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Novel Mass Spectrometry-Based Tool for Genotypic Identification of Mycobacteria

Michael Lefmann,[1] Christiane Honisch,[2] Sebastian Böcker,[2] Niels Storm,[3] Friedrich von Wintzingerode,[1] Cord Schlötelburg,[1]† Annette Moter,[1] Dirk van den Boom,[2]* and Ulf B. Göbel[1]

Institut für Mikrobiologie und Hygiene, Universitätsklinikum Charité, Humboldt-Universität zu Berlin, 10117 Berlin,[1] and SEQUENOM GmbH, 22761 Hamburg,[3] Germany, and SEQUENOM Inc., San Diego, California 92121[2]

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Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) after base-specific cleavage of PCR amplified and in vitro-transcribed 16S rRNA gene (rDNA) was used for the identification of mycobacteria. Full-length 16S rDNA reference sequences of 12 type strains of *Mycobacterium* spp. frequently isolated from clinical specimens were determined by PCR, cloning, and sequencing. For MALDI-TOF MS-based comparative sequence analysis, mycobacterial 16S rDNA signature sequences (~500 bp) of the 12 type strains and 24 clinical isolates were PCR amplified using RNA promoter-tagged forward primers. T7 RNA polymerase-mediated transcription of forward strands in the presence of 5-methyl ribo-CTP maximized mass differences of fragments generated by base-specific cleavage. In vitro transcripts were subsequently treated with RNase T1, resulting in G-specific cleavage. Sample analysis by MALDI-TOF MS showed a specific mass signal pattern for each of the 12 type strains, allowing unambiguous identification. All 24 clinical isolates were identified unequivocally by comparing their detected mass signal pattern to the reference sequence-derived in silico pattern of the type strains and to the in silico mass patterns of published 16S rDNA sequences. A 16S rDNA microheterogeneity of the *Mycobacterium xenopi* type strain (DSM 43995) was detected by MALDI-TOF MS and later confirmed by Sanger dideoxy sequencing. In conclusion, analysis of 16S rDNA amplicons by MS after base-specific cleavage of RNA transcripts allowed fast and reliable identification of the *Mycobacterium tuberculosis* complex and ubiquitous mycobacteria (mycobacteria other than tuberculosis). The technology delivers an open platform for high-throughput microbial identification on the basis of any specific genotypic marker region.

Mycobacteria are important pathogens that cause disease in humans and animals. The most important mycobacterial disease, tuberculosis, remains a global public health problem, with an estimated 8.4 million new cases in 1999 and 3 million deaths each year (41). Mycobacteria other than tuberculosis have become increasingly important as opportunistic pathogens, causing severe disease in patients with or without immune dysfunction (1, 2, 9, 11, 25, 27, 28). Since the introduction of broth culture systems for the isolation of mycobacteria, time for identification has decreased substantially. However, species identification by conventional phenotypic traits is still time-consuming and may frequently result in erroneous identification (33). Chromatographic analysis of cell wall lipids such as high-performance liquid chromatography and gas-liquid chromatography are technically demanding and expensive and therefore available in very few specialized clinical laboratories only (6, 21, 22).

Laboratories have begun to rely on amplification-based methods for genotypic characterization of mycobacteria (3, 18, 20, 23, 26, 31). The 16S rRNA gene (rDNA) is the most widely accepted gene used for bacterial identification of both cultured and as-yet-uncultured bacteria, with high impact on the discovery of new species of the *Mycobacterium* genus. 16S rDNA sequences constitute one of the largest gene-specific data sets, with constantly increasing numbers of entries in publicly available databases.

New genotypic techniques for fast identification of microorganisms have been developed during the past few years. High-density oligonucleotide arrays (DNA chip) were able to identify mycobacteria and detected rifampin resistance in a single assay (14, 36). Using fluorescence in situ hybridization with peptide nucleic acid probes the *M. tuberculosis* complex and mycobacteria other than tuberculosis were differentiated by direct visualization, which is especially useful in cases of mixed mycobacterial infections (10). Nucleic acid probes for hybridization-based identification are capable of identifying a narrow range of mycobacterial species (29). They are widely used, but problems with sensitivity and specificity have been described (7, 12).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been widely applied for the phenotypic characterization of bacterial whole cells, simple cell lysates, or bacterial products such as lipopolysaccharides and proteins (4, 5, 32, 37, 40). The potential to analyze bacterial DNA and RNA has been shown during the last few years. Hurst et al. (17) described a protocol for fast and accurate differentiation of PCR products from methanotrophic bacteria by MALDI-TOF MS according to their length. Rapid analysis of PCR products and restriction fragment length polymorphism patterns of microbial samples by MALDI-TOF MS

* Corresponding author. Mailing address: SEQUENOM Inc., 3595 John Hopkins Court, San Diego, CA 92121. Phone: (858) 202-9066. Fax: (858) 202-9084. E-mail: dvandenboom@sequenom.com.

† Present address: VDI/VDE-Technologiezentrum Informationstechnik GmbH, 14513 Teltow, Germany.

was reported by Taranenko and coworkers (35) using UV MALDI for size determination of double-stranded amplicons and restriction fragments. However, these approaches are limited by length heterogeneities of specific marker genes, which diminish their discriminatory power. Kirpekar et al. (19) monitored posttranscriptional modifications of bacterial RNA after G- and pyrimidine-specific digestion, using MALDI-TOF MS for direct analysis of the digest.

Rapid bacterial identification via base-specific cleavage of amplified 16S rDNA and MS was described by von Wintzingerode et al. (39) for the first time. Amplification of 16S rDNA signature sequences in the presence of dUTP instead of dTTP was followed by strand separation and uracil-DNA-glycosylase (UDG)-mediated cleavage at each T-specific site. Fragment pattern detection was performed by MALDI-TOF MS and resulted in unambiguous identification of a panel of cultured *Bordetella* spp. and as-yet-uncultured bacteria of anaerobic, organochlorine-reducing microbial consortia.

Here we present an alternative homogeneous in vitro transcription-RNase cleavage system followed by MALDI-TOF MS as a method for rapid 16S rDNA-based identification of mycobacteria to the species level. PCR amplification of mycobacterial 16S rDNA was combined with in vitro RNA transcription and subsequent G-specific cleavage of the RNA transcript. The resulting fragment pattern was analyzed by MALDI-TOF MS. The obtained mass signal pattern allows unambiguous and reliable identification of each species. Our concept combines the discriminatory power of 16S rDNA sequence information with the accuracy and data acquisition speed of MALDI-TOF MS.

TABLE 1. Mycobacterial strains used in this study and typing results of repeated MALDI-TOF MS analysis of clinical isolates

Species	Source	EMBL accession no.
Reference strains (n = 12)		
Mycobacterium abscessus	DSM 44196	AJ536038
Mycobacterium avium subsp. *avium*	DSM 44156	AJ536037
Mycobacterium celatum	DSM 44243	AJ536040
Mycobacterium fortuitum subsp. *fortuitum*	DSM 46621	AJ536039
Mycobacterium gordonae	DSM 44160	AJ536042
Mycobacterium intracellulare	DSM 43223	AJ536036
Mycobacterium kansasii	DSM 44162	AJ536035
Mycobacterium marinum	DSM 44344	AJ536032
Mycobacterium scrofulaceum	DSM 43992	AJ536034
Mycobacterium smegmatis	DSM 43758	AJ536041
Mycobacterium tuberculosis	H37RV	AJ536031
Mycobacterium xenopi	DSM 43995	AJ536033
Clinical isolates[a] (n = 24)		
Mycobacterium aurum[b]	MT1323	
Mycobacterium avium	MT1367	
Mycobacterium avium	MT1951	
Mycobacterium chelonae	MT1297	
Mycobacterium fortuitum	MT2014	
Mycobacterium gordonae	MT804	
Mycobacterium gordonae	MT2843	
Mycobacterium interjectum[b]	MT1223	
Mycobacterium intracellulare	MT1881	
Mycobacterium intracellulare	MT2544	
Mycobacterium kansasii	MT1619	
Mycobacterium marinum	MT3065	
Mycobacterium paraffinicum[b]	MT1423	
Mycobacterium smegmatis	MT3344	
Mycobacterium tuberculosis	MT1551	
Mycobacterium tuberculosis	MT1598	
Mycobacterium tuberculosis	MT1692	
Mycobacterium tuberculosis	MT1905	
Mycobacterium tuberculosis	MT2068	
Mycobacterium tuberculosis	MT2125	
Mycobacterium tuberculosis	MT2271	
Mycobacterium tuberculosis	MT2924	
Mycobacterium xenopi	MT309	
Mycobacterium xenopi	MT2236	

[a] Species determined by partial 16S rDNA sequencing and phenotypic methods (*M. chelonae*, *M. kansasii*, *M. marinum*, and *M. tuberculosis*). All clinical isolates were correctly identified by repeated MALDI-TOF MS (in triplicate) unless otherwise indicated.

[b] Correct identification after addition of calculated-mass signal pattern from published 16S rDNA sequences into the database.

MATERIALS AND METHODS

Bacterial strains. Twelve type strains purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and the Institute for Standardization and Documentation in Medical Laboratories (Instand e.V., Düsseldorf, Germany) as well as 24 mycobacterial isolates from our clinical microbiology department were used in the present study (Table 1). Mycobacteria were grown in liquid medium (MGIT liquid medium; Becton Dickinson Europe, Le Pont de Claix, France) with enrichment supplement (MGIT system oleic acid-albumin-dextrose-citric acid) and antimicrobial supplement (MGIT system PANTA [polymyxin B, nalidixic acid, trimethoprim, and azlocillin]) at 37 and 30°C (for *M. marinum*), respectively. When an MGIT vial was detected as positive, mycobacteria were concentrated in 0.5 ml of broth by centrifugation (3,300 × *g*, 20 min) followed by DNA extraction using a commercially available respiratory specimen preparation kit (AMPLICOR; Roche Molecular Systems, Inc., Branchburg, N.J.). In brief, 100 μl of resuspended mycobacterial pellet was transferred into a 1.5-ml polypropylene tube, washed with washing solution (500 μl) provided with the kit, and centrifuged (14,000 × *g*) for 10 min. The supernatant was discarded, and the bacterial pellet was suspended in a lysis reagent (100 μl). After incubation in a heating block at 60°C for 45 min the lysate was neutralized with the neutralizing reagent (100 μl) provided with the kit and stored at 4°C.

Identification by PCR and sequencing. Full-length 16S rRNA genes of the 12 type strains were analyzed as described in detail elsewhere (38). Briefly, 16S rDNA was amplified using eubacterial primers TPU1 (AGA GTT TGA TCM TGG CTC AG; corresponding to *Escherichia coli* positions 8 to 27) and RTU8 (AAG GAG GTG ATC CAK CCR CA; corresponding to *E. coli* positions 1541 to 1522), according to a standard protocol. PCR products were inserted into pCR2.1 (TA Cloning Kit; Invitrogen, de Schelp, The Netherlands) and transformed into *E. coli* according to the manufacturer's instructions. Recombinant plasmid DNA was purified using the GFX plasmid preparation kit (Amersham Pharmacia, Freiburg, Germany) and used directly for cycle sequencing (Thermosequenase fluorescent labeled primer cycle sequencing kit; Amersham Pharmacia). Sequencing reaction mixtures were analyzed on a LICOR 4000L automated DNA sequencer (MWG-Biotech, Ebersberg, Germany). Alignments were generated with ARB software (http://www.arb-home.de/) (38).

Identification of mycobacteria from clinical sources was performed by PCR amplification of partial 16S rDNA and direct sequencing focusing on hypervariable regions A and B corresponding to *E. coli* 16S rDNA positions 129 to 267 and 430 to 500 according to a protocol of Springer et al. (33). Resulting sequences were compared to all 16S rRNA gene entries in EMBL and GenBank databases using BLASTN and FASTA of the Husar program package (version 4.0; Heidelberg Unix Sequence Analysis Resources, DKFZ, Heidelberg, Germany). Clinical isolates were identified to the species level on the basis of sequence identity in both hypervariable regions with a database entry and a total sequence identity of >99%, respectively.

Identification by PCR, base-specific cleavage, and MALDI-TOF. Experiments for each mycobacterial strain were run in triplicate to validate the reproducibility of the PCR, base-specific cleavage, and MALDI-TOF methods.

PCR. Fifty-microliter amplification mixtures contained 1× PCR buffer [Tris-HCl, KCl, $(NH_4)_2SO_4$, $MgCl_2$ [pH 8.7]; final $MgCl_2$ concentration of 2.5 mM], a

200 μM concentration of each deoxynucleoside triphosphate, 1 U of HotStarTaq (QIAGEN GmbH, Hilden, Germany), 10 pmol of primer Myko109-T7 (5′-GT AATACGACTCACTATAGGG ACG GGT GAG TAA CAC GT-3′; corresponding to *E. coli* 16S rDNA positions 105 to 121) and 10 pmol of primer R259-SP6 (5′-ATTTAGGTGACACTATAGAA TTT CAC GAA CAA CGC GAC AA-3′; corresponding to *E. coli* 16S rDNA positions 609 to 590) and 5 μl of DNA. The temperature profile consisted of 40 cycles of denaturation (1 min, 95°C), annealing (1 min, 58°C), and extension (1 min 30 s, 72°C) after an initial step of HotStarTaq activation (15 min, 95°C) (Thermocyler Goldblock; Biometra, Göttingen, Germany).

RNA transcription. Forward-strand RNA transcription was performed by incubation of 2.4 μl of PCR product, 10 U of T7 RNA polymerase (Epicentre), a 0.5 mM concentration of each nucleoside triphosphate, and 1× transcription buffer (6 mM $MgCl_2$, 10 mM dithiothreitol, 10 mM NaCl, 10 mM spermidine, 40 mM Tris-Cl [pH 7.9] at 20°C) at 37°C for 2 h. Ribo-CTP was replaced by its chemically modified analog 5-methyl ribo-CTP (ribo-CTP; Trilink, San Diego, Calif.).

RNase T1 cleavage. Complete G-specific cleavage was achieved by adding 20 U of RNase T1 and 1 U of shrimp alkaline phosphatase and incubating this mixture at 30°C for 30 min.

Sample conditioning. Each sample was diluted by adding 21 μl of H_2O. Conditioning of the phosphate backbone was achieved with 6 mg of SpectroCLEAN (SEQUENOM, San Diego, Calif.).

MALDI-TOF MS analysis. Samples were analyzed from SpectroCHIP arrays. Aliquots of 15 nl were dispensed robotically onto a silicon chip (SpectroCHIP; SEQUENOM). Mass spectra were recorded using a Biflex III mass spectrometer (Bruker Daltonik, Bremen, Germany). Exclusively positive ions were analyzed, and ~50 single-shot spectra were accumulated per sample. All samples were analyzed in linear TOF mode using delayed ion extraction and a total acceleration voltage of 20 kV. Analysis of all mass spectra and automated data interpretation were performed with in-house software developed by SEQUENOM, Inc.

Nucleotide sequence accession numbers. Full-length 16S rRNA gene sequences of the 12 type strains were deposited in the EMBL nucleotide sequence database under accession numbers AJ536031 to AJ536042 (Table 1).

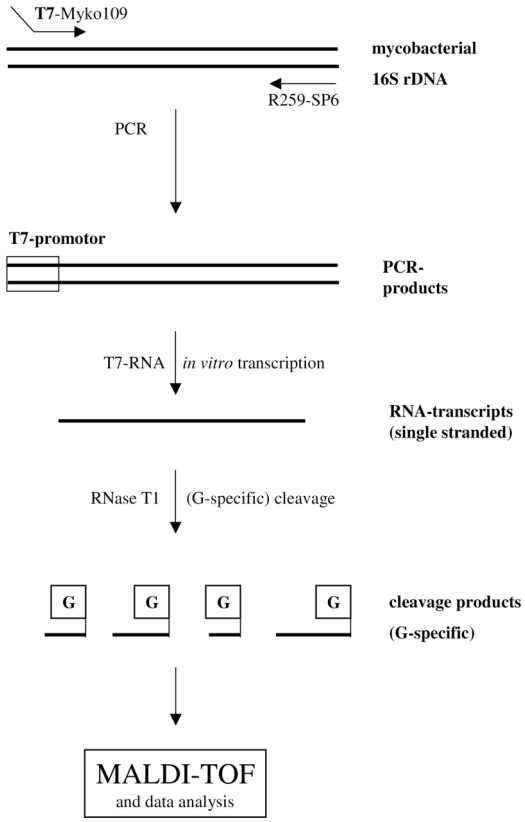


FIG. 1. Schematic representation of the base-specific cleavage of amplified and reverse-transcribed mycobacterial 16S rDNA.

RESULTS

An ~500-bp region of the 16S rRNA gene corresponding to *E. coli* 16S rDNA positions 105 to 609 was PCR amplified from all type strains and clinical isolates. RNA transcription and base-specific cleavage resulted in unique MALDI-TOF mass spectra for all tested type strains. The basic principle of the method is depicted in Fig. 1.

A representative mass spectrum of *M. tuberculosis* H37Rv is shown in Fig. 2a. The main cleavage products corresponding to peaks 1 to 27 are listed in Fig. 2b. Their nucleic acid composition and exact location within the uncleaved PCR amplicon are assigned. Reference mass signals have been calculated from the reference sequence by in silico cleavage at all positions of guanine and correlated to mass signals detected by MALDI-TOF MS. Calculated fragments with a mass difference smaller than 4 Da could not be separated in the linear, axial MALDI-TOF MS employed in this study. Corresponding detected cleavage products were assessed as one fragment only (peaks 2, 3, 4, 8, 9, 11, 12, and 18).

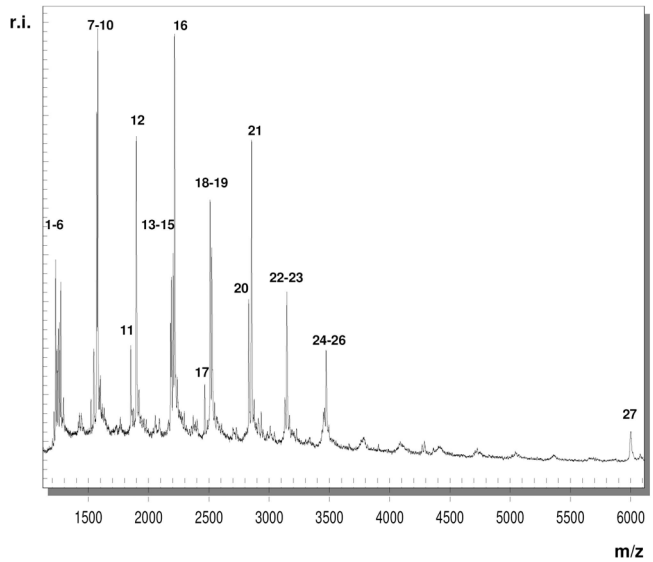
Mass signals 22, 24, and 25 (Fig. 2b) are classified LAST, because they represent cleavage products at the 3′ end of the transcript (all at position 510) differing by the addition of one 5-methyl-CTP (3′ fragment + 319.2 Da) or one ATP (3′ fragment + 329.2 Da), respectively. Nontemplated addition of a nucleotide to the 3′ end of the RNA transcript most probably reflects terminal transferase activity of T7-RNA polymerase, a feature well known for *Taq* DNA polymerases. The nontemplated addition of nucleotides to the terminal fragments has been included in the software-automated identification of fragments for all mycobacterial species to avoid misinterpretation.

Characteristic mass spectra of five representative mycobacterial type strains in a mass range between 1,500 and 2,600 Da are shown in Fig. 3. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. celatum* can be clearly differentiated by their unique mass spectra. *M. tuberculosis* is the only species lacking a fragment at 1,828 Da. *M. celatum* shows a signal at 1,884 Da not present within all other mass patterns. The spectrum of *M. kansasii* displays no signal at 2,180 Da. Mass spectra of *M. avium* and *M. intracellulare* differ from those of the other species by fragments at 2,532 and 2,157 Da, respectively.

In silico discriminatory peak patterns of all mycobacterial species used in this study are compiled in Table 2. The ranking was performed according to the number of missing and additional peaks compared to the mass spectrum of *M. tuberculosis*. Only discriminatory peaks that are not present throughout all *Mycobacteria* spp. are included. *M. tuberculosis* can be clearly differentiated from other species on the basis of multiple additional or missing mass signals. *M. celatum* and *M. kansasii* are the species closest to *M. tuberculosis*, showing one missing and three additional peaks or two missing and two additional peaks, respectively. *M. marinum* and *M. scrofulaceum* differ by only two fragments (2,453.5 and 2,795.8 Da). All calculated mass patterns have been confirmed experimentally. A comparison of all mass spectra resulted in unambiguous identification of all mycobacterial species.

In the case of the *M. xenopi* type strain, DSM 43995, com-

a)



b)

Peak No.	Detected mass signal in Da	Reference mass signal in Da	Ref type	Description
1	1215.86	1215.8	MAIN	5OH-TTCG-3OH @491; 5OH-CTTG-3OH @146
2	1228.94	1225.8	MAIN	5OH-ATTG-3OH @368
		1228.84	MAIN	5OH-CTCG-3OH @468; 5OH-CCTG-3OH @49; 5OH-TCCG-3OH @444; 5OH-TCCG-3OH @204; 5OH-CCTG-3OH @192; 5OH-CCTG-3OH @283; 5OH-TCCG-3OH @353
3	1240.04	1238.84	MAIN	5OH-CATG-3OH @90; 5OH-ACTG-3OH @219
		1241.89	MAIN	5OH-CCCG-3OH @131
4	1251.19	1248.84	MAIN	5OH-ATAG-3OH @74
		1251.89	MAIN	5OH-CCAG-3OH @408
5	1262.16	1261.88	MAIN	5OH-CAAG-3OH @279
6	1272.03	1271.88	MAIN	5OH-AAAG-3OH @105
7	1521.82	1521.97	MAIN	5OH-TCTTG-3OH @94
8	1547.61	1545.01	MAIN	5OH-ATCTG-3OH @25
		1548.06	MAIN	5OH-CCCTG-3OH @30
9	1570.81	1568.05	MAIN	5OH-ATACG-3OH @225
		1571.1	MAIN	5OH-CCCAG-3OH @231; 5OH-CACCG-3OH @391
10	1578.24	1578.05	MAIN	5OH-TAAAG-3OH @461; 5OH-ATAAG-3OH @44
11	1852.68	1851.18	MAIN	5OH-CTTTAG-3OH @111
		1854.22	MAIN	5OH-CCTTCG-3OH @316
12	1899.69	1897.26	MAIN	5OH-AAACTG-3OH @55
		1900.3	MAIN	5OH-ACCACG-3OH @79
13	2182.97	2183.43	MAIN	5OH-CACTTCG-3OH @35
14	2190.23	2190.39	MAIN	5OH-AATATTG-3OH @261
15	2203.56	2203.43	MAIN	5OH-TAATACG-3OH @421
16	2216.62	2216.47	MAIN	5OH-TAACACG-3OH @12; 5 OH-CACAATG-3OH @268
17	2466.45	2466.56	MAIN	5OH-TTCTCTCG-3OH @359
18	2511.28	2509.6	MAIN	5OH-AATTACTG-3OH @449
		2512.64	MAIN	5OH-CCTATCAG-3OH @138
19	2525.55	2525.69	MAIN	5OH-CCACACTG-3OH @209
20	2831.65	2831.86	MAIN	5OH-ACTCCTACG-3OH @236
21	2854.75	2854.9	MAIN	5OH-CCAACTACG-3OH @397; 5OH-CCTACCAAG-3OH @165
22	3131.39	3132.02	LAST	5OH-TCACCTAAAT-3OH @510
23	3148.06	3148.02	MAIN	5OH-TCTAATACCG-3OH @63
24	3451.22	3451.24	LAST	5OH-TCACCTAAAT-C-3OH @510
25	3461.86	3461.23	LAST	5OH-TCACCTAAAT-A-3OH @510
26	3474.06	3474.19	MAIN	5OH-AAATTCTATAG-3OH @497
27	6000.58	6001.8	MAIN	5OH-TAAACCTCTTTCACCATCG-3OH @327

FIG. 2. (a) *M. tuberculosis* mass spectrum displaying all signals generated by G-specific cleavage of the 16S rDNA amplicon after RNA transcription. To simplify identification, all mass signals corresponding to main cleavage products are marked with numbers. (b) List of detected mass signals corresponding to the spectrum depicted in panel a. For all signals the sequence and location within the uncleaved PCR amplicon is provided in the description column (position follows "@"). MAIN, main cleavage product; LAST, product at the 3′ end of the amplicon, often elongated by one nucleotide as a result of 3′-terminal transferase activity of T7 RNA polymerase.

parison of experimental and calculated mass patterns revealed an additional mass peak at 4,408.8 Da in MALDI-TOF analysis (Fig. 4a). Cloning of the respective *M. xenopi* 16S rDNA amplicon and repeated sequencing of several plasmids resulted in the detection of three sequence variants differing in 1 to 2 bp at *E. coli* position 198 (T/C) and 434 (T/C) (Fig. 4b). The sequence variation at *E. coli* position 198 is not detected in a G-specific cleavage reaction. The resulting dimeric fragments (5OH-TG-3p and 5OH-CG-3p) overlap with cleavage products of the same composition originating from different posi-

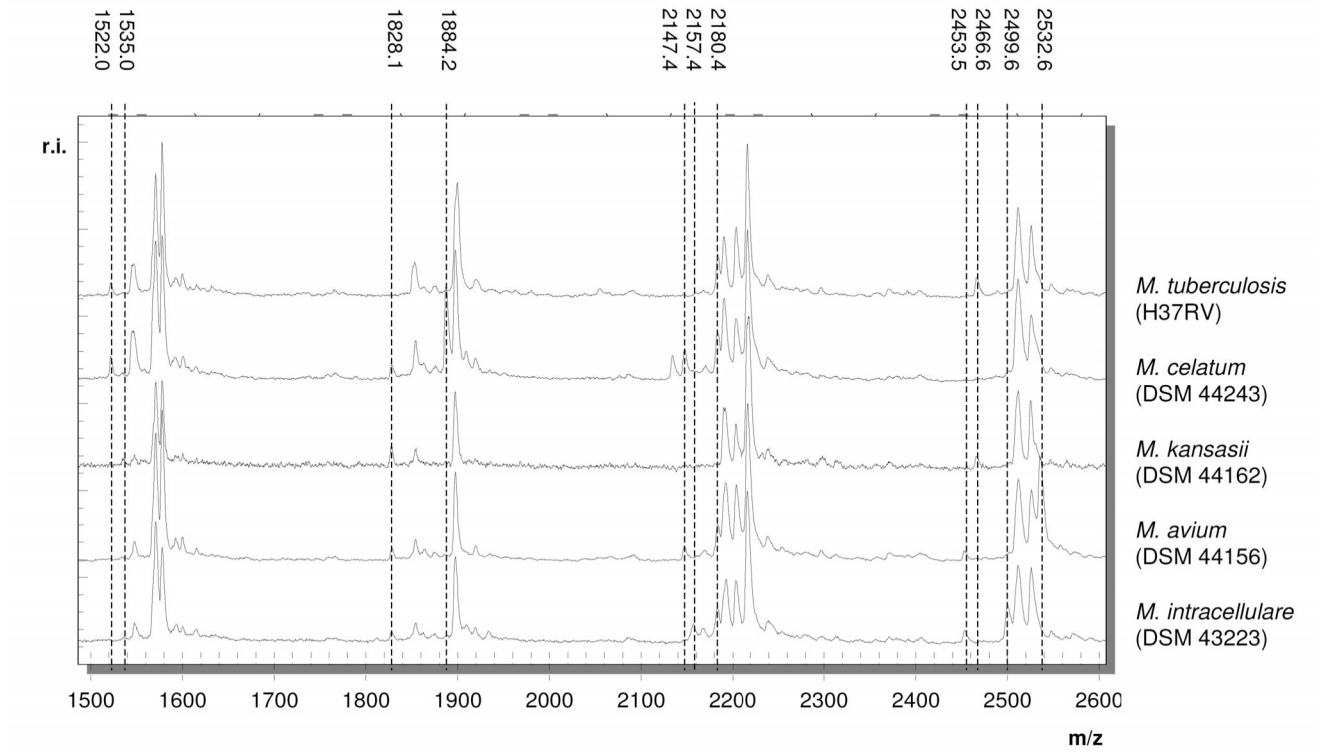


FIG. 3. Overlay of mass spectra of five representative mycobacterial strains in a mass range between 1,500 and 2,600 Da.

TABLE 2. Ranking of base-specific mycobacterial 16S rDNA cleavage mass patterns according to the number of identical and nonidentical mass peaks relative to the mass spectrum of *M. tuberculosis*[a]

Organism	Peak(s) with calculated mass(es) (Da) of:																											
	1,215.8	1,261.9	1,522.0	1,535.0	1,555.0, 1,558.0	1,591.0	1,828.1	1,864.2	1,877.3	1,884.2, 1,887.3	2,147.4	2,157.4	2,170.4	2,180.4, 2,183.4	2,203.4, 2,206.5	2,229.5	2,453.5	2,466.6	2,499.6	2,532.6, 2,535.7	2,795.8	2,808.8	2,818.8	2,841.9	2,867.9	3,490.3	4,079.6	4,408.8	4,421.8	4,764.1	5,373.4	6,001.8
M. tuberculosis	■	■	■											■	■			■														■
M. celatum	■	■	■				▒			▒	▒			■	■																	■
M. kansasii	■	■		▒			▒								■			■														■
M. marinum	■	■		▒			▒							■	■		▒															■
M. scrofulaceum	■	■		▒			▒							■	■						▒											■
M. paraffinicum	■	■		▒			▒							■	■							▒										■
M. avium	■	■					▒				▒			■	■		▒			▒												■
M. intracellulare	■	■					▒					▒		■	■		▒		▒													■
M. gordonae	■			▒			▒							■	■	▒	▒															■
M. xenopi	■	■			▒									■	■		▒						▒						▒			
M. interjectum	■	■		▒			▒	▒	▒					■	■													▒				
M. smegmatis	■	■					▒						▒		■											▒		▒				
M. fortuitum		■			▒		▒			▒			▒		■																▒	
M. abscessus		■			▒	▒	▒							■	■									▒	▒			▒		▒		
M. aurum	■	■					▒	▒		▒			▒							▒							▒					

[a] Only discriminating mass peaks are shown. Mass peaks identical to those of *M. tuberculosis* are shown as black boxes. Additional mass peaks are shown as gray boxes.

tions in the amplicon. Base-specific cleavage of an ~500-bp amplicon statistically results in all possible combinations of dimers represented multiple times. In addition, the mass range below 1,000 Da can be affected by background noise signals caused by matrix molecules, a feature specific to the use of 3-hydroxypicolinic acid matrices in MALDI-TOF MS.

Sequence variation at *E. coli* position 434 (T/C) affects a 14-bp G-specific cleavage product. The nucleotide mass difference between a T (corresponding to U in cleaved RNA) and a C diminishes the mass of the expected fragment by 13 Da. The detection of mass signals at both 4,408.8 and 4,421.8 Da leads to the conclusion that the analyzed amplicon of the type strain consists of a mixture of both sequence variants.

After establishing a database including the 12 mycobacterial type strains 24 clinical isolates have been analyzed automatically with MALDI-TOF MS. G-specific cleavage of RNA-transcribed 16S rDNA amplification products and MS led to unambiguous identification of 21 isolates. Results are presented in Table 1. All isolates representing species from the type strain database were identified correctly in repeated experiments. Three clinical isolates representing *M. aurum* (MT1323), *M. paraffinicum* (MT1423), and *M. interjectum* (MT1223) could not be identified after MALDI-TOF analysis of their RNA cleavage products. The database lacked the corresponding in silico mass pattern of all three species. An extension of the database with the species-specific mass signal pattern calculated from published 16S rDNA sequences of *M. aurum* (MT1323), *M. paraffinicum* (MT1423), and *M. interjectum* (MT1223) led to correct identification in all corresponding experiments.

DISCUSSION

The advantage of using molecular analysis based upon 16S rDNA sequence determination over using phenotypic methods for the identification of mycobacterial isolates has been extensively evaluated. In contrast to phenotypic (e.g., biochemical) features, the 16S rRNA sequence of a species is a stable property specific for most mycobacteria at the species level. Unfortunately members of the *Mycobacterium* tuberculosis complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*) share identical rDNA sequences. Instead, polymorphisms in the *gyrB* sequences facilitate the differentiation of all four species (24). Springer et al. found two 16S rDNA sequence regions, termed A and B, corresponding to *E. coli* 16S positions 129 to 267 and 430 to 500, suitable to identify most mycobacteria (33). RIDOM (http://www.ridom.de/) (15) and the MicroSeq 500 16S rDNA bacterial sequencing kit (PE Applied Biosystems) (8) employ the same regions of 16S rDNA in a stretch of 440 to 500 bp for mycobacterial identification.

In this study, we present comparative sequence analysis by MALDI-TOF MS as a novel tool for the analysis of a similar 500-bp region covering variable sequence regions A and B for 16S rDNA-based genotyping of mycobacteria. With the introduction of capillary systems, standard 16S rDNA sequencing has developed further during the past decade, and same-day turnaround times can be achieved. Although DNA extraction, PCR amplification, RNA transcription, and G-specific cleavage require similar time frames as processing steps in conventional sequencing, the speed and accuracy of MALDI-TOF MS are very appealing. Currently available mass spectrometers allow analysis of all cleavage products in less than 2 s. Sample processing can be automated (96- and 384-microtiterplate format), and analysis by MALDI-TOF MS can be completed within 4 h post-PCR processing using the described protocol.

According to Hartmer et al. (16) the small mass difference of 1 Da between uracil and cytosine is a serious limitation for the analysis of RNA fragments by MALDI-TOF MS. Therefore, CTP was exchanged for its 5-methyl base-modified ribonucleotide triphosphate analogue to increase the mass difference to

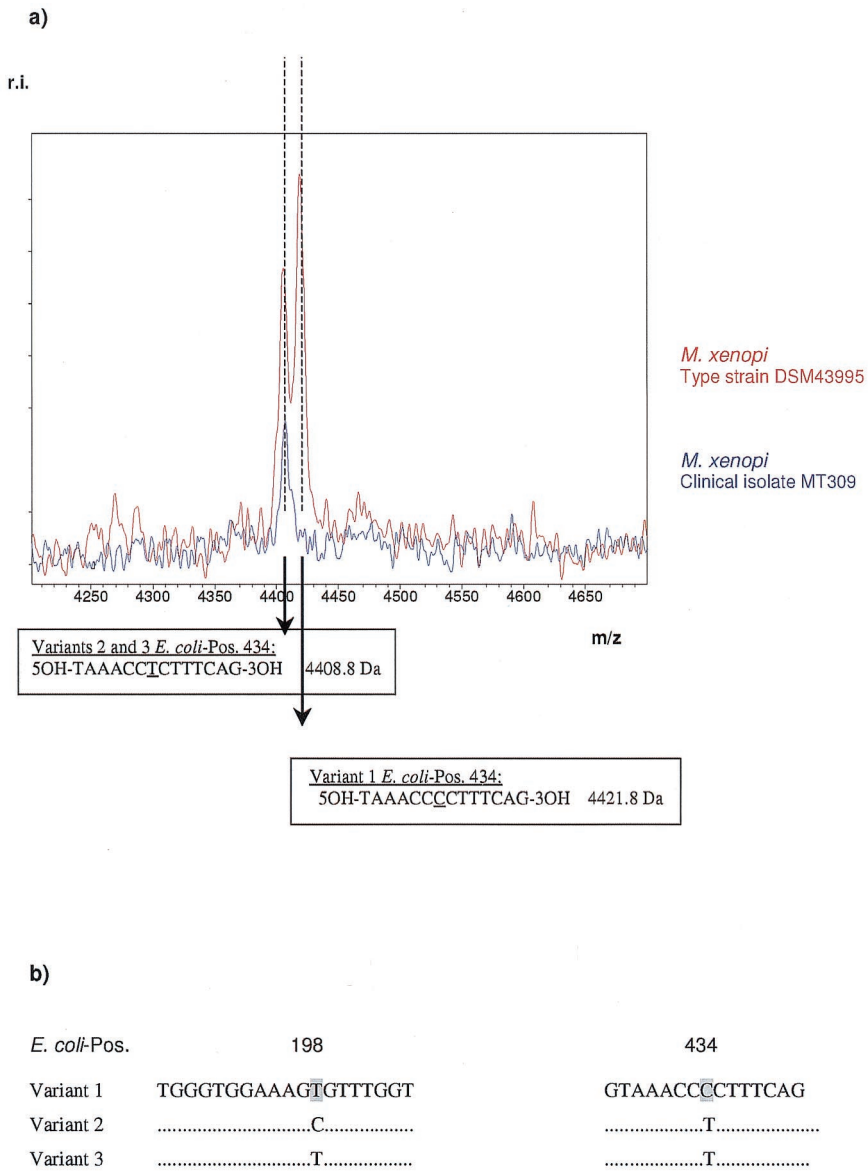


FIG. 4. Identification of *M. xenopi* variants. (a) Experimental mass pattern. (b) Sanger dideoxy sequencing of cloned 16S rDNA.

13 Da without loss in transcription yield. By using 5-methyl-CTP instead of CTP we were able to achieve good fragment separation with MALDI-TOF MS, limited only by fragments differing by less than 4 Da. RNA cleavage was performed with RNase T1, resulting in complete G-specific cleavage of the RNA transcripts (16). Addition of shrimp alkaline phosphatase leads to removal of 3′-phosphate groups at each fragment, further avoiding the development of 2′, 3′-cyclic phosphate groups as potential side products. Recently, von Wintzingerode et al. (39) described the use of MALDI-TOF MS for analysis of UDG-mediated base-specific cleavage of 16S rDNA PCR products for bacterial identification. After strand separation, UDG-mediated phosphate backbone cleavage resulted in a mixture of hydrolysis products (fragments applied for identification) and side products (nonspecific cleavage products without discriminatory power), increasing the complexity of the mass spectrum. Our approach generated no side products, and all resulting cleavage products were used for identification of the underlying sequence. The use of post-PCR in vitro RNA transcription mediated an additional amplification step resulting in a higher analyte concentration for MS analysis. Furthermore, the process generates a single-stranded nucleic acid, thus eliminating the need for strand separation. Moreover, the stability of RNA during the desorption-ionization process is higher than that of DNA due to the balancing effect of the 2′-hydroxy group on the polarization of the N-glycosidic bond of protonated bases (34).

MALDI-TOF analysis of base-specific 16S rRNA fragments resulted in unique mass spectra for each of the mycobacterial type strains. According to Fig. 3 and Table 1 some single mass peaks seem to be species specific (e.g., 2,157.4 Da for *M. intracellulare*, 2,229.5 Da for *M. gordonae*, and 3,490.3 Da for

M. smegmatis). However, the number of unique mass signals is expected to decrease within a growing database. In consequence, analysis based on single species-specific peaks could lead to erroneous assignments of sample spectra to the corresponding reference spectra. Hence, the process for identification of species was based on a comparison of whole mass peak patterns.

Based on a database of in silico patterns derived from in-house sequences and database 16S rDNA sequences, 24 mycobacterial isolates were identified correctly, and repeated experiments showed a high degree of reproducibility. This illustrates the potential of enlarging the database with more mycobacterial species-specific cleavage patterns of published 16S rDNA sequences. Even species differing in only three bases of their reference sequence, like *M. paraffinicum* and *M. scrofulaceum*, are unambiguously identified by G-specific cleavage and the corresponding mass signal pattern.

MALDI-TOF MS of cleavage products derived from *M. xenopi* type strain DSM 43995 resulted in discrepancies between expected mass signal patterns and sequencing data, illustrating the capabilities of our method, but also the limitations when only a single cleavage reaction is employed. A single nucleotide sequence variation embedded in a 14-mer cleavage product resulted in the unambiguous detection of an additional mass signal, whereas a second sequence variation as part of a 2-bp fragment did not induce a discernible change in the detected mass signal pattern. Due to overlapping cleavage products of the same composition and due to the small mass, the latter could not be detected. An opportunity to overcome this issue is alteration of the fragment compositions by a change of the cleavage base and by performing additional cleavage reactions (e.g., base-specific cleavage at C or T; unpublished data). This facilitates detection, identification, and localization of sequence changes and microheterogeneities (30).

Identification of the detected sequence variations in RIDOM (http://www.ridom.de/) showed 100% similarity with three sequevars of *M. xenopi* (variant 1 = DSM 43995, variant 2 = S88, variant 3 = S91). Variants S91 and S88 differ from type strain DSM 43995 by 1 to 2 bp and exhibit a 4- or 5-bp insertion in the 16S-23S rDNA internal transcribed spacer sequence (31). Interestingly, patient isolates MT309 and MT2236 revealed a missing signal at 4,421.8 Da and an additional signal at 4,408.8 Da, corresponding to the reference sequences of variants 2 and 3 of type strain *M. xenopi* DSM 43995 (Fig. 4). Results of partial 16S rDNA sequencing of both isolates verified the sequence variation (T instead of C) at *E. coli* position 434 and detected a C at *E. coli* position 198, resulting in 100% sequence identity with *M. xenopi* strain S88 as derived from the RIDOM database.

The method presented here uses 16S rDNA for identification of cultured mycobacterial strains. As described recently (39), 16S rDNA-based identification can be extended to yet-uncultured bacteria, and the application can be utilized for phylogenetic classification and typing of unknown bacteria. In contrast, protein- or whole-cell-based identification by MS requires cultivation and is prone to growth-dependent variations, resulting in problems of low reproducibility (4, 5, 35). Compared to conventional 16S rDNA sequencing for identification of bacteria, the absence of any time- and cost-consuming electrophoresis step is an important advantage. The experimental procedure for PCR amplification, RNA transcription, and subsequent base-specific cleavage is straightforward, and a homogeneous format avoids any purification steps.

So far we have restricted our approach to the analysis of ~500-bp amplicons. Comparative sequence analysis of larger target regions (e.g., 1,500 bp) covering the whole 16S rDNA appears to be promising. Further experiments will show whether a larger number of cleavage products will result in higher discriminatory power or will obscure important peaks by increasing cross talk and background noise due to a higher number of overlapping mass signals.

Resequencing of nucleic acids by base-specific cleavage after virtually all four bases has been described elsewhere (30). A single-stranded copy of the target sequence is cleaved to completion in four separate reactions at positions corresponding to each of the four bases. After MALDI-TOF MS each resulting peak can be unambiguously identified using a reference sequence. Changes in sequence have discernible effects on a maximum of four cleavage patterns. With reference sequences available for many bacterial pathogens, comparative sequence analysis with validation by MALDI-TOF seems to be an ideal tool for phylogenetic and diagnostic purposes as well as microheterogeneity detection and localization.

The platform is not restricted to 16S rDNA identification, but can be extended to other genotypic markers, e.g., *gyrB* sequence polymorphism analysis for *M. tuberculosis* complex differentiation, multidrug-resistant regions or multilocus-sequence typing, which further broadens its application.

The approach presented here is a promising prototype for high-throughput applications in clinical diagnostics as well as pharmaceutical and environmental microbiology, combining the accuracy of MALDI-TOF MS with the phylogenetic information of genotypic marker regions.

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