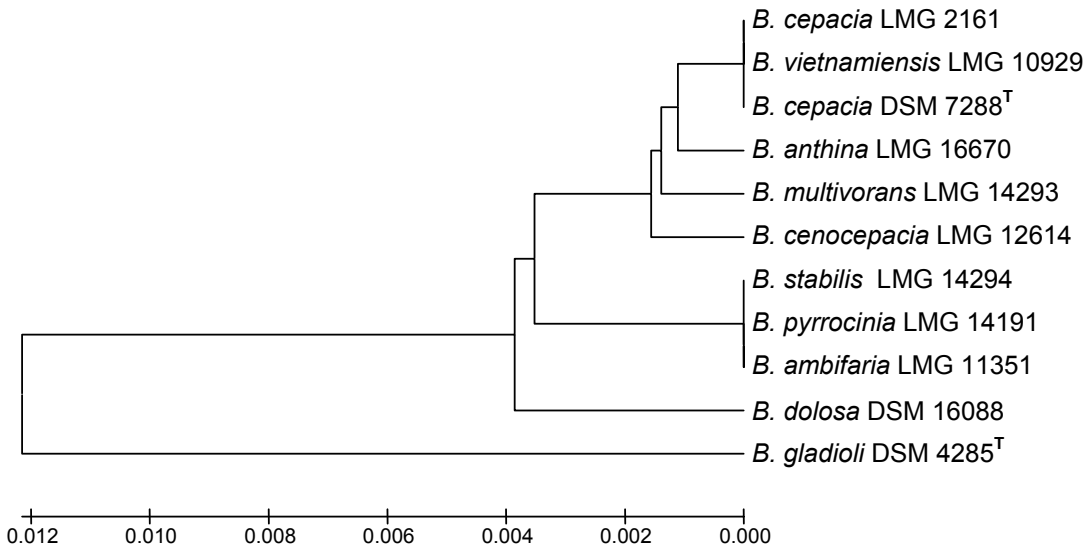
Figures

Fig. 1.



485 **Fig. 2.**

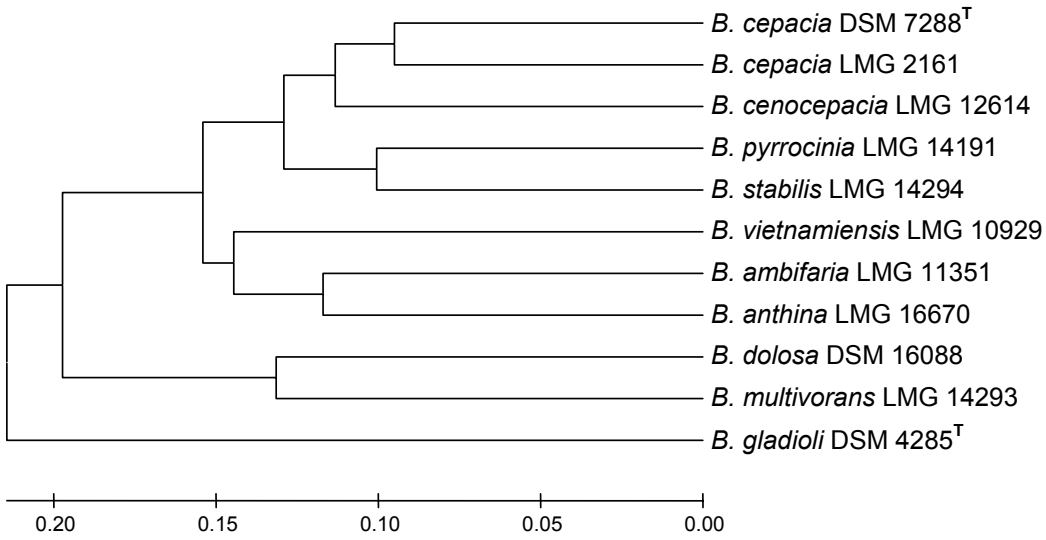
486 (A)



487

488

489 (B)



490