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Rapid identification of oral anaerobic bacteria cultivated from subgingival biofilm by MALDI-TOF-MS

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Introduction: To facilitate the identification of anaerobes cultivated from periodontal disease, whole cell bacterial identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was evaluated.
Methods: A total of 84 strains (nine reference strains and 75 recent clinical isolates from 33 patients with aggressive periodontitis) previously identified with phenotypic methods were used. All the references and 10 clinical isolates belonging to the same species as the reference strains were genotypically identified by sequence analysis of the 16S ribosomal RNA gene. All the strains were then analyzed using MALDI-TOF-MS.
Results: The reference strains of anaerobic bacteria used showed characteristic MALDI-TOF-MS spectra with peaks between m/z 2000 and up to about m/z 13,000. On visual inspection, the similarity of spectra produced by strains of a single genus could be recognized. Obvious differences between spectra produced by strains of different species were also easily noticed. The reproducibility of the method was proved by the similarity of spectra belonging to the same species. The spectra of the *Prevotella intermedia* strains identified with MALDI clustered together and clustered separately from the spectra of *Prevotella nigrescens*, proving that MALDI-TOF-MS is an accurate method that is capable of separating these two species. The quality of clustering was characterized by calculating an inconsistency coefficient (Mathworks:/Matlab Reference Manual v2007a/, Statistical toolbox).
Conclusion: Our results suggest that MALDI-TOF-MS might become a useful method for the identification of anaerobic bacteria, especially for those that cannot be readily identified by biochemical analysis. It may become an attractive system even for the routine identification of clinical isolates.

Key words: MALDI-TOF-MS; oral anaerobes

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Anaerobic bacteria are a major part of the endogenous flora of man. In the oral cavity, they outnumber aerobic bacteria by between 10 : 1 and 1000 : 1 (9). They are also opportunistic pathogens in many different types of infection, including oral infections (e.g. periodontal disease and endodontic infections) (9). Isolation and identification of anaerobic bacteria by conventional methods is often cumbersome and time-consuming and for this reason is often neglected by clinical laboratories. Molecular methods have been suggested, especially for the detection of the chief pathogens in periodontal disease, but they are often expensive and quantitative results might be misleading because live and dead bacteria cannot be distinguished. To facilitate the identification of cultivated anaerobes taken from periodontal disease, whole cell bacterial

identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was evaluated in the present study. This technique is a soft ionization method, which allows desorption of peptides and proteins from both whole different cultured bacteria and crude bacterial extracts (17). Ions are separated and detected according to their molecular masses and charges. Each mass peak corresponds to a molecular fragment released from the cell surface during laser desorption (8). Using this method, bacteria can be identified by comparing their mass spectrum (which is obtained within seconds) with those obtained from known reference strains using methods of multivariate statistical analysis.

Several bacteria, including viridans streptococci, bacteria from the Enterobacteriaceae family, *Mycobacterium* spp., *Helicobacter pylori*, and spores of *Bacillus cereus*, have been analyzed by MALDI-TOF-MS (4, 7, 10, 14, 16, 18, 19). It has also been used in bacterial chemotaxonomy (2, 17, 20). The present study aimed to evaluate the use and value of MALDI-TOF-MS for rapid identification of different species of anaerobic bacteria cultivated from the subgingival biofilm.

Materials and methods

Bacterial strains

A total of 84 bacterial strains were used in this study. Nine were reference strains: *Actinomyces odontolyticus* DSM 43331, *Actinomyces meyeri* MCCM 01956, *Parvimonas micra* ATCC 33270, *Peptostreptococcus anaerobius* ATCC 27337, *Porphyromonas gingivalis* Boston 381, *Tannerella forsythia* ATCC 43037, *Prevotella intermedia* DSM 20706, *Prevotella nigrescens* DSM 13386, *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25286. The other 75 strains were recent clinical isolates from the subgingival biofilm of 33 patients (mean age 39.39 ± 10.47 years) with aggressive periodontitis. The genotypes of all the reference strains and of 10 clinical isolates, selected on the basis of coherent previous identification with phenotypic and genotypic methods and belonging to the same species as the references strains, were identified by sequence analysis of the 16S ribosomal RNA (rRNA) gene. These species were used to establish the anaerobic database. All the other clinical isolates were biochemically identified using the rapid ID 32 A system (bioMerieux, Lyon, France).

MALDI-TOF-MS sample preparation

Individual colonies of each isolate or reference strain were subcultured on Columbia blood agar supplemented with vitamin K1 and hemin for 4 days at 37°C in an anaerobic chamber (Heraeus Instruments, Hanau, Germany). Colonies from half plates were suspended in 1 ml DNase-free water (Sigma, Taufkirchen, Germany). Bacterial suspensions were centrifuged at 6000 ***g*** for 15 min. The sediment was washed twice with water and then dissolved in 50 µl of 80% trifluoroacetic acid (TFA; Merck, Darmstadt, Germany) and left for 10 min at room temperature. Then, 150 µl of DNase-free water was added, followed by 200 µl acetonitrile (ACN; Sigma). The samples were stored at −20°C. After thawing, the samples were centrifuged at 10000 ***g*** for 2 min. The supernatant was transferred into a 1.5-ml reaction tube (Eppendorf, Hamburg, Germany) and dried in a vacuum centrifuge. The pellet was dissolved in 20 µl of 2.5% TFA/50% ACN. One microliter was pipetted onto a stainless steel MALDI target plate. For each strain, 10 spots were applied. After drying, the spots were covered with 1 µl of matrix (α-cyano-4-hydroxycinnamic acid, saturated solution in 2.5% TFA/50% ACN). The matrix/sample spots were crystallized by air-drying. To prove reproducibility, we repeated the sample preparation for every strain on a different day, starting with a new culture.

MALDI-TOF-MS parameters

All mass spectra were acquired using an Autoflex II (Bruker Daltonics, Bremen, Germany) MALDI-TOF-MS with a nitrogen laser (337 nm) operated in positive linear mode (delay 150 ns, voltage 20 kV, mass range 2–20 kDa) under the FLEXCONTROL software version 2.4 (Bruker Daltonics GmbH). Each spectrum was obtained by averaging 500 laser shots acquired in automatic mode at the minimum laser power necessary for the ionization of the sample. The spectra were externally calibrated using the standard calibrant mixture, Protein Calibration Standard I, supplied by Bruker Daltonics. The data files were transferred to FLEXANALYSIS version 2.4 (Bruker Daltonics GmbH) for automated peak extraction.

MALDI-TOF-MS statistical analysis

Using the FLEXANALYSIS software, 40 peaks were automatically labeled in each spectrum according to their appearance above the background (threshold ratio 1.5). Correct labeling was controlled by visual inspection. Peak lists containing masses and intensities were exported as ASCII files. Similarity analysis between lists of peaks was carried out using a hierarchical clustering procedure performed with MATLAB 7.3 (The MathWorks Inc., Natick, MA). To identify corresponding peaks in different spectra, a mass window was defined around each peak that considered the differences of masses assigned to identical peaks in different samples. A mass-dependent size of the mass window was chosen according to mass window size = size_{abs} + (mass × size_{rel}). Typically, size_{abs} of 1 Da and a size_{rel} of 0.001 were applied. Peaks originating from different spectra and occurring in the same window were assigned to one cluster. If neighboring clusters overlapped, they were omitted from further calculations. The similarity between spectra was determined by pairwise comparison of spectra, counting the number of clusters to which the two spectra contributed. By this procedure, a symmetric matrix of pairwise similarities was formed (peak-mass-based similarity matrix). In addition, a similarity matrix ($\sigma_{i,j}$), which considers peak masses and differences in the peak intensities was calculated according to:

$$\sigma_{i,j} = \sum_k \left(1 - \left(\frac{w_i^k - w_j^k}{w_i^k + w_j^k}\right)^2\right)$$

The similarity of samples i and j was obtained by a summation over all clusters k contributing to either sample i or sample j. w_i^k represents the intensity of peak i in the spectrum k.

Distance matrices ($\delta_{i,j}$) were calculated from normalized similarity matrices according to:

$$\delta_{i,j} = 1 - \sigma_{i,j}$$

Dendrograms were calculated on the basis of the distance matrices using a complete linkage function.

Results

The reference strains of anaerobic bacteria used showed characteristic MALDI-TOF-MS spectra (typical examples are given in Fig. 1). The sequence analysis of the 16S rRNA gene of *A. meyeri* MCCM 01956 suggested that *Actinobaculum schaali* [an *Actinomyces*-like bacterium described in 1997 (12)] was the correct identification. All other sequence analyzes of reference

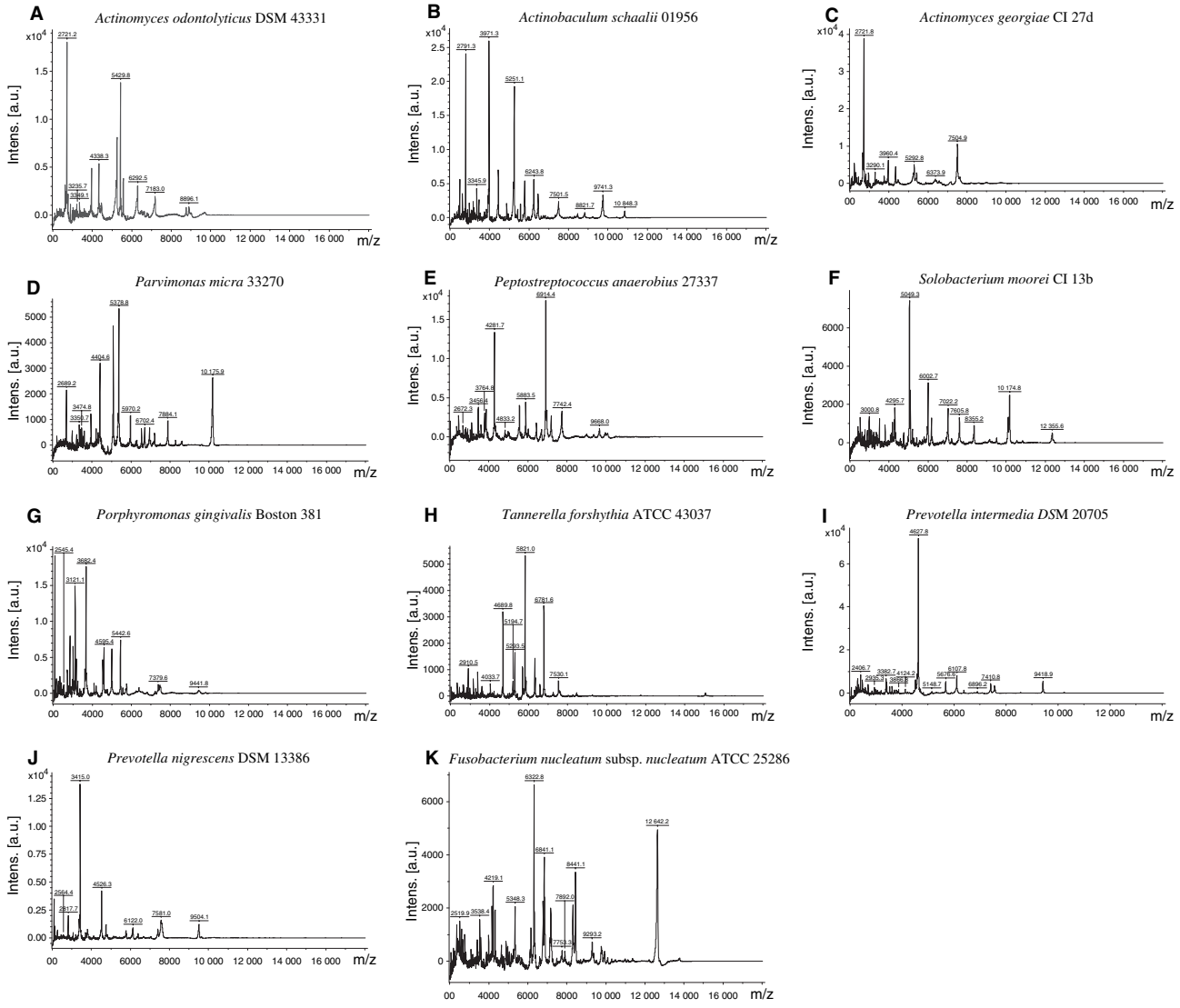

Fig. 1. MALDI-TOF mass spectra of different anaerobic strains: (A) *Actinomyces odontolyticus* DSM 43331, (B) *Actinobaculum schaalii* MCCM 01956, (C) *Actinomyces georgiae* CI 27d, (D) *Parvimonas micra* 33270, (E) *Peptostreptococcus anaerobius* 27337, (F) *Solobacterium moorei* CI 13b, (G) *Porphyromonas gingivalis* Boston 381, (H) *Tannerella forsythia* ATCC 43037, (I) *Prevotella intermedia* DSM 20706, (J) *Prevotella nigrescens* DSM 13386, (K) *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25286.

strains supported the phenotypic identification. The peaks obtained with the MALDI-TOF-MS were from the lower limit of the detection window (m/z 2000) up to about m/z 13,000. Peaks with m/z >10,000 were observed in *P. micra* ATCC 33270 and *F. nucleatum* subsp. *nucleatum* ATCC 25286. All other anaerobic strains showed peaks with m/z <10,000.

From a visual inspection, the similarity of spectra produced by strains of a single genus could be recognized. Obvious differences between spectra produced by strains of different species were also easily noted. The reproducibility of the method was proved by the similarity of spectra belonging to the same species. Similarity analysis using the peak lists obtained from the spectra of reference strains and some clinical isolates, which had been previously identified by sequence analysis of the 16S rRNA gene, showed that all the spectra belonging to one species clustered together (see Fig. 2). No outliers were observed. The quality of clustering was characterized by calculating an inconsistency coefficient (Mathworks:/Matlab Reference Manual v2007a/, Statistical toolbox). This inconsistency coefficient characterized each link in a cluster tree by comparing its length with the average length of other links at the same hierarchical level. The higher the value of this coefficient, the less similar were the objects connected by the link. The different species were found on significantly different subtrees (inconsistency coefficient >0.75). The spectra of all the *P. intermedia* strains identified with MALDI clustered together into two subtrees, which were significantly different from those of *P. nigrescens*, the strains of which also fell into two different subtrees (Fig. 3).

Discussion

All anaerobic bacteria analyzed had an m/z range between 2000 and 13,000. The viridans streptococci have an m/z range below 10,000 (4), coagulase-negative staphylococci have an m/z range below 7000 (1), and mycobacteria have a wider range, up to m/z 15,000 (16).

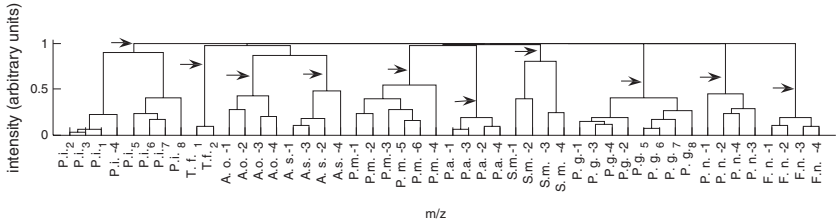

Fig. 2. Results of similarity analysis of anaerobic reference strains and some additional clinical isolates. The dendrogram was calculated from a peak-mass-based similarity matrix. A.o. 1–4, *Actinomyces odontolyticus* DSM 43331; A.s. 1–4, *Actinobaculum schaalii* MCCM 01956; P.m. 1–4, *Parvimonas micra* 33270; P.m. 5,6, *Parvimonas micra* clinical isolate 20c; P.a. 1–4, *Peptostreptococcus anaerobius* 27337; S.m. 1–4, *Solobacterium moorei* clinical isolate 13b; P.g.1–4, *Porphyromonas gingivalis* Boston 381; P.g. 5,6, clinical isolate 4c; T.f. 1–4, *Tannerella forsythia* ATCC 43037; P.i. 1–3, *Prevotella intermedia* DSM 20706; P.i. 4–6, clinical isolate 1474; P.i. 7,8, clinical isolate 1364; P.n. 1–4, *Prevotella nigrescens* DSM 13386; F.n. 1–4, *Fusobacterium nucleatum* ATCC 25286. Significant trees (inconsistency coefficient >0.75) are marked by arrows.

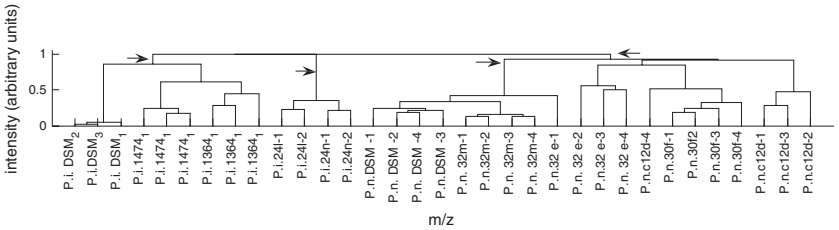

Fig. 3. Results of similarity analysis of anaerobic reference strains and some additional clinical isolates. The dendrogram was calculated from a peak-mass-based similarity matrix. P.i., *Prevotella intermedia* DSM 20706, CI 1474, CI 1364, CI 24l, CI 24n; P.n., *Prevotella nigrescens* DSM 13386, CI 32e, CI 32m, CI 30f, CI c12d. Significant trees (inconsistency coefficient >0.75) are marked by arrows.

These results made it possible to create a database of MALDI-TOF-MS spectra for anaerobes using the spectra of the reference strains. Similarity analysis with selected clinical isolates, identified by sequence analysis of their 16S rRNA gene, showed that almost all the spectra of the same species clustered together. One exception was generated by a strain of *Solobacterium moorei*. Rapid ID 32 A identified this strain as *P. anaerobius* and only sequencing of the 16S rRNA gene allowed the correct identification. This taxon was not yet part of the MALDI-TOF-MS spectra database so it was also not identified by this procedure. However, *S. moorei* clustered separately from all other strains analyzed here. This fact further proved that this method was reliable.

Identification of other clinical strains using this reference database showed coherence with the previous biochemical identification with very few exceptions. Only two strains identified by Rapid ID 32 A as *P. intermedia/P. nigrescens* proved not to be either of these two species in MALDI-TOF-MS. Further sequencing of these two strains showed that they belonged to the *Selenomonas* and *Campylobacter* genera, which were not yet included in the database. Another strain identified by Rapid ID 32 A as *P. anaerobius* was unambiguously identified as *S. moorei*. The samples of a strain that had been biochemically identified as *A. meyeri* could not be identified because the reference *A. meyeri* MCCM 01956 turned out to be *A. schaalii* and there was no *A. meyeri* in the MALDI-TOF-MS database. One isolate that was identified by Rapid ID 32 A as *A. meyeri* showed a 16S rRNA gene sequence described for *Actinomyces georgiae* (not included in the database), and its MALDI-TOF-MS spectrum was more similar to that of *A. odontolyticus* DSM 43331 (the only *Actinomyces* species from the database) than other anaerobic species.

The *P. intermedia* group bacteria are black-pigmented gram-negative anaerobic rods that are commonly found in dental biofilm. This group includes two phenotypically indistinguishable species *P. intermedia* and *P. nigrescens* (3). Routine biochemical tests and gas–liquid chromatography are incapable of separating them (6, 9). Instead, methods like restriction endonuclease analysis, rRNA gene restriction analysis (ribotyping), multilocus enzyme electrophoresis, multiplex polymerase chain reactions, or sodium dodecyl sulfate–polyacrylamide gel electrophoresis must be used (15, 21, 22). However, these techniques are time-consuming and some of them have low levels of reproducibility. Therefore, the capability of MALDI-TOF MS to distinguish between *P. intermedia* and *P. nigrescens* was explored. A total of 37 strains (including two reference strains) were used. The clinical strains were very well identified as *P. intermedia/P. nigrescens* by the Rapid ID 32 A system and species differentiation was performed using sequence analysis of the 16S rRNA gene. Then, MALDI-TOF-MS was performed. Although the MS spectra of the two species are visually similar, the mathematical similarity analysis could differentiate between them. This proved that MALDI-TOF-MS is a new and accurate method that is capable of separating these two species. This result is also of clinical importance because there are several studies that associate *P. intermedia* with periodontal disease and *P. nigrescens* with a healthy periodontium (5). However, there are also opposing data (11, 13, 21). The availability of a straightforward method to identify the two species might be helpful to clarify this problem.

Several mass-spectrometry-based approaches have been developed to rapidly differentiate bacteria. Among them, MALDI-TOF-MS of whole cells or cell extracts is exceptionally straightforward. Based on peptides and small proteins, MALDI-TOF-MS generates complex spectra that often contain unique m/z signatures for different microorganisms. Identification and differentiation of microorganisms can be achieved by pattern analysis of the mass spectra using mathematical tools based on non-linear statistics.

The costs for consumables and chemicals are very low for MALDI-TOF-MS analysis. Also, hands-on time is short, especially if acquisition of the spectra can be performed automatically. However, at least until now, the high initial outlay for the MALDI-TOF-MS instruments seemed to prevent the method from widespread application. There are increasing numbers of MALDI-TOF mass spectrometers in many research institutions worldwide, they all run around-the-clock, and it would be difficult to use them to their full capacity with research work alone. Sharing them is supported by the facts that bacterial analysis requires only seconds per sample and makes only moderate demands on the performance of the mass spectrometer. Furthermore, dried bacterial extracts can be stored and transported at ambient temperature for weeks without compromising the quality of the spectra. However, because of the number of variables encountered with MALDI and the current lack of standardization it seems quite possible that the spectra of bacteria might not be directly comparable between laboratories. Hence, the necessity to build reliable databases in a particular laboratory may be an important step in devising a routine diagnostic algorithm for the identification of bacteria from subgingival biofilm.

In conclusion, our results suggest that MALDI-TOF-MS might become a useful method for the identification of anaerobic bacteria, especially for those that cannot readily be identified by biochemical analysis. With databases for various groups of bacteria expanding, MALDI-TOF-MS becomes an attractive system even for the routine identification of clinical isolates.

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