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Differentiation of *Streptococcus pneumoniae* from Nonpneumococcal Streptococci of the *Streptococcus mitis* Group by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry

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The differentiation of species within the *Streptococcus mitis* group has posed a problem in the routine diagnostic microbiology laboratory for some time. It also constitutes a major weakness of recently introduced matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) fingerprinting systems. As the phylogenetic resolution of the spectral similarity measures employed by these systems is insufficient to reliably distinguish between the most closely related members of the group, the major pathogen *Streptococcus pneumoniae* is frequently misidentified. In this study, a comparative analysis of MALDI-TOF spectra of several species from the *S. mitis* group has been performed in order to identify single peaks that could be used to improve mass spectrometry-based identification of the respective species. A characteristic peak profile could be identified that unambiguously distinguished the 14 *S. pneumoniae* isolates studied from 33 nonpneumococcal isolates of the *S. mitis* group. In addition, specific peak combinations could be assigned to other members of the group. The findings of this study suggest that it is possible to distinguish different species of the *S. mitis* group by close analysis of their mass peak profiles.

Streptococcus pneumoniae (the pneumococcus) is a major human pathogen. It causes a wide spectrum of diseases, including pneumonia, bacteremia, meningitis, sinusitis, and acute otitis media.

The diagnosis of pneumococcal disease is often complicated by the fact that the pathogen belongs to a group of closely related commensal streptococci, the *Streptococcus mitis* group. In particular, *Streptococcus pseudopneumoniae* has been incorrectly identified as *S. pneumoniae* (1, 13). Bacteria of this group are naturally competent for transformation, and it is thought that exchange of genetic material by homologous recombination was an important factor in their evolution (4, 6, 10, 14). As a consequence, many physiological and molecular traits, which are used for the identification of *S. pneumoniae*, are shared by other members of the group (15, 22). According to the analysis of 16S rRNA and housekeeping gene sequences, *S. pneumoniae* is most closely related to *S. pseudopneumoniae*, *S. mitis*, and *Streptococcus oralis*. Secure differentiation between these species represents a common challenge for the routine clinical microbiology laboratory, and misidentifications occur with all routinely employed identification systems (3, 11, 22).

Recently, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) fingerprinting has been introduced as a routine microbial identification tool and found widespread acceptance thanks to its broad species coverage and superior turnaround time (19). Pathogen identification by mass spectrometry is based on the generation of whole-cell mass spectra from bacterial cells or cell extracts which are compared to reference spectra from well-characterized isolates by means of whole-spectrum similarity measures (8). Commercially available systems provide reliable identification for the majority of clinically relevant species, and the underlying algorithms have been successfully applied to viridians streptococci (9). Nevertheless, the technique so far also failed at differentiating between *S. pneumoniae* and its closest relatives (21). On the other hand, several studies have shown ways to increase the phylogenetic resolution of mass spectrometry by focusing the analysis on a predefined subset of discriminatory peaks (5, 24). The present study investigated the potential of this method to improve the differentiation between the pneumococcus and other closely related streptococci by MALDI-TOF MS.

MATERIALS AND METHODS

Bacterial isolates. Two *S. pneumoniae* type strains (ATCC 33400 and 49169), four *S. pneumoniae* serotypes (types 6B, 19A, 19F, and 14; Staten Seruminstitut, Copenhagen), one strain of *S. pseudopneumoniae* (ATCC BAA-960; type strain), one isolate of a nonpneumococcal strain of the *S. mitis* group (no. 1), and one strain of *S. oralis* (NZ isolate SID 08/60) were provided by the New Zealand Reference Culture Collection at the Institute of Environmental Science & Research (ESR) and included in the analysis. All the other 38 strains were clinical isolates that had been collected and stored at −80°C at Canterbury Health Laboratories, Christchurch, New Zealand. Of those, 8 were clinical *S. pneumoniae* blood culture isolates (2 each of 6B, 19A, 19F, and 14, serotyped by ESR), and 17 were clinical isolates identified as nonpneumococcal isolates belonging to the *S. mitis* group by ESR by nonmolecular methods. Thirteen were clinical isolates of *S. pseudopneumoniae* (226/432, 490/944, 499/962, 785/1491, 773/1479, 169/320, 474/913, 747/1438, 705/1359, 554/1066, 22/57, 35/48, 83/152) that had been identified on the basis of their variable optochin sensitivity (resistance in 5% CO_2 and susceptibility in ambient air), bile insolubility, DNA probe hybridization test positivity (AccuProbe *Streptococcus pneumoniae*; GenProbe, San Diego, CA), NOW *S. pneumoniae* immunochromatographic antigen test (Binax, Portland, ME)

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positivity, pneumolysin gene (*ply*) positivity by PCR, and absence of a capsule (Quellung test).

Sequence-based identification. As the limited ability of current phenotypic identification procedures to differentiate the *S. mitis* group has been well documented (7), we sought definitive sequence-based identification for all study isolates presumably identified as *S. pseudopneumoniae* or nonpneumococcal isolates belonging to the *S. mitis* group by conventional methods. The bacterial recombinase A gene (*recA*), which has been shown to allow for phylogenetic differentiation within the *S. mitis* group (25), has been chosen as the target gene. DNA sequencing was performed as described previously (14, 21). The DNA was extracted from isolated colonies on 5% sheep blood agar by inoculating 500 μl of a detergent-based buffer (unpublished) and then boiling for 10 min. Cellular debris was removed by centrifugation, and 5 μl of DNA extract (supernatant) was added to 20 μl of a PCR mixture containing 1× PCR buffer with $MgCl_2$ (2.0 mM final concentration), 100 μM deoxynucleoside triphosphate (dNTP) (Roche Diagnostics, Indianapolis, Indiana, USA), 1.0 μM each of primers *recA* 2F [5′-GCTT(T/C)ATCGATGC(C/T/G)GA(G/A)CA-3′] and *recA* 5R [5′-GTTTCCGG(G/A)TT(A/T/G)CC(G/A)AACAT-3′] (Zbinden 2011) (Invitrogen New Zealand Ltd., Mulgrave, Victoria, Australia), and 1.25 U of FastStart *Taq* polymerase (Roche Diagnostics, Indianapolis, IN). PCR cycling parameters included an initial denaturation for 10 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C; and a final extension for 5 min at 72°C. Amplicons were purified with the High Pure PCR product purification kit (Roche Diagnostics, Indianapolis, IN) by following the manufacturer's instructions. The partial *recA* gene was sequenced using primers *recA* 2F and *recA* 5F and BigDye Terminator chemistry version 3.1 (Invitrogen New Zealand Ltd., Mulgrave, Victoria, Australia). The DNA sequencing products were analyzed on ABI genetic analyzer Avant 3130xl (Invitrogen New Zealand Ltd., Mulgrave, Victoria, Australia). The DNA sequences were analyzed by Web-based alignment at http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi, and the cutoff for *recA*-based species identification was set at 99% sequence similarity.

MALDI-TOF MS. Bacterial isolates were grown on 5% sheep blood agar at 37°C with 5% CO_2. Bacteria were harvested with a 10-μl loop at several different time points. The two pneumococcal ATCC isolates and all *S. pseudopneumoniae* isolates were harvested for mass spectrometry at 10, 16, 26, and 32 h, and all other bacterial isolates were harvested at 20 and 26 h. The bacterial colonies were suspended in 300 μl of sterile water, vortexed as per the manufacturer's instructions, mixed with 900 μl of 100% ethanol, vortexed again, and centrifuged at 7,550 × *g* for 2 min at room temperature. The supernatant was discarded, and the ethanol was removed by pipetting after a second centrifugation and a 5-min drying time of the pellet at room temperature. The pellet was resuspended in 50 μl of 70% formic acid, and then 50 μl of acetonitrile was added and mixed with the suspension, followed by centrifugation at 7,550 × *g* for 2 min. One microliter of each bacterial extract was spotted onto a ground steel target (Bruker Daltonik GmbH, Bremen, Germany) and air dried for 15 min. Each spot was overlaid with 1 μl of matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid) and allowed to air dry for 15 min prior to MALDI-TOF MS. Additionally, a selection of strains was prepared as direct bacterial smears transferred onto the ground steel plate plus the addition of 1 μl of 70% formic acid per spot prior to the addition of the matrix solution. Spectrum acquisition was performed on a Microflex LT benchtop instrument with the flexControl 3.3 software (Bruker Daltonik GmbH, Bremen, Germany) in linear positive mode at a laser frequency of 60 Hz over a mass range of 2,000 to 20,000 Da. Voltages of ion sources 1 and 2 were set at 20 and 18.13 kV, respectively. Spectra were accumulated from 160 laser shots per spot. Between 20 and 24 good-quality spectra were obtained for each bacterial extract. A bacterial test standard (Bruker Daltonik GmbH, Bremen, Germany) was included on each ground steel target for instrument calibration.

Analysis of mass spectra. Analysis of the mass spectra was performed using the spectrum view of flexAnalysis and MALDI Biotyper 3.0 software (Bruker Daltonik GmbH, Bremen, Germany). All spectra underwent smoothing and baseline subtraction according to standard settings deposited in the flexAnalysis MBT-Standard.FAMS method. Afterward, peaks that facilitated the differentiation between *S. pneumoniae* and other *S. mitis* group streptococci were identified by visual comparison of all spectra. The presence (+) or absence (−) of these discriminatory peaks in the mass spectra of each study isolate was noted in a peak profile (peak presence was assumed if a peak was observed in all spectrum replicates of a given isolate). Each peak profile was additionally cross-checked by visual examination in the gel view of MALDI Biotyper 3.0. Isolate grouping by peak profile was compared to conventional identification results.

Nucleotide sequence accession numbers. Sequences were reported to GenBank and are listed in Table 1.

RESULTS

Reference identification of clinical isolates. This study aims at the identification of MALDI-TOF signals to discriminate *S. pneumoniae* from closely related *S. mitis* group isolates. Reference identification of study isolates was achieved using a polyphasic approach (20) requiring consistency of *recA* sequencing results and biochemical identification by the Rapid ID32 Strep identification kit (bioMérieux, Marcy l'Etoile, France) for species-level identification. Nine of the 33 nonpneumococcal study isolates showed inconsistent findings and were thus classified as *S. mitis* group (Table 1, first column). The sequences that obtained 99% sequence similarity by *recA*-based species identification were submitted to GenBank (www.ncbi.nlm.nih.gov/GenBank/), and the assigned accession numbers are included in Table 1. The *recA* sequence-based identifications listed in the table without an accession number signal less than 99% sequence similarity.

Identification of discriminatory *m/z* values. Peaks obtained with the abbreviated extraction method did not reliably produce "good spectra". All results described here are based on peaks obtained with the longer extraction method described above.

Discriminatory peaks were detected at *m/z* 2,625, 2,911, 2,937.5, 5,253, 5,824, 5,877, and 6,955. Spectra obtained in quintuplicate and at different time points showed only a minor degree of variation regarding the presence or absence of these peaks. Peak position variation between acquisitions despite calibration should not exceed 500 ppm, which translates to about ±3 Da at *m/z* 5,000 (flexAnalysis 3.3 User Manual; Bruker Daltonik GmbH, Bremen, Germany). Three peak pairs (*m/z* 5,877 and *m/z* 2,937.5, *m/z* 5,824 and *m/z* 2,911, *m/z* 5,253 and *m/z* 2,625) showed a high degree of correlation and are assumed to each represent differently charged states of a single protein.

Analysis of peak profiles with respect to isolate reference identification demonstrated that characteristic peak patterns could be attributed to different streptococcal species (Table 1). *S. pneumoniae* isolates consistently showed presence of the correlated peak pair *m/z* 2,937.5 and *m/z* 5,877 and absence of peaks at *m/z* 2,625, 2,911, 5,253, 5,824, and 6,955. Of notice, none of the nonpneumococcal isolates exhibited a peak profile associated with the *S. pneumoniae* isolates in this study (MALDI profile 1 [MP1]).

S. pseudopneumoniae isolates were also characterized by the presence of the correlated peak pair *m/z* 2,937.5 and *m/z* 5,877. In addition, correlated peaks at *m/z* 2,625 and *m/z* 5,253 were consistently found (MP2).

Isolated peaks at *m/z* 2,911, 5,824, and 6,955 were frequently observed in the spectra of *S. oralis* isolates (MP4). Additional profiles were recorded for nonpneumococcal isolates 22/57 (MP3), SMIT 1, SORA 7, SORA 9 (MP5), and SMIT 7 (MP6).

TABLE 1 MALDI profiles and *recA* sequencing results of different streptococcal species[c]

Isolate	*recA* sequence (GenBank accession no.)	Conventional identification	*m/z* value[a]							MALDI profile
			2,625	2,911	2,937.5	5,253	5,824	5,877	6,955	
S. pneumoniae ATCC 33400	*S. pneumoniae*	*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pneumoniae ATCC 40619	*S. pneumoniae*	*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pneumoniae 6B (type strain)		*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pneumoniae 19A (type strain)		*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pneumoniae 19F (type strain)		*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pneumoniae 14 (type strain)		*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pneumoniae 6B (clinical isolate)		*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pneumoniae 19A (clinical isolate)		*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pneumoniae 19F (clinical isolate)		*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pneumoniae 14 (clinical isolate)		*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pneumoniae 6B (clinical isolate)		*S. pneumoniae*	−	−	+	−	−	+	−	MP1
*S. pneumoniae*19A (clinical isolate)		*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pneumoniae 19F (clinical isolate)		*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pneumoniae 14 (clinical isolate)		*S. pneumoniae*	−	−	+	−	−	+	−	MP1
S. pseudopneumoniae BAA-960	*S. pseudopneumoniae*	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. pseudopneumoniae 226/432	*S. pseudopneumoniae* (JQ957541)	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. pseudopneumoniae 490/944	*S. pseudopneumoniae* (JQ957514)	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. pseudopneumoniae 499/944	*S. pseudopneumoniae* (JQ957524)	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. pseudopneumoniae 785/1491	*S. pseudopneumoniae* (JQ957517)	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. pseudopneumoniae 773/1479	*S. pseudopneumoniae* (JQ957519)	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. pseudopneumoniae 169/320	*S. pseudopneumoniae* (JQ957515)	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. pseudopneumoniae 474/913	*S. pseudopneumoniae* (JQ957520)	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. pseudopneumoniae 747/1438	*S. pseudopneumoniae* (JQ957516)	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. pseudopneumoniae 705/1359	*S. pseudopneumoniae* (JQ957518)	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. pseudopneumoniae 554/1066	*S. pseudopneumoniae* (JQ957522)	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. pseudopneumoniae 35/48	*S. pseudopneumoniae* (JQ957523)	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. pseudopneumoniae 83/252	*S. pseudopneumoniae* (JQ957521)	*S. pseudopneumoniae*	+	−	+	+	−	+	−	MP2
S. mitis group[b] 22/57	*S. mitis/S. pneumoniae*	*S. pseudopneumoniae*	+	−	−	+	−	+	−	MP3
S. mitis group[b] *#1*	*S. oralis*	*S. mitis*	−	+	−	−	+	−	+	MP4
S. mitis group[b] (SMIT 1)	*S. sanguinis*	*S. mitis*	−	−	−	−	−	−	+	MP5
S. mitis group[b] (SMIT 2)	*S. oralis* (JQ957525)	*S. mitis*	−	+	−	−	+	−	+	MP4
S. mitis group[b] (SMIT 3)	*S. oralis* (JQ957526)	*S. mitis*	−	+	−	−	+	−	+	MP4
S. mitis group[b] (SMIT4)	*S. mitis/S. pneumoniae*	*S. mitis*	−	+	−	−	+	−	+	MP4
S. mitis group[b] (SMIT 5)	*S. oralis* (JQ957527)	*S. mitis*	−	+	−	−	+	−	+	MP4
S. mitis group[b] (SMIT 6)	*S. pseudopneumoniae*	*S. mitis*	−	+	−	−	+	−	+	MP4
S. mitis (SMIT 7)	*S. mitis*	*S. mitis*	+	−	+	−	−	+	+	MP6
S. oralis SID 08/60	*S. oralis* (JQ957528)	*S. oralis*	−	+	−	−	+	−	+	MP4
S. oralis (SORA 1)	*S. oralis* (JQ957529)	*S. oralis*	−	+	−	−	+	−	+	MP4
S. oralis (SORA 2)	*S. oralis* (JQ957530)	*S. oralis*	−	+	−	−	+	−	+	MP4
S. oralis (SORA 3)	*S. oralis* (JQ957531)	*S. oralis*	−	+	−	−	+	−	+	MP4
S. oralis (SORA 4)	*S. oralis* (JQ957532)	*S. oralis*	−	+	−	−	+	−	+	MP4
S. oralis (SORA 5)	*S. oralis* (JQ957533)	*S. oralis*	−	+	−	−	+	−	+	MP4
S. oralis (SORA 6)	*S. oralis* (JQ957534)	*S. oralis*	−	+	−	−	+	−	+	MP4
S. oralis (SORA 7)	*S. oralis* (JQ957535)	*S. oralis*	−	−	−	−	−	−	+	MP5
S. mitis group[b] (SORA 9)	*S. sanguinis*	*S. oralis*	−	−	−	−	−	−	+	MP5
S. oralis (SORA 10)	*S. oralis*	*S. oralis*	−	+	−	−	+	−	+	MP4
S. oralis (SORA 11)	*S. oralis* (JQ957536)	*S. oralis*	−	+	−	−	+	−	+	MP4

[a] −, absence of peak; +, presence of peak at a given *m/z* value.
[b] *S. mitis* group other than *S. pneumoniae.*
[c] The typing (conventional identification) performed by the Institute of Environmental Science & Research, New Zealand (ESR), was based on nonmolecular methods.

The presence or absence of the *m/z* peaks in the different species is illustrated in Fig. 1. The peaks shown in Fig. 1 are a "zoomed in" version of the much sharper peaks visualized in a whole spectrum; therefore, they appear broader.

DISCUSSION

MALDI-TOF MS was first proposed as a tool for bacterial identification in 1996 (12). A publication in 2000 suggested that mass spectra of spore cells of the *Bacillus cereus* group allow differentiation from other *Bacillus* species (17), streptococci of the mutans group were successfully differentiated by MALDI-TOF MS in 2005 (16), and viridians streptococci have been successfully discriminated by MALDI-TOF MS in 2007 (9). By now, dedicated mass spectrometry systems are established for routine species identification in many laboratories that achieve discrimination of even closely related species by the comparison of spectrum fingerprints acquired within minutes from tiny amounts of colony material to exhaustive reference databases. Still, current algorithms produce variable results for streptococci of the *S. mitis* group, and misidentifications occur frequently, in particular for *S. pneumoniae*, *S. mitis*, and *S. oralis* (18). This is of importance considering that all members of the *S. mitis* group can be pathogens and their presence in blood cultures, in particular in immunocompromised patients, can be clinically significant and often requires treatment strategies that differ from *S. pneumoniae* due to high penicillin resistance rates in the *S. mitis* group (2). In contrast to

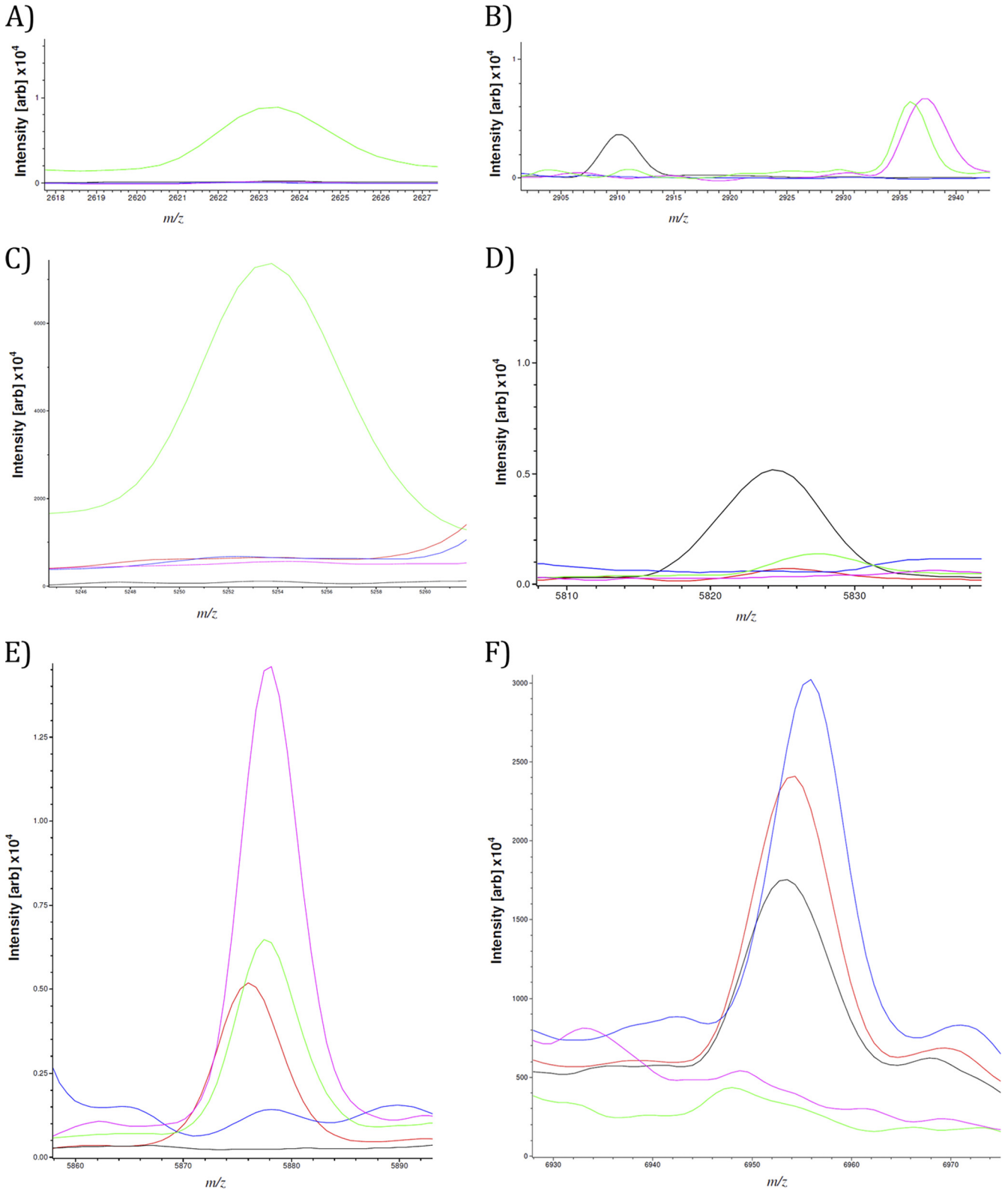

FIG 1 Individual *m/z* values as they are visible on the flexAnalysis software. Spectra are overlaid to ensure consistency with measured intensities. The chosen spectra were from strains with sequence-confirmed identification. *S. pneumoniae*, pink; *S. pseudopneumoniae*, green; *S. oralis*, black; *S. sanguinis*, blue; and *S. mitis*, red. (A) *m/z* 2,625; (B) *m/z* 2,911and 2,937.5; (C) *m/z* 5,253; (D) *m/z* 5,824; (E) *m/z* 5,877; and (F) *m/z* 6,955.

misidentifications of *Brucella* spp., *Pseudomonas* spp., and *Acinetobacter* spp., also repeatedly reported from evaluations of commercially available MS fingerprinting systems, this weakness, which persisted through multiple database updates, can apparently not be attributed to gaps or classification errors in the available collections of reference spectra but seems to result from true limits of phylogenetic resolution of the whole-spectrum similarity measures employed by these systems: in a study by Williamson et al., clustering by whole-spectrum similarity allowed for the correct recognition of isolates belonging to an *S. pneumoniae* conjunctivitis outbreak but failed to separate nonoutbreak *S. pneumoniae* isolates from *S. mitis* and *S. oralis* strains (23). However, our analysis of a limited set of isolates identified seven peaks with significant interspecies variability that could be utilized as species-specific biomarkers to improve mass spectrometry-based differentiation within the *S. mitis* complex. These seven peaks gave 6 different MALDI profiles. MP1 (containing peaks *m*/*z* 2,937.5 and 5,877) was representative for *S. pneumoniae*, MP2 (containing peaks *m*/*z* 2,625, 2,937.5, 5,253, and 5,877) and MP3 (containing peaks *m*/*z* 2,625, 5,253, and 5,877) were representative of *S. pseudopneumoniae*, while MP4 (containing peaks *m*/*z* 2,911, 5,824, and 6,955), MP5 (containing peak *m*/*z* 6,955), and MP6 (containing peaks *m*/*z* 2,625, 2,937.5, 5,877, and 6,955) were representative of other nonpneumococcal species belonging to the *S. mitis* group. Importantly, no nonpneumococcal isolates could be assigned to MP1. To strengthen the robustness of these findings, it is necessary to investigate a larger number of genetically well-characterized *S. mitis* group isolates.

In 10 strains, conventional identification and *recA* sequencing were inconsistent. In 6 of those strains, the MALDI-TOF MS result conferred with the sequencing result; in the remaining 4 strains, the MALDI profile was neither indicative of the sequence nor the conventional identification.

It is likely that the currently available methods, including single target sequencing, are still insufficiently discriminatory for the *S. mitis* group. To find characteristic MALDI-TOF peaks distinguishing between *S. pneumoniae* and nonpneumococcal isolates belonging to the *S. mitis* group is encouraging. Until then, a quick visual check for the presence of the seven peaks identified in this study could help to rapidly distinguish between *S. pneumoniae* and other *S. mitis* group species.

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