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Evaluation of Matrix-Assisted Laser Desorption Ionization–Time-of-Flight Mass Spectrometry in Comparison to 16S rRNA Gene Sequencing for Species Identification of Nonfermenting Bacteria▽

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Nonfermenting bacteria are ubiquitous environmental opportunists that cause infections in humans, especially compromised patients. Due to their limited biochemical reactivity and different morphotypes, misidentification by classical phenotypic means occurs frequently. Therefore, we evaluated the use of matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) for species identification. By using 248 nonfermenting culture collection strains composed of 37 genera most relevant to human infections, a reference database was established for MALDI-TOF MS-based species identification according to the manufacturer's recommendations for microflex measurement and MALDI BioTyper software (Bruker Daltonik GmbH, Leipzig, Germany), i.e., by using a mass range of 2,000 to 20,000 Da 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. By 16S rRNA gene sequencing, 57 of the 80 isolates produced a unique species identification (≥99% sequence similarity); 11 further isolates gave ambiguous results at this threshold and were rated as identified to the genus level only. Ten isolates were identified to the genus level (≥97% similarity); and two isolates had similarity values below this threshold, were counted as not identified, and were excluded from further analysis. MALDI-TOF MS identified 67 of the 78 isolates (85.9%) included, in agreement with the results of the reference method; 9 were misidentified and 2 were unidentified. The identities of 10 randomly selected strains were 100% correct when three different mass spectrometers and four different cultivation media were used. Thus, MALDI-TOF MS-based species identification of nonfermenting bacteria provided accurate and reproducible results within 10 min without any substantial costs for consumables.

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 immunocompromised patients (30). In the group of cystic fibrosis patients in particular, nonfermenting bacteria are the main causes of morbidity and mortality (18). Furthermore, the antibiotic resistance of some nonfermenting bacterial species often complicates therapy (25, 30). Accurate species identification is therefore critical not only because the prognosis for an infected patient differs significantly depending on the species identified (33) but also because in some cases, e.g., infections with *Burkholderia cepacia* genomovar III (5), strict infection control measures must 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, antibiotic resistance, 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 patients with chronic infections often loose their characteristic phenotypes (12, 26). To overcome these drawbacks, genotypic identification methods have become widely used, and most of them are based on the polymorphism of the 16S rRNA genes. Species-specific PCRs, restriction patterns, and more recently, partial DNA sequencing are used for species identification. Nowadays, sequencing of the 16S rRNA gene is accepted as the reference method for species identification, and several studies have shown its superiority to phenotypic methods for the identification of various groups of bacteria, including nonfermenting bacteria (2, 3, 8, 9, 14, 15, 27). However, a prerequisite for the retrieval of valid identification results is the use of an extensive and comprehensive quality-controlled database (6, 16).

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS), which can be used to

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analyze the protein composition of a bacterial cell, has emerged as a new technology for species identification. By measuring the exact sizes of peptides and small proteins, which are assumed to be characteristic for each bacterial species, it is possible to determine the species within a few minutes when the analysis is started with whole cells, cell lysates, or crude bacterial extracts (13, 17, 22). However, due to difficulties with the reproducibility of results because of the use of different cultivation conditions and the limited availability of reference data sets, MALDI-TOF MS has not yet been widely used for species identification.

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

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MATERIALS 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 to 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), the 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 described previously (27). Four microliters of the extract was used in each PCR. PCR was performed in a total volume of 40 μl containing 1× FastStart DNA Master Plus SYBR green (Roche Diagnostics Corp., Indianapolis, IN), 500 nM each primer 16S-27f and 16S-519r (27), and 4 mM Mg^{2+}. The thermal cycling reactions were performed with a 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) and then a single final extension (2 min at 72°C). Negative controls, which contained water instead of template DNA, were run in parallel in each run. The PCR product was purified by an enzymatic method, modified from the method of Dugan et al. (11), with exonuclease I (New England Biolabs GmbH, Frankfurt-Hoechst, Germany) and shrimp alkaline phosphatase (Amersham Pharmacia Biotech, Freiburg, Germany). Briefly, 5 μl of the PCR product was incubated with 1 U of each enzyme at 37°C for 30 min. The enzymes were then inactivated at 80°C for 15 min, and the PCR products were stored at 4°C. The amplicons were sequenced with an ABI Prism BigDye Terminator (version 3.0) ready reaction cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequencing reaction required 0.5 μl of premix from the kit, 1.8 μl Tris-HCl–$MgCl_2$ buffer (400 mM Tris-HCl, 10 mM $MgCl_2$), 10 pmol of the sequencing primers (which were the same as the PCR primers), and 2 μl of the cleaned PCR product in a total volume of 10 μl.

For the sequencing chemistry mixtures, the same primer used for PCR (primer 16S-27f or 16S-519r) was used to obtain forward and reverse sequence data for partial 5′ 16S rRNA gene sequencing. All sequencing reactions were performed with a standard thermocycler to complete 25 cycles of denaturation (10 s at 96°C), annealing (5 s at 53°C), and extension (4 min at 60°C). The sequencing products were purified with Centri-Sep spin columns (Princeton Separations, Adelphia, NJ), followed by preparation for analysis on an ABI Prism 310 or a 3100 Avant genetic analyzer, in accordance with the instructions of the manufacturer (Applied Biosystems). The double-stranded sequences corresponding to *Escherichia coli* 16S rRNA gene positions 54 to 510 were analyzed in accordance with the procedure described for the Ribosomal Differentiation of Medical Micro-Organisms (RIDOM) database (16).

Sample preparation for MALDI-TOF MS, spectrum generation, and data analysis. A colony of a fresh overnight culture was used for sample preparation before measurement. The material was thoroughly suspended in 300 μl double-distilled water, 900 μl ethanol was added, and the components were mixed well. Prior to shipment to the place of measurement, the samples in ethanol-water were centrifuged, the supernatant was removed, and the pellets were dried. For sample extraction, 50 μl of formic acid (70% in water) was added to the bacterial pellet, the components were mixed thoroughly, and 50 μl of acetonitrile was added. After centrifugation at 13,000 × *g* for 2 min, 1 μl of the supernatant containing the bacterial extract was transferred to a sample position on a ground steel MALDI target plate and allowed to dry at room temperature. Subsequently, the sample was overlaid with 2 μl of MALDI matrix (a saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile–2.5% trifluoroacetic acid) and dried again.

For database construction and validation, measurements were performed with a microflex LT (Bruker Daltonik GmbH, Leipzig, Germany) bench-top mass spectrometer equipped with a 20-Hz nitrogen laser (parameter settings: ion source 1 (IS1), 20 kV; IS2, 18.5 kV; lens, 8.5 kV; detector gain, 2,650 V; and gating, none). Spectra were recorded in the positive linear mode for the mass range of 2,000 to 20,000 Da at the maximum laser frequency. The database references (main spectra) for the newly investigated bacteria were constructed by using the automated functionality of the MALDI BioTyper (version 1.1) software package (Bruker Daltonik GmbH). Briefly, for each database entry, 20 individually measured mass spectra were imported into the software. After smoothing of the spectra, baseline correction, and peak picking, the resulting peak lists were used by the program to calculate and to store a main spectrum containing the average peak mass, average peak intensity, and frequency information.

For microorganism identification, the raw spectra of the unknown bacteria were imported into the MALDI BioTyper software and analyzed by standard pattern matching (with default parameter settings) against the main spectra of 2,506 microorganisms, used as reference data, in the BioTyper database (these spectra are an integrated part of the BioTyper software). The reference database consisted of the 248 newly created main spectra for the nonfermenting bacteria investigated and other clinical, veterinary, and environmental bacterial strains. Preparation of samples of the 248 nonfermenter reference strains was performed at the Institute for Hygiene, University Hospital Muenster (Muenster, Germany). The clinical nonfermenting strains being evaluated were prepared at the ARUP Institute for Clinical and Experimental Pathology.

Preparation of the ground steel plate, MS of 96 samples, and a search of the database for similarity for species identification took about 3 h with the microflex instrument. A single sample could be identified in approximately 10 min.

MALDI-TOF MS reproducibility testing. Complementary to the already determined intraspecies (4, 36) and interlaboratory (37) reproducibilities of MALDI-TOF MS-based species identification, the spectra of 10 randomly chosen nonfermenter strains (*Brevundimonas aurantiaca* DSM 4731, *Brevundimonas intermedia* DSM 4732, *Brevundimonas andropogonis* DSM 9511, *Brevundimonas caribensis* DSM 13236, *Flavobacterium johnsoniae* DSM 2064, *Flavobacterium mizutaii* DSM 11724, *Pseudomonas aeruginosa* DSM 50071, *Pseudomonas beteli* LMG 978, *Pseudomonas boreopolis* LMG 979, *Pseudomonas extremorietalis* DSM 15824) were determined under different conditions. First, the testing was done by parallel measurement on three different MALDI-TOF MS instruments (the microflex LT instrument, the autoflex II TOF/TOF instrument with a 50-Hz nitrogen laser, and the ultraflex III TOF/TOF instrument with a 200-Hz smartbeam laser, all from Bruker Daltonik GmbH) to test the comparabilities of the results obtained with the different instruments. The parameter settings for the additional instruments were as follows: IS1, 20 kV; IS2, 18.7 kV; lens, 8.0 kV; detector gain, 1,756 V; and gating, maximum, 1,500 Da, for the autoflex instrument and IS1, 25 kV; IS2, 23.45 kV; lens, 6.0 kV; detector gain, 1,650 V; and gating, maximum, 1,500 Da, for the ultraflex instrument. Second, the influence of different cultivation conditions was characterized by cultivation of the 10 strains on four different media (Columbia blood agar, chocolate agar, Mueller-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 the bacterial cultures was investigated by analyzing three strains (*B. aurantiaca* DSM 4731, *B. caribensis* DSM 13236, *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.

TABLE 1. Nonfermenter culture collection strains used to establish the reference database for MALDI-TOF MS-based species identification

Genus	Strain[a]
Achromobacter	*Achromobacter 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	*Acidovorax 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	*Acinetobacter 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	*Alcaligenes faecalis* subsp. *faecalis* DSM 30030, *A. faecalis* subsp. *parafaecalis* DSM 13975
Alishewanella	*Alishewanella fetalis* DSM 16032
Arsenophonus	*Arsenophonus nasoniae* DSM 15247
Arthrobacter	*Arthrobacter monumenti* DSM 16405
Balneatrix	*Balneatrix alpica* CIP 103589
Bergeyella	*Bergeyella zoohelcum* LMG 8351
Blastomonas	*Blastomonas natatoria* DSM 3183, *B. ursincola* DSM 9006
Brevundimonas	*Brevundimonas aurantiaca* DSM 4731, *B. diminuta* DSM 7234, *B. intermedia* DSM 4732, *B. nasdae* DSM 14572, *B. subvibrioides* DSM 4735, *B. vesicularis* DSM 7226
Burkholderia	*Burkholderia 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	*Chryseobacterium joostei* LMG 18212, *C. scophthalmum* LMG 13028
Comamonas	*Comamonas aquatica* LMG 2370, *C. kerstersii* DSM 16026, *C. nitrativorans* DSM 13191, *C. terrigena* DSM 7099, *C. testosteroni* DSM 50244
Delftia	*Delftia acidovorans* DSM 39
Elizabethkingia	*Elizabethkingia meningoseptica* DSM 2800, *E. miricola* DSM 14571
Empedobacter	*Empedobacter brevis* LMG 4011
Flavobacterium	*Flavobacterium 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	*Inquilinus limosus* DSM 16000
Malikia	*Malikia spinosa* DSM 15801
Microbulbifer	*Microbulbifer elongatus* DSM 6810
Myroides	*Myroides odoratimimus* LMG 4029, *M. odoratus* DSM 2811
Novosphingobium	*Novosphingobium aromaticivorans* DSM 12444, *N. rosa* DSM 7285, *N. subarcticum* DSM 10700, *N. subterraneum* DSM 12447
Ochrobactrum	*Ochrobactrum anthropi* DSM 6882, *O. gallinifaecis* DSM 15295, *O. grignonense* DSM 13338, *O. intermedium* LMG 3301, *O. tritici* DSM 13340
Pandoraea	*Pandoraea apista* LMG 16407, *P. norimbergensis* DSM 11628, *P. pnomenusa* LMG 18817, *P. pulmonicola* LMG 18106
Pannonibacter	*Pannonibacter phragmitetus* LMG 5414, *P. phragmitetus* LMG 5430
Pseudomonas	*Pseudomonas 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* DSMZ 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	*Ralstonia eutropha* DSMZ 531, *R. mannitolilytica* LMG 6866, *R. pickettii* DSM 6297, *R. syzygii* DSM 7385
Rhizobium	*Rhizobium radiobacter* DSM 30147, *R. rubi* DSM 6772, *R. tropici* DSM 11418
Shewanella	*Shewanella algae* DSMZ 9167, *S. baltica* DSM 9439, *S. fidelis* LMG 20552, *S. frigidimarina* DSM 12253, *S. profunda* DSM 15900, *S. putrefaciens* DSM 6067
Sphingobacterium	*Sphingobacterium faecium* DSM 11690, *S. mizutaii* DSM 11724, *S. multivorum* DSM 11691, *S. spiritivorum* DSM 11722, *S. thalpophilum* DSM 11723
Sphingobium	*Sphingobium chlorophenolicum* DSM 7098, *S. herbicidovorans* DSM 11019, *S. xenophagum* DSM 6383
Sphingomonas	*Sphingomonas 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	*Sphingopyxis macrogoltabida* DSM 8826, *S. terrae* DSM 8831
Stenotrophomonas	*Stenotrophomonas acidaminiphila* DSM 13117, *S.africana* CIP 104854, *S. maltophilia* DSM 50170, *S. nitritireducens* DSM 12575, *S. rhizophila* DSM 14405
Terrimonas	*Terrimonas ferruginea* DSM 30193
Weeksella	*Weeksella virosa* LMG 12995
Wolinella	*Wolinella succinogenes* DSM 1740

[a] A total of 248 nonfermenter culture collection strains were used. Abbreviations: 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 Ghent, Ghent, Belgium.

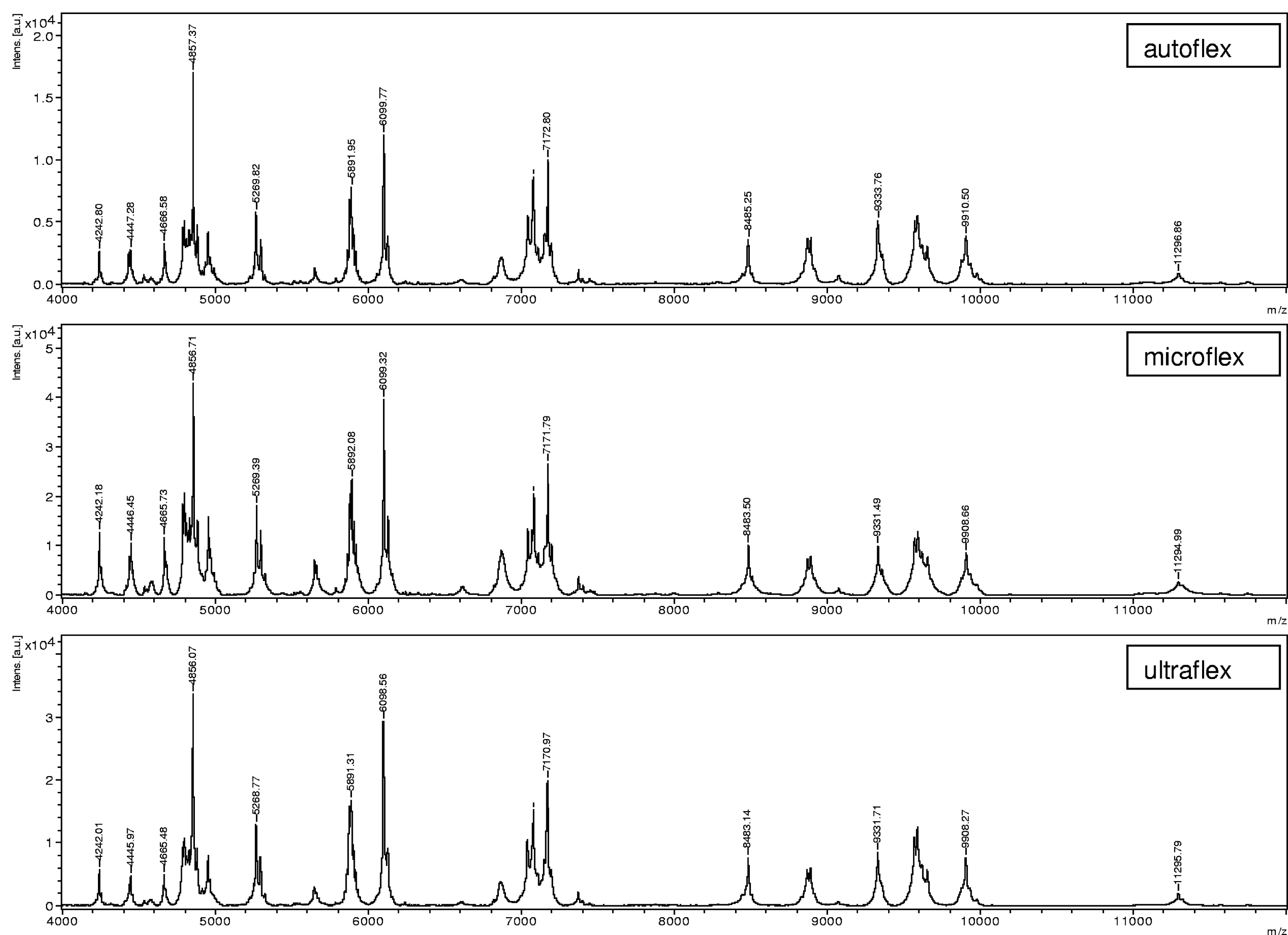

FIG. 1. Comparison of mass spectra for one exemplary nonfermenting strain (strain Galv12) generated on three different MALDI-TOF MS instruments (autoflex, microflex, ultraflex). Exemplary masses (in daltons) are depicted. Intens. [a.u.], intensity (in arbitrary units).

Evaluation of MALDI-TOF MS-based species identification by use of clinical isolates. To evaluate the MALDI-TOF MS reference database, 80 blind-coded clinical nonfermenters were analyzed. As the reference method for species designation, partial 16S rRNA gene sequencing was used. The reference partial 16S rRNA gene sequence database (*E. coli* 16S rRNA gene positions 54 to 510) included all 248 nonfermenter culture collection strains used as a reference for MALDI-TOF MS (Table 1). Sequence data were stored and analyzed by using the RIDOM framework (27). Sequence similarity of ≥99% was used for identification to the species level, and sequence similarity of ≥97% was used for identification to the genus level (3). Further differentiation to the species level was made between unique and ambiguous similarity search results (multiple top-scoring results); the latter were rated as identification to the genus level only. Sequence similarities below 97% were rated as not identifiable. MALDI-TOF MS results based on the log(score) values calculated by the BioTyper software were compared to the 16S rRNA gene sequence similarity search results. BioTyper software requires log(score) values of ≥2.0 for identification to the species level and values of between <2 and ≥1.7 for identification to the genus level. Results based on log(score) values of <1.7 were rated as not identifiable by the software. These BioTyper thresholds were empirically determined on the basis of information in an in-house database with data for more than 2,800 bacterial strains that were either culture collection strains or well-characterized clinical strains.

To determine the discriminatory ability of 16S rRNA gene sequencing and MALDI-TOF MS within certain groups of strains, the pairwise distances were calculated and displayed in a tree created by the unweighted pair group method with arithmetic averaging by using MEGA software (version 4.0) (37).

RESULTS

The cell extracts from all 248 culture collection strains, which represented the majority of all clinically relevant nonfermenter species, gave sufficient spectra and were included in the MALDI-TOF MS reference database (database version 1.0).

To investigate the reproducibility of the spectra generated with different mass spectrometers, 10 culture collection strains were randomly chosen to be analyzed with three different mass spectrometers. For all 10 strains, the log(score) results gave the same species identification result irrespective of the mass spectrometer used, with all log(score) values being above 2.0. Figure 1 shows representative results for strain Galv12 measured with the three different spectrometers. To determine the influence of different cultivation media on the quality of the spectra, the 10 cultured strains were also analyzed after cultivation on four different media. Furthermore, three strains were analyzed after up to 7 days storage on Columbia blood agar at room temperature. In all cases, MS resulted in identical, correct identification results relative to those in the reference database.

TABLE 2. Identification results for the 80 clinical nonfermenter isolates obtained by MALDI-TOF MS in comparison to those obtained by partial 16S rRNA gene sequence-based species identification[a]

Level of ID and isolate	16S rRNA gene sequencing[b]		MALDI-TOF MS	
	Species ID	Level of ID[c]	Species ID	Log(score)[d]
Concordance				
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. multivorans*	2.379
W12	*P. synxantha/P. mucidolens/ P. libanesis/P. gessardii*	Four different species possible	*P. tolaasii*	2.148
Galv17	*P. monteilii/P. putida*	Two different species possible	*P. putida*	2.477
Galv23	*P. psychrotolerans/P. oryzohabitans*	Two different species possible	*P. oryzihabitans*	1.704
W06	*P. monteilii/P. putida*	Two different species possible	*P. putida*	2.127
W10	*P. monteilii/P. putida*	Two different species possible	*P. monteilii*	2.011
W14	*P. monteilii/P. putida*	Two different species possible	*P. monteilii*	1.941
W23	*P. monteilii/P. putida*	Two different species possible	*P. putida*	2.048
W26	*Achromobacter xylosoxidans* subsp. *xylosoxidans/A. ruhlandii*	Two different species possible	*Achromobacter ruhlandii*	2.395
W29	*P. monteilii/putida*	Two different species possible	*P. putida*	2.124

Continued on facing page

TABLE 2—*Continued*

Level of ID and isolate	16S rRNA gene sequencing[b]		MALDI-TOF MS	
	Species ID	Level of ID[c]	Species ID	Log(score)[d]
Discrepant				
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*/*B. intermedia*/*B. vesicularis*	Three different species possible	*Arthrobacter castelli*	1.800
None				
Neb38	No ID	No ID possible	*Acinetobacter* sp. strain DSM 30009	2.283
NF08	No ID	No ID possible	*Acinetobacter* sp. strain DSM 30009	1.922

[a] Abbreviations: *S.*, *Stenotrophomonas*; *P.*, *Pseudomonas*; *B.*, *Burkholderia*; subsp., subspecies; spp., species; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ID, identification.
[b] Sequences were compared by the RIDOM procedure (16).
[c] 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 to the species level, and sequence similarities of ≥97% were used for identification to the genus level. A further differentiation to the species level was made between sequences with unique and ambiguous similarity search results (multiple top-scoring results); the latter were rated as identification to the genus level only. Sequence similarities below 97% were rated as not identifiable and the numbers of different species possible are given.
[d] Log(score) values of ≥2.0 were required for the identification to the species level, and values of ≥1.7 were required for identification to the genus level. Log(score) values below 1.7 were rated as not identifiable.

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

The identification results obtained by MALDI-TOF MS and 16S rRNA gene sequencing, used as the reference method, are shown in Table 2, together with the achievable levels of identification. In total, MALDI-TOF MS identified 67 of the 78 isolates (85.9%), concordant with the sequencing results; of these, 47 of 57 (82.5%) were identified to the species level [log(score), ≥2.0] and 20 of 21 (95.2%) were identified to the genus level [log(score), between <2.0 and ≥1.7] (Table 3). Of the remaining 11 isolates, 4 isolates (isolates Neb14, Neb20, W15, and W18) had log(score) values of ≥2.0 and were correctly identified to the genus level but had discordant species designations in comparison to the results of the reference method. For four isolates (isolates W03, W08, W22, and W28), the correct genus was determined by the MALDI BioTyper software, with log(score) values of between 1.7 and 2.0, whereas 16S rRNA gene sequencing gave a species identification. For isolate NF18, the genus determination result was discordant with the 16S rRNA gene sequencing result. Finally, the two isolates with log(score) values of <1.7 (isolates Neb37 and W16) were rated as nonidentifiable by MALDI-TOF MS (Table 3).

To investigate the discriminatory ability of closely related species by MALDI-TOF MS in comparison to that by 16S rRNA gene sequencing, nine species of the former *Burkholderia cepacia* complex were investigated in detail. By partial 16S rRNA gene sequencing, three strains, *B. cepacia* DSM 7288 (*B. cepacia* type strain), *B. cepacia* LMG 2161 (the former reference strain for *B. cepacia* complex genomovar I), and *Burkholderia vietnamiensis* LMG 10929 (formerly genomovar V), were indistinguishable (Fig. 2A). Likewise, three other strains, *Burkholderia stabilis* LMG 14294 (formerly genomovar IV),

TABLE 3. Aggregated identification results for 78 clinical nonfermenter isolates obtained by MALDI-TOF MS in comparison to those obtained by partial 16S rRNA gene sequencing as the reference method

Level of identification (no. of isolates) by 16S rRNA gene	No. (%) of isolates with the following MALDI-TOF MS ID[a]:		
	Concordance	Discrepant	None
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)

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

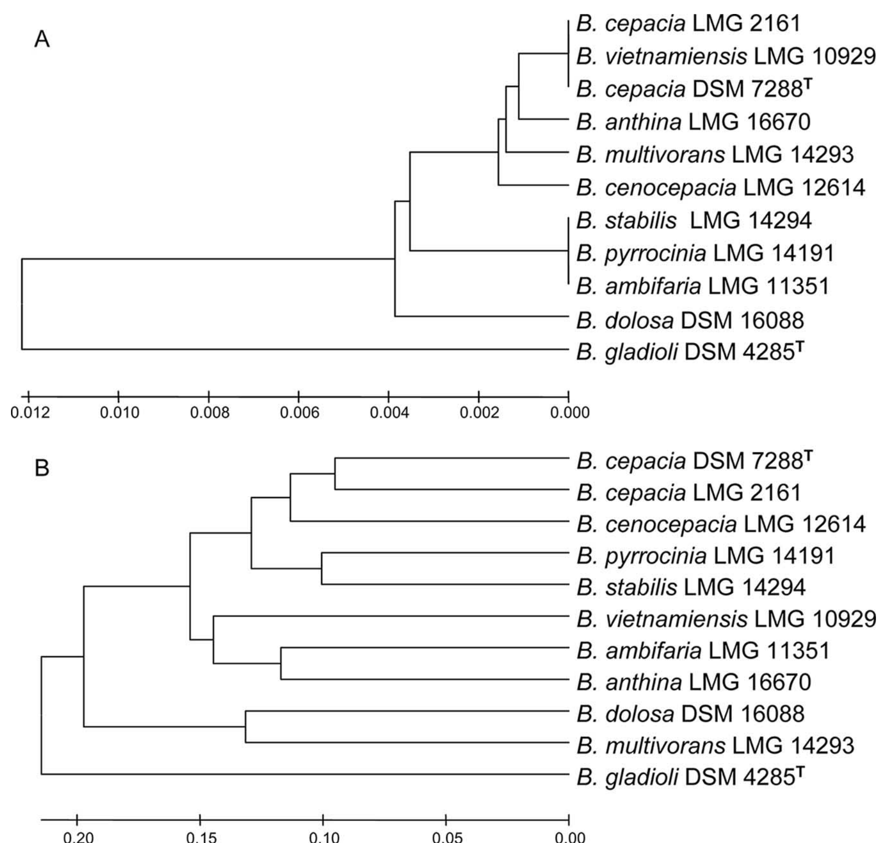

FIG. 2. Comparison of discriminatory abilities of partial 16S rRNA gene sequencing and MALDI-TOF MS for strains within the former *Burkholderia cepacia* complex. Nine reference strains of the former *B. cepacia* complex, a *B. cepacia* type strain (DSM 7288), and *Burkholderia gladioli* DSM 4285 (which was used as the outgroup) are shown on a rooted tree created by the unweighted pair group method with arithmetic averaging. MEGA software (version 4.0) was used for tree construction (34). (A) Tree based on partial 16S rRNA gene sequences (453 bp, *E. coli* gene positions 54 to 510). The sequence distances are given as the percent difference. (B) Tree based on the MALDI-TOF MS results. The log(score)-derived distances are given in percent. Abbreviations: LMG, culture collection of the Laboratorium voor Microbiologie, Universiteit Ghent, Ghent, Belgium; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Burkholderia pyrrocinia LMG 14191 (formerly genomovar IX), and *Burkholderia ambifaria* LMG 11351 (formerly genomovar VII), were also indistinguishable. In contrast, MALDI-TOF MS 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 from the patient's environment is of major importance for optimal patient management and the establishment of 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 by using blind-coded nonfermenter isolates from clinical specimens (Table 2). With 82.5% isolates correctly identified to the species level and 95.2% isolates correctly identified to the genus level (Table 3), the results of MALDI-TOF MS-based identification showed a high concordance to those of the reference method. In contrast, the use of two current phenotypic identification methods (the API 20NE and Vitek 2 systems) resulted in correct identification rates of only 61% and 54%, respectively, in a different study with nonfermenters (3).

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

This analysis corroborated the higher discriminatory ability of MALDI-TOF MS in comparison to that of 16S rRNA gene sequencing (36) and enabled the valid identification of all members of the former *B. cepacia* complex.

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

In contrast to earlier work (7), which applied a mass range of 550 to 2,200 Da, a range of 2,000 to 20,000 Da was used in this study. This mass range represents predominantly ribosomal proteins obtained from whole bacteria or crude bacterial extracts (24, 32). These proteins are abundant in the cell and are positively charged, which favors their measurement by MALDI-TOF MS and which results in a relative robustness under different culture conditions. Another reason for the improved reproducibility is the use of a dedicated algorithm of 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 pattern matching to compare unknown mass spectra with reference data stored in a database. In the first step, the software extracts a list of the mass spectrum peaks after smoothing and baseline subtraction. This list of peaks is compared with each entry in the reference database, and thereby, the unknown peak in the 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. On the basis of the peak matches and intensity correlations, a score and the logarithm of this score are calculated, with values from 0 to 3 indicating 0 to 100% pattern matches, respectively. After comparison of an unknown spectrum with all main spectra in the database, all log(score) values are ranked. Finally, MALDI-TOF MS-based species identification was hampered in the past due to the lack of a comprehensive reference database built with data for 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 database of spectra for 248 reference nonfermenting bacteria was initially established and subsequently evaluated by using blind-coded clinical isolates.

One advantage to the current study is that the same reference strains were used both in 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 the reference method of identification. This method was not able to identify all clinical isolates to the species level (≥99% sequence similarity). Ideally, a polyphasic approach to identification, with the combined use of phenotypic and genotypic characteristics, should be considered. However, due to the diversity among the species of nonfermenting bacteria and the current changes in bacterial nomenclature within this group, there is no clearly defined strategy for the identification of nonfermenting bacteria. Moreover, 16S rRNA gene sequencing has already been found to be superior to phenotypic identification techniques for nonfermenting bacteria (3, 14). It is noteworthy that for 12 of the 21 isolates identified to the genus level only by 16S rRNA gene sequencing in this study, MALDI-TOF MS gave species results [with log(score) values of ≥2.0]. Although it is difficult to prove, MALDI-TOF MS most likely outperformed the reference method in these cases.

For nonfermenting bacteria, the MALDI-TOF MS method achieved a high degree of reproducibility of measurements in a mass range that included characteristic signals, which are little influenced by culture conditions. In addition, a new algorithm for pattern matching was developed, and a high-quality reference database was created. The more stringent control of cultivation conditions might, however, be necessary for other bacterial strains that accumulate storage products (36), sporulate (e.g., for *Bacillus* species), or show autolysis during long-time storage (e.g., *Streptococcus* species).

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

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M. Kostrzewa and T. Maier have declared potential conflicts 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 report. All other authors have declared that no competing interests exist.

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