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Identification of *Francisella tularensis* by Whole-Cell Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry: Fast, Reliable, Robust, and Cost-Effective Differentiation on Species and Subspecies Levels[▽]

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***Francisella tularensis*, the causative agent of tularemia, is a potential agent of bioterrorism. The phenotypic discrimination of closely related, but differently virulent, *Francisella tularensis* subspecies with phenotyping methods is difficult and time-consuming, often producing ambiguous results. As a fast and simple alternative, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was applied to 50 different strains of the genus *Francisella* to assess its ability to identify and discriminate between strains according to their designated species and subspecies. Reference spectra from five representative strains of *Francisella philomiragia*, *Francisella tularensis* subsp. *tularensis*, *Francisella tularensis* subsp. *holarctica*, *Francisella tularensis* subsp. *mediasiatica*, and *Francisella tularensis* subsp. *novicida* were established and evaluated for their capability to correctly identify *Francisella* species and subspecies by matching a collection of spectra from 45 blind-coded *Francisella* strains against a database containing the five reference spectra and 3,287 spectra from other microorganisms. As a reference method for identification of strains from the genus *Francisella*, 23S rRNA gene sequencing was used. All strains were correctly identified, with both methods showing perfect agreement at the species level as well as at the subspecies level. The identification of *Francisella* strains by MALDI-TOF MS and subsequent database matching was reproducible using biological replicates, different culture media, different cultivation times, different serial *in vitro* passages of the same strain, different preparation protocols, and different mass spectrometers.**

Francisella tularensis is the causative agent of the zoonotic disease tularemia. Beside its medical and veterinary impact, it possesses a high potential to be used for bioterrorist attacks (7). The World Health Organization (WHO) has modeled a bioterrorist attack with *Francisella tularensis* leading to very high costs for society ($5.4 billion if 100,000 individuals are affected) due to the extreme infectivity of the pathogen. Tularemia appears in various clinical forms depending on the virulence of the involved *F. tularensis* strain as well as the route and dose of inoculation.

The genus *Francisella* was recently rearranged (12, 23) and is currently divided into four species, including *F. tularensis* and *F. philomiragia.* The species *Francisella tularensis* is further subdivided into four subspecies, *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *mediasiatica*, and *F. tularensis* subsp. *novicida*, all four showing significant differences in virulence regarding animal and human infections (28). *F. tularensis* subsp. *tularensis* (type A), which is almost exclusively found in North America, and *F. tularensis* subsp. *holarctica* (type B), which is endemic in areas all over the northern hemisphere, represent the two most virulent and clinically relevant subspecies. Several strains of *F. tularensis* subsp. *mediasiatica* have been isolated in Central Asia, but little is known about their virulence in humans. *F. tularensis* subsp. *novicida* is rarely isolated from human specimens but can cause a tularemia-like disease in immunocompromised individuals (5).

Differentiation of the highly virulent *F. tularensis* subsp. *tularensis* from the less virulent *F. tularensis* subsp. *holarctica* is of substantial clinical interest (30) but may be even more important regarding the potential use of *F. tularensis* as a biological warfare agent.

Identification and differentiation of bacteria based on the polymorphism of the 16S and 23S rRNA genes have become widely accepted (18). Although 16S rRNA gene sequencing is accepted as the reference method for species identification (3, 6, 8), 23S rRNA gene sequencing seems to be more suitable for the identification of *F. tularensis* subspecies (W. Splettstoesser, E. Seibold, E. Zeman, K. H. Trebesius, and A. Podbielski, submitted for publication).

Cultivation of *F. tularensis* is fastidious. It has high requirements for the growth media used for its cultivation (e.g., Thayer-Martin and cysteine heart agar [CHA]), and cultivation may take up to 10 days (29). Identification and differentiation of *Francisella* strains by traditional phenotyping are difficult, often leading to ambiguous results. Additionally these methods include prolonged cultivation, thereby increasing the risk for laboratory-acquired tularemia (29).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has been successfully applied to identify bacterial species (2, 10, 13, 15, 20, 22, 24, 25) by measuring the mass of peptides and small proteins from

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whole cells. The resulting protein spectra are postulated to be characteristic for each bacterial species. The available literature regarding the identification of and discrimination between subspecies or even single strains by MALDI-TOF MS is limited (1, 24). *Francisella tularensis* subspecies were successfully discriminated with surface-enhanced laser desorption ionization–time of flight MS (SELDI-TOF MS) (19, 26), a modified MALDI-TOF MS method which allows selective absorption of proteins on a chromatographic array surface prior to MS (31, 32). Compared to SELDI-TOF MS, MALDI-TOF MS is less time-consuming and more cost-effective. Furthermore, there are several databases available for MALDI-TOF MS, which allow for matching spectra of unknown isolates with reference spectra.

Although there are still concerns regarding the reproducibility of MALDI-TOF MS under different cultivation conditions, recent publications showed good reproducibility for various cultivation conditions in several bacterial groups (22, 24). A recent international study achieved 98.75% interlaboratory reproducibility regarding the identification of 60 blind-coded nonfermenting bacterial samples (21).

The aim of our study was to identify and differentiate strains of the genus *Francisella* by the use of MALDI-TOF MS at the subspecies level by combining MALDI-TOF MS with dedicated bioinformatics and statistical methods, i.e., database search, pattern-matching algorithm, cluster analysis, and principal component analysis (PCA). Initial spectra from representative strains of each *Francisella* species and subspecies were used to set up database entries for reidentification of *F. tularensis* strains. This database was evaluated with 45 blind-coded *Francisella* strains that were also analyzed by 23S rRNA gene sequencing. Reproducibility of the method was tested with various growth media, cultivation times, numbers of strain passages, protein extraction protocols, and mass spectrometers.

MATERIALS AND METHODS

Bacterial strains. The *Francisella* strains used in this study comprised field isolates from Austria (kindly provided by E. Hofer) and Germany and strains obtained from the *Francisella* strain collection of the Swedish Defense Research Agency (kindly provided by M. Forsman) (Table 1). The strains have been characterized by fragment analysis of the RD1 PCR (4) and multiple-locus variable-number-tandem-repeat analysis (MLVA) (14). Unless stated differently, *Francisella* cultures were initiated directly from frozen stocks and grown for 72 h on CHA (Becton Dickinson, Heidelberg, Germany) at 37°C and 5% CO_2.

Sample preparation for MALDI-TOF MS, spectrum generation, and data analysis. For protein extraction, two different protocols were used. Unless explicitly stated differently, a trifluoroacetic acid (TFA)-based protocol was used (16). Briefly, an equivalent of three wire loops of bacterial biomass was suspended in 1 ml 80% TFA and incubated for 30 min at 4°C. A total of 200 μl of this suspension was transferred into a new tube and mixed with 600 μl aqua dest and 800 μl acetonitrile (AN). After centrifugation of this extract at 13,000 × *g* for 5 min, the supernatant was used for MS.

After extraction, 1 μl of each of the respective samples was placed onto a polished steel MALDI target plate and allowed to dry at room temperature. Subsequently, each sample was overlaid with 1 μl of matrix (10 mg/ml α-cyano-4-hydroxy-cinnamic acid in 50% AN-2.5% TFA) and dried at room temperature.

MALDI-TOF MS analyses of all strains were performed on a microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with a 20-Hz nitrogen laser. Instrument parameter settings were as follows: IS1, 20 kV; IS2, 18.5 kV; lens, 8.5 kV; PIE, 250 ns; no gating; detector gain, 2,650 V. A subset of samples was measured with an Ultraflex III TOF/TOF mass spectrometer (IS1, 25 kV; IS2, 23.45 kV; lens, 6 kV; PIE, 100 ns; gating at 1,500 Da; detector gain, 1,704 V). Spectra were collected in the linear positive mode within a mass range of 2,000 to 20,000 Da and at maximum laser frequency. Before the sample measurements were recorded, the mass spectrometer was calibrated using a bacterial test standard (Bruker Daltonics, Bremen, Germany). Bacterial strains investigated for the first time were recorded as reference spectra (synonymous with main spectra), using the automated functionality of the MALDI Biotyper 2.0 software package. Recording of the averaged spectrum was followed by smoothing, baseline correction, external calibration, and automated peak selection (synonymous with peak picking). The resulting peak list was used for generating a corresponding reference spectrum, containing the average mass and the average intensity of the selected peaks as well as information about the frequency of the selected peaks in multiple measurements.

To identify unknown bacterial strains, their spectra were used for pattern matching against all reference spectra in a database, which is part of MALDI Biotyper 2.0 software package. The highest match was used for the identification of an unknown bacterial strain. The database contained 3,287 reference spectra of a broad variety of microorganisms. Reference spectra of five *Francisella* reference spectra generated as a part of this study were added to this database.

Pattern matching was determined by calculation of a score, considering the proportion of matching peaks between the unknown spectrum and the reference spectra of the database as well as the consistency of the peak intensities between these spectra. The logarithmic score values (here abbreviated as log score) range from 0 (no similarity) to 3 (absolute identity). Log score values of ≥2.0 are rated as a firm identification at species or subspecies level. Log score values of ≥1.7 and <2.0 are rated as identification of microorganisms at least at genus level. Log score values of <1.7 are rated as not suitable for identification by the MALDI Biotyper 2.0 software package. These thresholds were empirically determined with data of more than 3,000 well-characterized bacterial strains.

PCA and hierarchical cluster analysis were conducted with the integrated tools of the MALDI Biotyper 2.0 software package, using default settings.

23S rRNA gene sequencing and sequence analysis. Sequencing of the 23S rRNA gene was used as a reference method for species and subspecies designation. Sequenced data were stored and analyzed using Bionumerics 5.0 (Applied Math, Belgium). Identification of species and subspecies was performed by identification of single-nucleotide polymorphisms.

DNA of bacterial strains was extracted with the QIAamp DNA mini kit according to manufacturer's manual (Qiagen, Hilden, Germany). The full 23S rRNA gene was amplified with three PCRs, which generated overlapping fragments. The primer sequences are summarized in Table 2. For each PCR, 2 μl of the extracts were used. PCR was performed in a total volume of 50 μl containing 20 μl 5 Prime MasterMix (2.5×; 5 Prime, Hamburg, Germany) and 0.2 μM each the forward and reverse primers. The PCRs were performed with a GenAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA). Cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 30 s, amplification at 72°C for 90 s, and a single final extension at 72°C for 5 min. The PCR products were purified with the QIAquick PCR purification kit according to the manufacturer's manual (Qiagen, Hilden, Germany).

The purified PCR products were sequenced with an BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The total volume of the sequencing reaction mix was 10 μl containing 4 μl of the ready mix (BigDye Terminator v3.1 cycle sequencing RR-100) from the kit, 3 μl of the purified PCR product, and 0.2 μM the respective sequencing primer. Identical primers were used in both the amplification and sequencing PCR. All sequencing reactions were performed with the same thermocycler as described above. Cycler conditions were as follows: initial denaturation step at 96°C for 1 min, followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. The sequencing products were purified with Centri-Sep spin columns (Princeton Separations, Adelphia, NJ) and subsequently analyzed on a 3130 genetic analyzer (Applied Biosystems, Foster City, CA) in accordance with the instructions of the manufacturer.

MALDI-TOF MS reproducibility testing. For reproducibility testing, the spectra of selected *Francisella* strains were determined under different conditions. Technical variance was determined by measuring eight (technical) replicates of a given bacterial whole-cell lysate on the same MALDI target plate in the same mass spectrometer run. Thereafter, the variance of biological replicates was determined. Biological replicates are here defined as repeated samples of the same strain, cultivated and prepared on different days, but under the standard conditions as described above. Preparations of three biological replicates for each selected strain were measured at the same time on the same mass spectrometer (Microflex LT). In addition, we tested the influence of different cultivation conditions. Therefore, the selected strains were either cultivated on a

TABLE 1. *Francisella* strains used in this study

Species or origin	Strain	Alternative designation(s)
Francisella tularensis subsp. *tularensis* (type A)		
Squirrel; GA	FL3/1[a]	FSC033, SnMF, CDC standard
Hare; NV; 1953 (type A II)	FL3/5	FSC054, Nevada 14
Human; OH; 1941 (type A I)	FL3/6	FSC237, Schu S4
Tick; BC, Canada; 1935	FL3/2	FSC041
Avirulent variant of Schu S4	FL3/4	FSC043
Unknown	FL3/7	Schu 164
Human; UT; 1920 (type A II)	F066	ATCC 6223
Francisella tularensis subsp. *holarctica* (type B)		
Tick; Moscow area, Russia; 1949	F124	FSC257, strain 503/840
Hare; Austria; 1994	F006	
Hare; Austria; 1995	F008	
Hare; Austria; 1997	F019	
Hare; Austria; 1997	F030	
Human; Austria; 1997	F036	
Live vaccine strain; Russia	F049[a]	ATCC 29684
Sweden	F123	FSC200
Callithrix jacchus; Lower Saxony, Germany; 2004	F092	Sen7055
Macaca mulatta; Lower Saxony, Germany; 2005	F099	DPZ 7131 M-4
Macaca fascicularis; Lower Saxony, Germany; 2002	F100	
Macaca fascicularis; Lower Saxony, Germany; 2002	F101	
Arvicola terrestris; Lower Saxony, Germany; 2005	F105	
United States; 1958	F077	KF479
Hare; Châteauroux, France; 1952	F078	
Human, Japan	F119	FSC20
Monkey; Bern, Switzerland; 2002	F102	
Hare; Germany; 2006	F110	
France	F127	
France	F128	
Hare; Bavaria, Germany; 2007	F154	
Human; Lower Saxony, Germany; 2008	F155	
Hare; Baden Württemberg, Germany; 2007	F156	
Hare; Lower Saxony, Germany; 2008	F157	
Human; North Rhine-Westphalia, Germany; 2007	F181	
Human; Saxony, Germany; 2008	F182	
Kosho, Japan; 1965	F076	03-01294
Austria; 1994	F053	5008/94
Francisella tularensis subsp. *mediasiatica*		
Tick; Central Asia; 1982	F064	FSC148
Midday gerbil; Central Asia, Kazakhstan; 1965	F063[a]	FSC147
Unknown	F065	FSC149
Unknown	F070	117/16
Francisella tularensis subsp. *novicida*		
Human; TX; 1995	F060	FSC159
Human; United Kingdom; 2003	F125	FSC595
Water; UT; 1950	F048	ATTC 15482
Human; TX; 1991	F059	FSC156
Water; UT; 1950	F061[a]	FSC040, ATTC 15482
Francisella philomiragia		
Water; UT	F093	ATCC 25017
Water; UT	F050[a]	ATCC 25016
Muskrat; UT; 1959	F051	ATCC 25015
Abscess, human; Gothenburg, Sweden; 1982	F184	CCUG 12603
Blood, human; Zürich, Switzerland; 1979	F185	CCUG 13404
Water; UT; 1960	F094	ATCC 25018

[a] Reference strain, used for generating reference spectra.

different agar (Thayer-Martin; Becton Dickinson, Heidelberg, Germany) at 37°C for 48 h or cultivated on CHA at 37°C for 96 h and 120 h. The influence of a different preparation protocol prior to MALDI-TOF MS was also tested. This second preparation protocol is based on the one described by Mellmann and colleagues (22). Briefly, an equivalent of three wire loops of bacterial biomass was suspended in 1.2 ml 75% ethanol and incubated for 30 min at room temperature. After centrifugation of the suspension at 13,000 × *g* for 5 min, the supernatant was removed thoroughly, and the pellet was dried. For sample extraction, the pellet was resuspended in 50 μl of 70% formic acid. After addition of 50 μl AN and mixing, the extract was centrifuged at 13,000 × *g* for 5 min. Only

TABLE 2. Primer sequences for amplification of the 23S rRNA gene

Primer name	Sequence	Position[a]
23sFtul_1nf	5′-ATG AAG GAC GTG ATA ATC TGC G-3′	56–76
23sFtul_2r	5′-AAC CTC CTG GCT GTC TGG G-3′	1022–1040
23sFtul_2f	5′-TGT ATA ACT CAT TGG GGT AAA GC-3′	821–842
23sFtul_3r	5′-TAC CCA CCA TAC ACT GTC CTC-3′	2188–2200
23sFtul_3f	5′-GAA GTA TAG GGT GTG ACG CC-3′	1788–1807
23sFtul_4r	5′-CAC ACC CAG CCT ATC AAC G-3′	2792–2810

[a] Positions designate the localization of the primers within a consensus sequence of the 23S rRNA gene of the genus *Francisella*.

the supernatant of the extract was used for MS. Finally the testing was done by measuring the influence of different MALDI-TOF instruments. Therefore, samples were measured in parallel on a Microflex LT and an Ultraflex III TOF/TOF instrument with the settings described above.

RESULTS

MALDI-TOF MS protein profiling and reference database. Five *Francisella* strains which are representative for *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *mediasiatica*, *F. tularensis* subsp. *novicida*, and *F. philomiragia* where chosen to generate reference spectra. MALDI-TOF MS with cell extracts from these strains resulted in spectra of sufficient quality to generate reference spectra, which were included into an existing database containing spectra of 3,287 bacterial strains, mainly of clinical relevance. The visual inspection of the five reference spectra (Fig. 1a) revealed a high overall similarity. The majority of differences are found between the *F. philomiragia* (F050) strain and the *F. tularensis* strains. For example, two peaks (at 6,153 Da and 7,757 Da) in the spectrum of F050 are shifted to masses different from the other reference strains. Some differences between the spectra of the *F. tularensis* subspecies can also be observed. The highly virulent *F. tularensis* subsp. *tularensis* (FSC033) for instance, exhibits a unique peak at 6,730 Da, whereas *F. tularensis* subsp. *holarctica* exhibits a discriminating peak at 7,800 Da.

To ascertain that variability between the reference spectra is suitable for discrimination between the different subspecies, a PCA with spectra of eight technical replicates of each of the five strain preparations was performed (Fig. 1b). Within the PCA, all technical replicates of a given reference spectrum form discrete clusters according to their strain, indicating that there is only a little variance between technical replicates of spectra of a particular strain but enough variation between the spectra of the reference strains to constitute discrimination between them (technical variability $<$ biological variability).

After establishing spectra for the *Francisella* reference strains, the MALDI Biotyper 2.0 database was extended by these reference spectra. To prove the suitability of the extended MALDI Biotyper 2.0 database for routine identification of and discrimination between *F. tularensis* subspecies, spectra of 45 blind-coded *Francisella* strains were matched against it. The set of blind-coded *Francisella* strains included laboratory strains as well as clinical and field isolates. All blind-coded strains were correctly identified according to their designated species and subspecies, with no nonidentification or false identification with the other 3,287 reference spectra contained in the database. The log scores of spectra of the blind-coded *Francisella* strains were all higher than 2.0 (Table 3).

Beside the full discrimination between all blind-coded strains with the log score approach, which is sufficient for the clinical setting, we were interested in analyzing the capability of MALDI-TOF MS to display the phylogenetic relationships of the strains. Therefore, a cluster analysis with all strains (reference as well as blind-coded strains) (Fig. 2) based on a correlation matrix was performed, using the integrated tools of the MALDI Biotyper 2.0 software package. The resulting dendrogram (Fig. 3) shows discrete clusters for *F. philomiragia*, *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, and *F. tularensis* subsp. *novicida*. The *F. tularensis* subsp. *mediasiatica* strains did not form a single cluster but did partially group within the *F. tularensis* subsp. *tularensis* cluster. Thus, in contrast to the full discrimination between the *Francisella* species and subspecies by matching spectra with reference spectra in the MALDI Biotyper 2.0 database, the cluster analysis predominantly, but not fully, resolved the *F. tularensis* subspecies. Furthermore, the *F. tularensis* subsp. *novicida* group did not cluster together with the other *F. tularensis* subspecies but did cluster with the *F. philomiragia* group.

As a reference method, sequencing of the 23S rRNA gene was used for all strains. All 45 blind-coded strains were unambiguously identified to the species and subspecies levels ($\geq$99% sequence similarity) by full 23S rRNA gene sequencing and detection of species- or subspecies-specific single-nucleotide polymorphisms. The comparison of the identification results of both methods shows perfect agreement on species as well as subspecies level (Table 4).

The overall high reproducibility of the spectra was investigated with several approaches. We first analyzed the reproducibility of subspecies identification for biological replicates by analyzing three repeated cultivations of five different strains. The spectra of all biological replicates were correctly identified within the reference database. Comparing the spectra of the respective biological replicates with each other by pattern matching resulted in log scores between 2.5 and 2.76. In the subsequent cluster analysis, biological replicates formed separate clusters according to their designated species and subspecies (Fig. 3).

Furthermore, we tested the influence of a different growth medium (Thayer-Martin agar). Four strains cultivated on Thayer-Martin agar were identified according to their species and subspecies, with log scores ranging from 2.39 and 2.72 compared with spectra of the same strains grown on CHA. Similar results were obtained when a single strain (F049) was harvested after different cultivation times (72 h, 96 h, and 120 h). F049 was correctly identified as *F. tularensis* subsp. *holarctica* at any time point, with log scores of 2.65 and 2.6 for 96 h and 120 h, respectively, compared with the spectrum of F049 after a 72-h cultivation time. Additionally, the influence of cultivation passages of a strain on the stability of the MALDI-TOF MS profiles was determined. Therefore, an *F. tularensis* subsp. *holarctica* strain (F053) was passaged 30 times on CHA. Extracts from the passage numbers 1, 2, 3, 5, 10, 15, 20, and 30 were profiled and matched against each other and the reference database. Again, the spectra of all passages re-

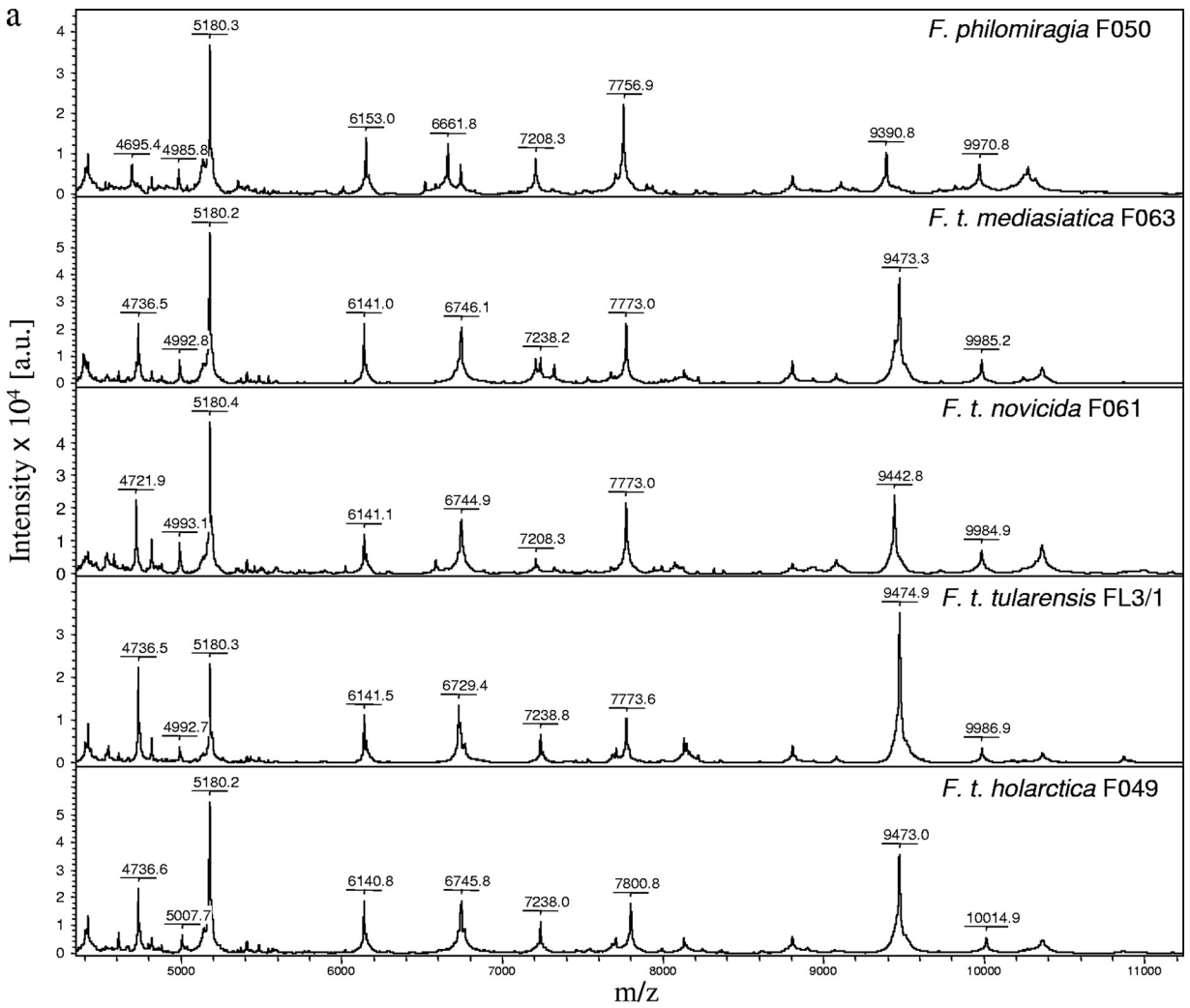

FIG. 1. (a) Comparison of the mass spectra of the five *Francisella* reference strains in the range from 3,800 to 11,200 Da. Exemplary masses (in daltons) are depicted. a.u., arbitrary units. (b) Three-dimensional PCA plot of the technical replicates of the five *Francisella* strains. Every dot represents a single technical replicate. The spectra of a particular *Francisella* reference strain are color coded: *F. tularensis* subsp. *tularensis* in red, *F. tularensis* subsp. *holarctica* in green, *F. tularensis* subsp. *mediasiatica* in blue, *F. tularensis* subsp. *novicida* in yellow, and *F. philomiragia* in pink.

TABLE 3. MALDI Biotyper 2.0 identification results summarized for 45 blind-coded *Francisella* strains on different taxonomic levels[b]

Species	Subspecies	Correct ID[a]	Log score value		
			Reference	Highest	Lowest
F. tularensis	*tularensis*	6/6	2.95	2.89	2.29
	holarctica	27/27	2.94	2.74	2.03
	mediasiatica	3/3	2.94	2.73	2.33
	novicida	4/4	2.94	2.64	2.23
F. philomiragia		5/5	2.94	2.69	2.24

[a] Proportion of correct identifications of MALDI-TOF MS spectra by matching against the reference database.

[b] The log score values for the five reference strains (reference) matched against themselves as well as the highest and lowest log scores for matches of the blind-coded strains sorted by species and subspecies are shown. The maximum log score achievable is 3.0. Log score values of ≥2.0 are rated as a correct identification of strain on species or subspecies level.

sulted in the correct identification of *F. tularensis* subsp. *holarctica*, with log scores ranging from 2.26 to 2.85 compared with the spectrum of the first passage, indicating high similarity between the spectral profiles.

The spectra also yielded reproducible identifications when we used a different protocol for preparation of the bacteria, based on ethanol and formic acid. All five strains prepared with this alternative protocol were correctly identified when matched to spectra generated with the TFA method, with a range of 2.13 to 2.24. These log score values are the overall lowest observed for all variations tested.

Finally, we compared reproducibility on two different mass spectrometers. Therefore, five *Francisella* strains were selected and analyzed on a Microflex LT mass spectrometer and in parallel on an Ultraflex III TOF/TOF mass spectrometer. All strains were identified according to their designated species and subspecies irrespective of the mass spectrometer used, with log scores ranging from 2.33 to 2.56 for spectra generated on an Ultraflex III TOF/TOF mass spectrometer and compared to their corresponding spectra from a Microflex LT mass spectrometer.

DISCUSSION

Fast and reliable identification of *F. tularensis* from clinical specimens or environmental samples is of major concern in the context of clinically severe tularemia or in the context of an intentional release of *F. tularensis*. For a rapid identification at the species or subspecies level, classical bacteriological methods are not well suited because they are slow and cumbersome and pose a significant risk for laboratory personnel. In this study, we evaluated the potential of MALDI-TOF MS to overcome these well-known problems to identify *F. tularensis* and to distinguish single isolates at the subspecies level. All 45 blind-coded strains tested in this study were correctly identified according to their designated species and subspecies, showing no misidentification compared to other clinical relevant bacteria within the database (containing spectra of 3,287 bacterial strains). The method is fast (10 to 30 min), does not need a high level of staff training, and reduces the risk of laboratory-associated infections because minimal handling of living culture material is needed for sample preparation. Biosafety of MALDI-TOF MS can be further improved by using a preparation protocol that ensures inactivation of bacteria, as done in this study. We are not aware of any phenotypic identification system showing a similar fast, reliable, and safe performance. Modern, semi- or fully automated identification systems like Phoenix (BD, Heidelberg, Germany) or the Vitek 2 automated microbiology system (bioMérieux, Marcy l'Etoile, France), which are widely used in clinical microbiology, may be able to identify *Francisella tularensis* strains; however, detailed data on their validity are not published, and misidentification may occur (17). Furthermore, these systems are not able to discriminate between *Francisella* species or between subspecies of *Francisella tularensis*. In contrast to this, phenotypic identification by MALDI-TOF MS or genetic characterization as 23S rRNA gene sequencing seems to be equally suitable for subspecies identification and differentiation within the genus *Francisella*, resulting in perfect agreement when both methods were employed in parallel.

Identification of bacteria with sequence data of rRNA genes (16S or 23S) is less prone to variation, needs no prior cultivation, and is principally applicable to mixed cultures. On the other hand, identification based on MALDI-TOF MS data is likely to achieve a higher taxonomic resolution than that achieved by rRNA gene sequencing, can be performed in high-throughput screening, and, most importantly, is less time-consuming and less expensive (27), provided that bacteria are isolated prior to identification. A recent publication compared molecular typing methods with high resolution for their ability to distinguish between *Francisella* subspecies (9). Ribotyping, pulsed-field gel electrophoresis, amplified fragment length polymorphism, and MLVA were able to provide identification to subspecies level within 12 to 24 h after obtaining an isolate, and the last method provided identification below subspecies level. The tested Raman spectrometry accomplished identification within 10 min after obtaining an isolate but could not discriminate between *F. tularensis* subspecies. In contrast to these results, MALDI-TOF MS identifies *Francisella* strains at least on subspecies level in the same time range as does Raman spectrometry.

Currently, the timeliness of diagnosis by MALDI-TOF MS is not ensured for presumptive identification, as this method is not yet directly applicable to clinical or environmental specimens as occurring during a natural tularemia outbreak or after a potential bioterrorist attack. However, preliminary data suggest that MALDI-TOF MS might be successfully applied to these specimens as well (11). Although PCR-based methods are suitable for the direct and thereby fast identification of microorganisms from such specimens, they can be inhibited by components within a specimen, provide no evidence concerning the viability of the detected microorganisms, and may possess a rather low clinical sensitivity. For these reasons, diagnostic results from PCR are usually regarded as preliminary and should be verified by other methods based on the prior isolation of the microorganism. Clinical routine diagnostic as well as definitive confirmation of a bioterrorist attack predominantly relies on prior isolation of microorganisms from clinical and environmental specimens, respectively. In this setting, i.e., starting identification after obtaining bacterial isolates, MALDI-TOF MS probably is one of the fastest diagnostic tools available to identify *Francisella* species and subspecies.

Based on log score values, all tested strains could be fully

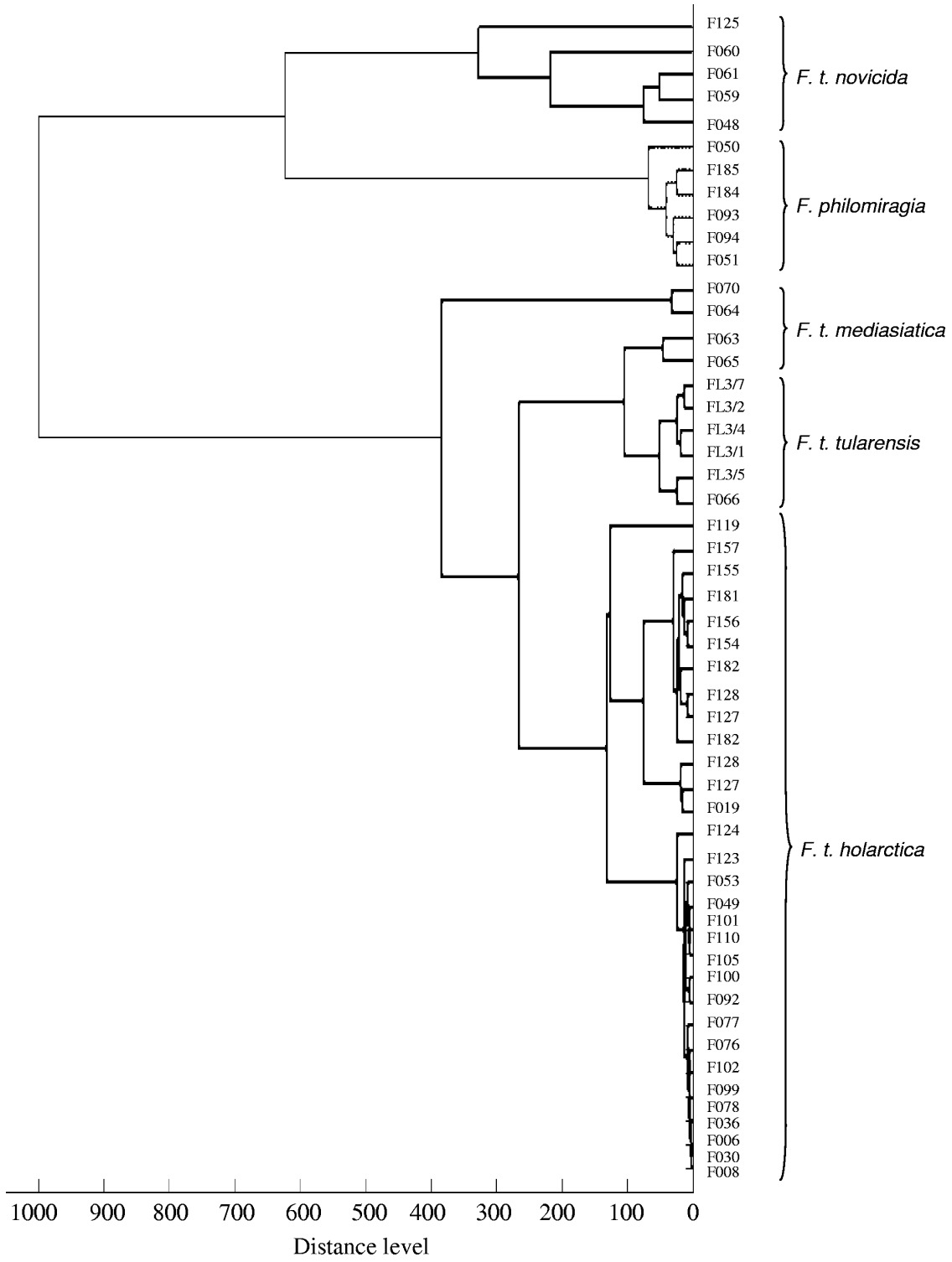

FIG. 2. Cluster analysis of MALDI-TOF MS spectra of all *Francisella* strains. Distance is displayed in relative units.

discriminated by the use of MALDI-TOF MS according to their species and subspecies, respectively. Cluster analysis based on a matrix of pairwise correlation values for all *Francisella* spectra resulted in two separate clusters on the species level, one including all *F. philomiragia* and all *F. tularensis* subsp. *novicida* strains and the other including all strains of the *F. tularensis* subspecies *tularensis*, *holarctica*, and *mediasiatica*. Within this second cluster, strains of the respective subspecies formed separate clusters, with the exception of strains of *F. tularensis* subsp. *mediasiatica*, which partially clustered to-

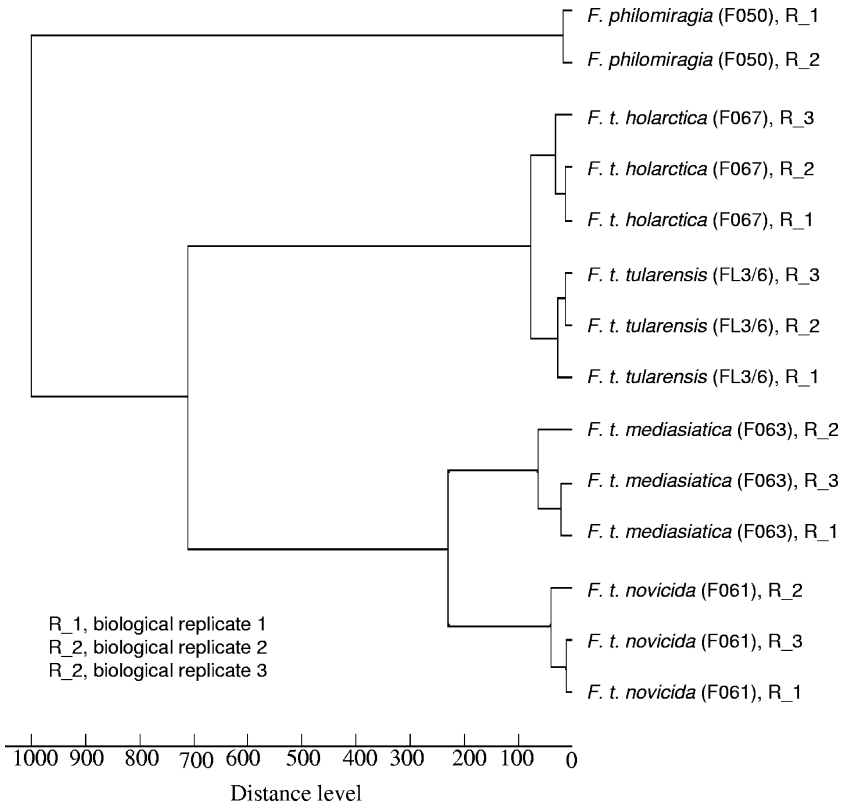

FIG. 3. Cluster analysis of MALDI-TOF MS spectra derived from biological replicates of five representative *Francisella* strains. Distance is displayed in relative units. R_1, R_2, and R_3 represent the biological replicates.

gether with *F. tularensis* subsp. *tularensis* strains. In summary, the full discrimination with the identification routine by the use of log score values was only partially reflected in cluster analysis, regarding the species as well as subspecies level. The difference between the discrimination power of log score-based identification and that of the correlation-based cluster analysis of the MALDI Biotyper 2.0 software package may be explained by the limitations of the latter method, which is not dedicated to resolve large and complex clusters at this level of similarity.

The widespread use of MALDI-TOF MS in clinical microbiology has been hampered by difficulties regarding reproducibility associated with initial approaches and the limited availability of reference databases. The limitations due to reproducibility seem to correspond to the mass range used. Recent publications about using a mass range based on the concept of measuring presumably ribosomal proteins have shown that MALDI-TOF MS protein fingerprints are not significantly influenced by variability in growth conditions (2, 22). We also tested the reproducibility of the method to identify *Francisella* strains under various conditions, including biological replicates, different agars, different time periods of cultivation, different cultivation passages of the same strain, different preparation protocols, and different mass spectrometers. Under all conditions, the spectrum of any tested *Francisella* strain was correctly identified according to its species and subspecies, respectively. The queries to the reference database resulted in log score values of ≥ 2 for all samples, with the majority of samples having log scores of >2.3. This supports a high degree of uniformity between the spectra. We conclude a high reproducibility of the method independent from various conditions of cultivation, preparation and measuring of *Francisella* strains. Interestingly the observed stability of spectral profiles after 30 passages of an *F. tularensis* subsp. *holarctica* strain may indirectly give some support to the underlying concept, that the majority of measured peaks resemble ribosomal proteins, as it seems that most selected peaks of the spectra are highly conserved.

We showed that the application of MALDI-TOF MS in combination with a reference database and a pattern recognition algorithm, which results in a log score, is a robust methodological approach that is able to reproducibly identify and discriminate *F. tularensis* strains at least at the subspecies level. The standard pattern-matching approach uses a high number of peaks which are highly reproducible, found in a strain, and

TABLE 4. Comparison of identification by MALDI-TOF MS with 23S rRNA gene sequencing[c]

Identification by 23S rRNA sequence (no. of strains)	No. of isolates (relative %) identified by MALDI-TOF MS	
	Concordance	Discordance
Species (45)[a]	45 (100)	0 (0)
Subspecies (40)[b]	40 (100)	0 (0)

[a] Identification of all 45 *Francisella* strains at the species level.
[b] Identification of the subset of all 40 *Francisella tularensis* strains at the subspecies level.
[c] The maximum log score achievable is 3.0. Log score values of ≥ 2.0 are rated as a correct identification of a strain at the species or subspecies level.

identification is based on intraspecies similarity of these patterns. To achieve a higher taxonomic resolution, e.g., to discriminate single strains within a *Francisella tularensis* subspecies, a different approach may be required. More precisely it may be necessary to adapt the pattern recognition algorithm to use "less conserved" peaks, as the phylogenetic distance between strains decreases. In such a case, it is likely that more-stringent cultivation conditions must be followed and that a larger set of more similar *Francisella* strains has to be used. A future identification scheme may consist of a hierarchical approach applying the suitable algorithms for different taxonomic levels in a sequential mode.

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M.K. and T.M. declare potential conflicts of interest, as they are both employees of Bruker Daltonics GmbH, which produces the MALDI-TOF MS as well as the MALDI Biotyper 2.0 software package. All other authors declare that no competing interests exist.

REFERENCES

1. **Arnold, R. J., and J. P. Reilly.** 1998. Fingerprint matching of E. coli strains with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of whole cells using a modified correlation approach. Rapid Commun. Mass Spectrom. **12:**630–636.
2. **Barbuddhe, S. B., T. Maier, G. Schwarz, M. Kostrzewa, H. Hof, E. Domann, T. Chakraborty, and T. Hain.** 2008. Rapid identification and typing of listeria species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl. Environ. Microbiol. **74:**5402–5407.
3. **Becker, K., D. Harmsen, A. Mellmann, C. Meier, P. Schumann, G. Peters, and C. von Eiff.** 2004. Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of *Staphylococcus* species. J. Clin. Microbiol. **42:**4988–4995.
4. **Broekhuijsen, M., P. Larsson, A. Johansson, M. Byström, U. Eriksson, E. Larsson, R. G. Prior, A. Sjöstedt, R. W. Titball, and M. Forsman.** 2003. Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. J. Clin. Microbiol. **41:**2924–2931.
5. **Clarridge, J. E., III, T. J. Raich, A. Sjosted, G. Sandstrom, R. O. Darouiche, R. M. Shawar, P. R. Georghiou, C. Osting, and L. Vo.** 1996. Characterization of two unusual clinically significant *Francisella* strains. J. Clin. Microbiol. **34:**1995–2000.
6. **Cloud, J. L., P. S. Conville, A. Croft, D. Harmsen, F. G. Witebsky, and K. C. Carroll.** 2004. Evaluation of partial 16S ribosomal DNA sequencing for identification of nocardia species by using the MicroSeq 500 system with an expanded database. J. Clin. Microbiol. **42:**578–584.
7. **Dennis, D. T., T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, A. M. Friedlander, J. Hauer, M. Layton, S. R. Lillibridge, J. E. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, and K. Tonat.** 2001. Tularemia as a biological weapon: medical and public health management. JAMA **285:**2763–2773.
8. **Ferroni, A., I. Sermet-Gaudelus, E. Abachin, G. Quesne, G. Lenoir, P. Berche, and J. L. Gaillard.** 2002. Use of 16S rRNA gene sequencing for identification of nonfermenting gram-negative bacilli recovered from patients attending a single cystic fibrosis center. J. Clin. Microbiol. **40:**3793–3797.
9. **Fey, P. D., M. M. Dempsey, M. E. Olson, M. S. Chrustowski, J. L. Engle, J. J. Jay, M. E. Dobson, K. S. Kalasinsky, A. A. Shea, P. C. Iwen, R. C. Wickert, S. C. Francesconi, R. M. Crawford, and S. H. Hinrichs.** 2007. Molecular analysis of Francisella tularensis subspecies tularensis and holarctica. Am. J. Clin. Pathol. **128:**926–935.
10. **Holland, R. D., J. G. Wilkes, F. Rafii, J. B. Sutherland, C. C. Persons, K. J. Voorhees, and J. O. Lay, Jr.** 1996. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. **10:**1227–1232.
11. **Hsieh, S. Y., C. L. Tseng, Y. S. Lee, A. J. Kuo, C. F. Sun, Y. H. Lin, and J. K. Chen.** 2008. Highly efficient classification and identification of human pathogenic bacteria by MALDI-TOF MS. Mol. Cell. Proteomics **7:**448–456.
12. **Huber, B. E., R. Escudero, H. J. Busse, E. Seibold, H. C. Scholz, P. Anda, P. Kampfer, and W. D. Splettstoesser.** 25 September 2009, posting date. Description of Francisella hispaniensis sp. nov., isolated from human blood, reclassification of Francisella novicida (Larson et al. 1955) Olsufiev et al. 1959 as Francisella tularensis subsp. novicida comb. nov., and emended description of the genus Francisella. Int. J. Syst. Evol. Microbiol. [Epub ahead of print.] doi:10.1099/ijs.0.015941-0.
13. **Ilina, E. N., A. D. Borovskaya, M. M. Malakhova, V. A. Vereshchagin, A. A. Kubanova, A. N. Kruglov, T. S. Svistunova, A. O. Gazarian, T. Maier, M. Kostrzewa, and V. M. Govorun.** 2009. Direct bacterial profiling by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry for identification of pathogenic Neisseria. J. Mol. Diagn. **11:**75–86.
14. **Johansson, A., J. Farlow, P. Larsson, M. Dukerich, E. Chambers, M. Bystrom, J. Fox, M. Chu, M. Forsman, A. Sjostedt, and P. Keim.** 2004. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J. Bacteriol. **186:**5808–5818.
15. **Lartigue, M. F., G. Hery-Arnaud, E. Haguenoer, A. S. Domelier, P. O. Schmit, N. van der Mee-Marquet, P. Lanotte, L. Mereghetti, M. Kostrzewa, and R. Quentin.** 2009. Identification of *Streptococcus agalactiae* isolates from various phylogenetic lineages by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. **47:**2284–2287.
16. **Lasch, P., H. Nattermann, M. Erhard, M. Stammler, R. Grunow, N. Bannert, B. Appel, and D. Naumann.** 2008. MALDI-TOF mass spectrometry compatible inactivation method for highly pathogenic microbial cells and spores. Anal. Chem. **80:**2026–2034.
17. **Leelaporn, A., S. Yongyod, S. Limsrivanichakorn, T. Yungyuen, and P. Kiratisin.** 2008. Francisella novicida bacteremia, Thailand. Emerg. Infect. Dis. **14:**1935–1937.
18. **Ludwig, W., and K. H. Schleifer.** 1994. Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. FEMS Microbiol. Rev. **15:**155–173.
19. **Lundquist, M., M. B. Caspersen, P. Wikstrom, and M. Forsman.** 2005. Discrimination of Francisella tularensis subspecies using surface enhanced laser desorption ionization mass spectrometry and multivariate data analysis. FEMS Microbiol. Lett. **243:**303–310.
20. **Marklein, G., M. Josten, U. Klanke, E. Muller, R. Horre, T. Maier, T. Wenzel, M. Kostrzewa, G. Bierbaum, A. Hoerauf, and H. G. Sahl.** 2009. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates. J. Clin. Microbiol. **47:**2912–2917.
21. **Mellmann, A., F. Bimet, C. Bizet, A. D. Borovskaya, R. R. Drake, U. Eigner, A. M. Fahr, Y. He, E. N. Ilina, M. Kostrzewa, T. Maier, L. Mancinelli, W. Moussaoui, G. Prevost, L. Putignani, C. L. Seachord, Y. W. Tang, and D. Harmsen.** 2009. High interlaboratory reproducibility of matrix-assisted laser desorption ionization-time of flight mass spectrometry-based species identification of nonfermenting bacteria. J. Clin. Microbiol. **47:**3732–3734.
22. **Mellmann, A., J. Cloud, T. Maier, U. Keckevoet, I. Ramminger, P. Iwen, J. Dunn, G. Hall, D. Wilson, P. Lasala, M. Kostrzewa, and D. Harmsen.** 2008. 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. J. Clin. Microbiol. **46:**1946–1954.
23. **Mikalsen, J., and D. J. Colquhoun.** 25 September 2009, posting date. Francisella asiatica sp. nov. isolated from farmed tilapia (Oreochromis sp.) and elevation of Francisella philomiragia subsp. noatunensis to species rank as Francisella noatunensis comb. nov., sp. nov. Int. J. Syst. Evol. Microbiol. [Epub ahead of print.] doi:10.1099/ijs.0.002139-0.
24. **Moura, H., A. R. Woolfitt, M. G. Carvalho, A. Pavlopoulos, L. M. Teixeira, G. A. Satten, and J. R. Barr.** 2008. MALDI-TOF mass spectrometry as a tool for differentiation of invasive and noninvasive Streptococcus pyogenes isolates. FEMS Immunol. Med. Microbiol. **53:**333–342.
25. **Nagy, E., T. Maier, E. Urban, G. Terhes, and M. Kostrzewa.** 2009. Species identification of clinical isolates of Bacteroides by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry. Clin. Microbiol. Infect. **15:**796–802.
26. **Seibold, E., R. Bogumil, S. Vorderwulbecke, S. Al Dahouk, A. Buckendahl, H. Tomaso, and W. Splettstoesser.** 2007. Optimized application of surface-enhanced laser desorption/ionization time-of-flight MS to differentiate Francisella tularensis at the level of subspecies and individual strains. FEMS Immunol. Med. Microbiol. **49:**364–373.
27. **Seng, P., M. Drancourt, F. Gouriet, B. La Scola, P. E. Fournier, J. M. Rolain, and D. Raoult.** 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin. Infect. Dis. **49:**543–551.
28. **Sjöstedt, A.** 2007. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. Ann. N. Y. Acad. Sci. **1105:**1–29.
29. **Splettstoesser, W. D., H. Tomaso, S. Al Dahouk, H. Neubauer, and P. Schuff-Werner.** 2005. Diagnostic procedures in tularaemia with special focus on molecular and immunological techniques. J. Vet. Med. B Infect. Dis. Vet. Public Health **52:**249–261.
30. **Staples, J. E., K. A. Kubota, L. G. Chalcraft, P. S. Mead, and J. M. Petersen.** 2006. Epidemiologic and molecular analysis of human tularemia, United States, 1964–2004. Emerg. Infect. Dis. **12:**1113–1118.
31. **Tang, N., P. Tornatore, and S. R. Weinberger.** 2004. Current developments in SELDI affinity technology. Mass Spectrom. Rev. **23:**34–44.
32. **Wiesner, A.** 2004. Detection of tumor markers with ProteinChip technology. Curr. Pharm. Biotechnol. **5:**45–67.