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Differentiation of mutans streptococci by intact cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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It is difficult to distinguish mutans streptococci on the species level, and even more so on the subspecies level. Intact cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (ICM) was applied to reference strains of five of the species of the mutans group (*Streptococcus criceti*, *Streptococcus downei, Streptococcus mutans*, *Streptococcus ratti*, *Streptococcus sobrinus*), nonmutans streptococci (*Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus salivarius*, and *Streptococcus sanguinis*), and 177 mutans streptococci isolated from saliva of 10 children. From the analysis of the reference strains, readily distinguishable ICM mass spectra were obtained for the different species. Based on multivariate statistical analysis, a correct and unambiguous assignment was made of the spectra of 159 isolated mutans streptococci to *S. mutans* and 16 isolates to *S. sobrinus*. Two isolates were sorted out and were identified by sequencing of their 16S rRNA genes as *Streptococcus anginosus*. In addition, ICM indicated a misclassification for some reference strains (AHT, V 100 and E 49) and re-classified AHT and E 49 as *S. ratti* and V 100 as *S. sobrinus*. This was confirmed by 16S rDNA sequencing. Based on a statistical similarity analysis of the spectra of reference strains and a quantitative assessment of the reproducibility of ICM, the isolates identified as either *S. mutans* or *S. sobrinus* were phenotyped on the subspecies level. In the population of the clinical isolates, 14 unambiguously different *S. mutans* and three different *S. sobrinus* phenotypes were detected. ICM proved to be a powerful tool for a differentiation of mutans streptococci down to the subspecies level.

Key words: differentiation; intact cell MALDI-TOF-MS; mutans streptococci; phenotyping

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With the rapid development and improvement of mass spectrometry during the last decade (7, 26, 28), these methods became of particular interest for direct and rapid analysis of intact microorganisms (5, 8, 16, 18, 29, 30). In previous studies, cultured cells from different bacterial species were characterized by mass spectrometry based on soft laser desorption (3, 6, 9, 10, 33). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been applied for bacterial differentiation and identification using two different approaches: the isolation and identification of specific proteins (biomarkers; 1, 19), and direct intact cell analysis (ICM). The latter can be based either on the detection of specific peaks (13, 20, 23, 34) or on the analysis of the complete spectral pattern (4).

Streptococci are important components of the human oral bacterial flora (31). They comprise the human pathogenic mutans streptococci, which are crucial for the development of dental caries. The available methods for the identification and differentiation of mutans streptococci, like

Table 1. Strains of mutans and oral streptococci. Reclassified strains are given in bold (V 100, originally *Streptococcus mutans*; AHT, E 49, originally *Streptococcus criceti*)

Species	Strains
Streptococcus mutans	NCTC 10449[1], OMZ 125[2], GS 5[3], Ingbritt[3], JB 1600[5] LM7[4], SE 11[2], OMZ 175[4]
Streptococcus sobrinus	OMZ 65[4], OMZ 176[2], **V 100[4]**
Streptococcus downei	Isolated strain from a carious lesion[9]
Streptococcus criceti	OMZ 61[2]
Streptococcus ratti	FA I[3], OMZ 51[2], LB 2[8], **AHT[7]**, **E 49[3]**
Streptococcus mitis	OMZ 8[4]
Streptococcus sanguinis	OMZ 9S[2]
Streptococcus salivarius	OMZ 47[4]
Streptococcus oralis	NS 9[6], NS 30[6]

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cultivation, biochemical characterization and DNA sequencing of 16S rRNA genes, as well as DNA fingerprinting using arbitrarily primed (AP) or repetitive elements (rep) polymerase chain reaction (PCR) are laborious and time consuming (2, 14, 17, 21, 24, 25, 31).

The present investigation was focused on an exploration of the capability of ICM to assign clinical isolates of mutans streptococci to the correct species. As a prerequisite, reproducible and highly informative MALDI-TOF-MS spectra had to be acquired from well-characterized reference strains cultured under defined conditions. For the analysis of the spectra, hierarchical clustering and, as a control method, quantum clustering were applied. The discriminatory power of the ICM method was exploited to detect strains and to correct previously classified strains. In addition, the suitability of ICM for the differentiation of *Streptococcus mutans* and *Streptococcus sobrinus* at the subspecies level was studied in detail.

Material and methods

Bacterial strains and cultivation

Eighteen strains of mutans streptococci, five strains of oral nonmutans streptococci, and 177 mutans streptococci isolates from the saliva of 10 human donors obtained during the Erfurt caries risk assessment study (German Federal Ministry for Education, Science, Research and Technology; grant no. 01 ZZ 9502) were included. The strains and isolates were characterized by colony morphology, cultivation on selective media, biochemical reactions, and analysis of membrane fatty acids (27) (Table 1).

Bacteria were grown anaerobically under strictly identical conditions for 24 h at 35 ± 2°C (GasPack, BBL, Becton Dickinson, Cockeysville, MD) in 10 ml of Balmelli's broth (10 g bacto-tryptose, 5 g yeast extract, 5 g K_2HPO_4, 3 g beef extract, 50 g sucrose [all from Merck, Darmstadt, Germany]) and 1000 ml water pH 7.2. Cultures were centrifuged at 4000 × g for 5 min at 4°C (Megafuge 1.0, Heraeus-Christ, Osterode, Germany). The cell pellets were stored at −20°C.

All strains underwent species-specific *S. sobrinus* and *S. mutans* PCR (22). The reference strains AHT, E 49, and V 100 and two *Streptococcus anginosus* isolates were identified by 16S rDNA sequencing. All reference strains, all clinical isolates identified by PCR and ICM as *S. sobrinus*, and one isolate from each group of unambiguously different phenotypes of each patient were confirmed by 16S rDNA sequencing (Table 2).

ICM

Pellets containing 10^6–10^7 bacterial cells were washed twice with 200 µl HPLC grade H_2O and centrifuged at 10,000 × g for 1 min. The samples were resuspended in 10 µl matrix solution (alpha-cyano-4-hydroxycinnamic acid, 20 mg/ml in 0.1% trifluoroacetic acid (TFA)/acetonitrile 1 : 2) and 1, 2, or 3 µl of this mixture was applied onto a stainless steel target (scout 384). Samples were allowed to crystallize, washed twice with 0.1% TFA and recrystallized in 0.1% TFA/acetonitrile 1 : 2.

A Biflex III mass spectrometer (Bruker Daltonic, Bremen, Germany) supplied with an N_2-laser (wavelength 337 nm, pulse 5 ns, flight distance 1200 mm) was used. The measurements were carried out in linear positive mode (delay 400 ns, voltage 20 kV, mass range: 2–20 kDa).

For calibration, a peptide mixture containing somatostatin 28 (synthetic, $M+H^+ = 3148.0$ Da), insulin (bovine pancreas, $M+H^+ = 5733.5$ Da), cytochrome c (horse heart, $M+H^+ = 12360.0$ Da), myoglobin (horse heart, $M+H^+ = 16951.0$ Da, Sigma, Taufkirchen, Germany) was added. A three-step calibration procedure was carried out. First, the *x*-axis was calibrated using the peptide mixture (external calibration). Second, the peptide mixture was added to a bacterial sample and used for internal calibration. Third, selected sample specific peaks were used for internal calibration without peptide mixture. A total of 120 laser shots (4 cycles of 30 shots) was applied per spot to obtain a sum spectrum. From each culture, samples

Table 2. Strains isolated from MSB agar which were primarily identified as mutans streptococci by classical microbiology. Two of these strains were re-classified as *Streptococcus anginosus* by ICM and 16S rDNA sequencing. N: number of isolates of one species per proband; σ_1: maximal distance between ICM mass spectra of duplicates of reference strains; σ_2: minimal distance between spectra of different reference strains of the same species; a,b,c,d: different phenotypes of *Streptococcus mutans* in one proband

	Streptococcus mutans			*Streptococcus sobrinus*			*Streptococcus anginosus*		
Proband	N	$>\sigma_1$	$>\sigma_2$	N	$>\sigma_1$	$>\sigma_2$	N	$>\sigma_1$	$>\sigma_2$
P 1	23	3	2 (a, b)						
P 2	14	3	2 (a, b)						
P 3	26	6	4 (a, b, c, d)	1	1	1			
P 4	11	5	3 (a, b, c)						
P 5	16	1	1 (a)						
P 6	16	2	2 (a, b)	8	4	1			
P 7	9	4	3 (a, b, c)	7	2	1			
P 8	12	4	3 (a, b, c)						
P 9	10	2	2 (a, b)						
P 10	22	4	1 (a)				2	2	2

were prepared on 3 different days and multiple sum spectra were obtained. Six sum spectra were superimposed to one master spectrum (XMASS, Bruker Daltonic). From each spectrum 120 peaks were labeled automatically in the mass range of 2–20 kDa. Correct labeling was controlled manually. Peak lists were exported as ASCII files.

Statistical analysis

Classification and identification of peaks in ICM master spectra

Peak lists were imported into Microsoft EXCEL 2000 (Microsoft Corp., Redmond, WA) files. Applying MATLAB 7.0.1 (The MathWorks Inc., Natick, MA), the peak intensities of the master spectra were log-transformed followed by subtraction of the baseline using a Loess smoother (32). Each master spectrum was normalized by a linear transformation that mapped the 10th and 90th percentiles to 0 and 1, respectively. To align a set of normalized master spectra, a moving mass-window was applied taking into account the essential variability of m/z-values of identical fragments in different spectra. The centre of this window represents the consensus position of that peak in all spectra. The width of the window depends on the precision of the m/z determination and was assumed to be linearly related to the m/z-value (window width = absolute width + relative width * peak mass). Based on an analysis of repeated measurements of master spectra, an absolute width of 3 Da and a relative width of 10^4 were found to be appropriate. The mass window was assigned to each peak. All windows that contained the same group of peaks were considered together. These peaks were then regarded as originating from the same molecular ion and were represented by their mean mass and their normalized peak intensities. The remaining windows, which overlapped, were resolved by repeating this procedure using a halved window width. By this method, a matrix η_{ij} was constructed which contained the intensities of the peaks in the individual master spectra. Its rows (i) are the mean peak masses and its columns (j) are the master spectra. An element η_{ij} was set = 0 if a particular peak was absent in a master spectrum.

Construction of a distance matrix

The similarity of the master spectra was characterized by an Euclidean distance matrix d_{mn}. The Euclidean Distance Coef-

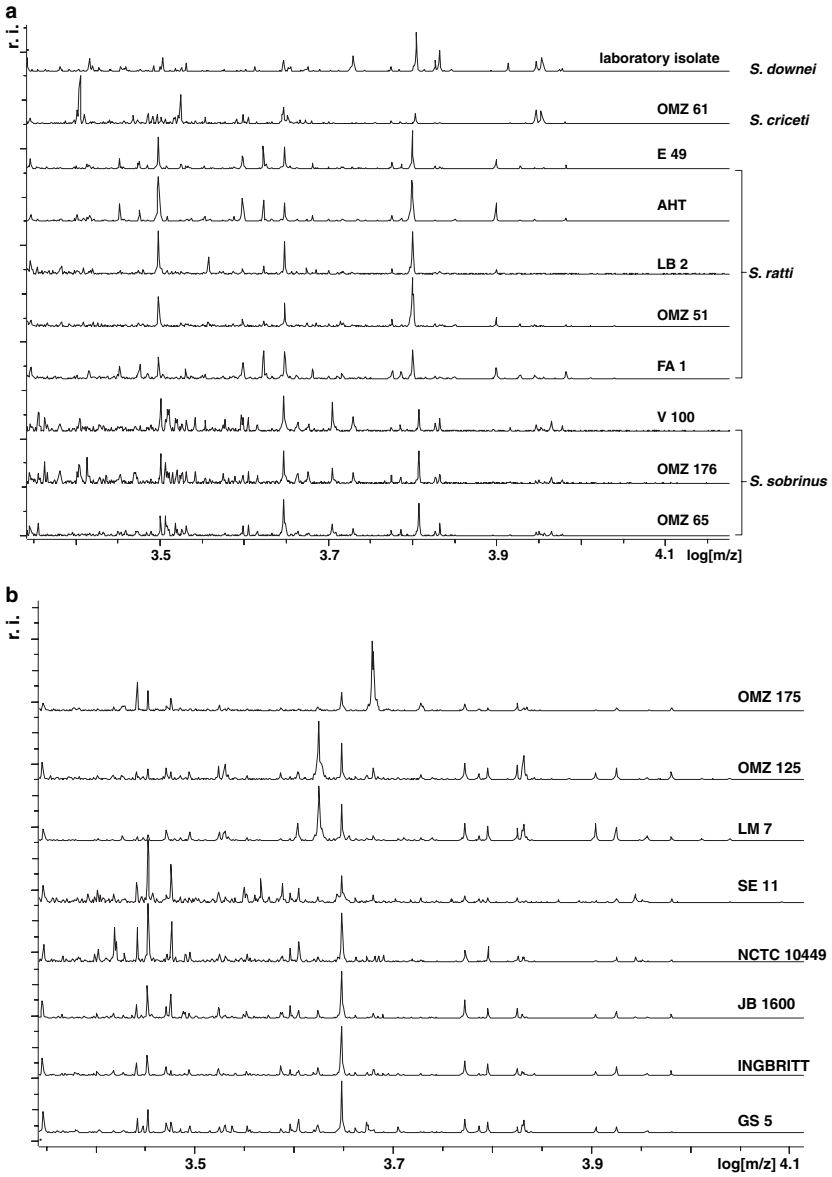

Fig. 1. ICM mass spectra of different mutans streptococci. a: Different reference strains of the indicated species and a laboratory isolate identified as *S. downei*. The original *S. criceti* strains AHT and E 49 and the *S. mutans* strain V 100 were reclassified as *S. ratti and S. sobrinus*, respectively, on the basis of ICM and 16S rDNA sequencing. b: Different reference strains of *S. mutans*.

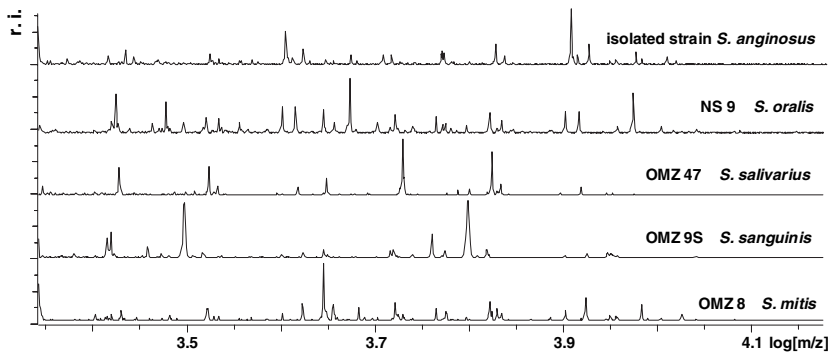

Fig. 2. ICM mass spectra of different oral nonmutans streptococci reference strains and an *S. anginosus* isolated from the saliva of proband 10.

ficient d_{mn} compared two master spectra m and n, and was defined as follows:

$$d_{mn}^2 = \frac{\sum_k w_{kmn}(\eta_{km} - \eta_{kn})^2}{\sum_k w_{kmn}}$$

where η_{kj} is the intensity of the idealized peak k in spectrum j, and w_{kmn} is a weight of 1 or 0 depending on whether at least one of the intensities for the kth peak mass in the spectra exceeds a predefined threshold value.

To consider the occurrence of a variable number of peaks per master spectrum, the distance coefficients d_{mn} were corrected according to:

$$\tilde{d}_{mn}^2 = \frac{n_m * n_n}{(n_m + n_n)} * d_{mn}^2$$

where $n_{m \text{ and }} n_n$ are the number of peaks in the normalized master spectra m and n which exceed the threshold value. This correction was applied to balance the impact of different numbers of peaks per master spectrum.

Analysis of the distance matrix by multivariate statistical analysis using unsupervised clustering procedures

Hierarchical clustering. The distance matrix was analyzed by Agglomerative Hierarchical Clustering Algorithms using Complete Linkage. To assess the quality of the clustering results, cophenetic correlation coefficients were calculated. If the clustering was valid, the distances between master spectra within a cluster correlated strongly with the distances in the distance matrix d_{mn}. The results were visualized as rooted dendrograms.

Quantum clustering. The large matrix h_{ij} was compressed by quantum clustering (12) and expressed initially by eigenvectors and eigenvalues. The Singular Value Decomposition (11) was then applied to h_{ij}. The truncation of the matrix was achieved by considering only the most significant eigenvalues. The master spectra were thus represented by vectors in a space of lower dimensionality. The parameter σ, which characterizes the relative cluster size, was adjusted on an empirical basis. Depending on its numerical value the number of clusters could vary from 1 to the number of ICM mass spectra. The method returned a variable number of clusters and assigned each mass spectrum to exactly one of them.

Determination of the level of similarity between bacterial strains

As a first step, the distances between repeatedly obtained ICM mass spectra of individual reference strains or isolates were determined. The maximal distance between these spectra (σ_1) characterized the lowest resolution level of the method. In a second step, the minimal distance between the ICM mass spectra of different reference strains of the same species (σ_2) was determined. Distances between spectra lower than σ_1 cannot be resolved. If spectra of different isolates obtained from one saliva sample differed by less than σ_1, the isolates were assumed to be identical. For further analysis they were replaced by their centroid spectrum. If spectra of different isolates obtained from different saliva samples differed by less than σ_1, the isolates were assigned to one phenotype. If the distance of two spectra was larger than σ_2, the corresponding isolates were classified as different phenotypes. Strains

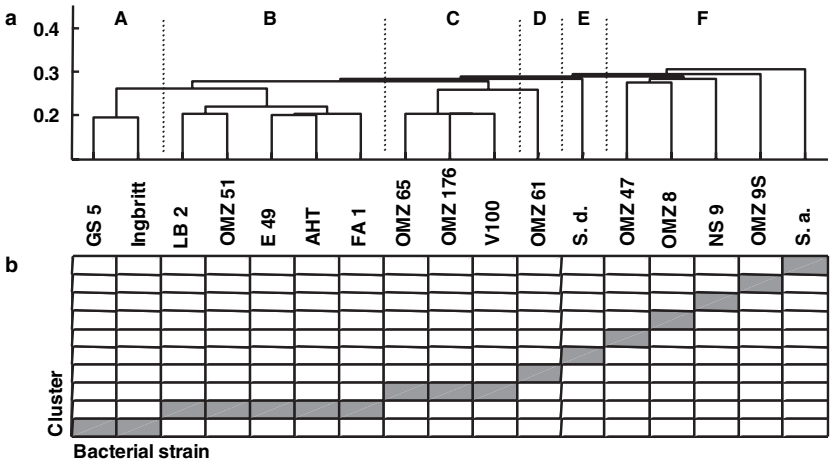

Fig. 3. Analysis of the distance matrix obtained from the spectra of oral streptococci by multivariate statistical analysis using unsupervised clustering procedures. A: *S. mutans*, B: *S. ratti*, C: *S. sobrinus*, D: *S. criceti*, E: *S. downei*, F: nonmutans streptococci, OMZ 47: *S. salivarius*, OMZ 8: *S. mitis*, NS 9: *S. oralis*, OMZ 9S: *S. sanguinis*, S.a.: *S. anginosus*. a: Results obtained by hierarchical clustering. b: Results obtained by quantum clustering in a truncated SVD (Singular Value Decomposition) space, dimensions = 10, $\sigma = 0.06$.

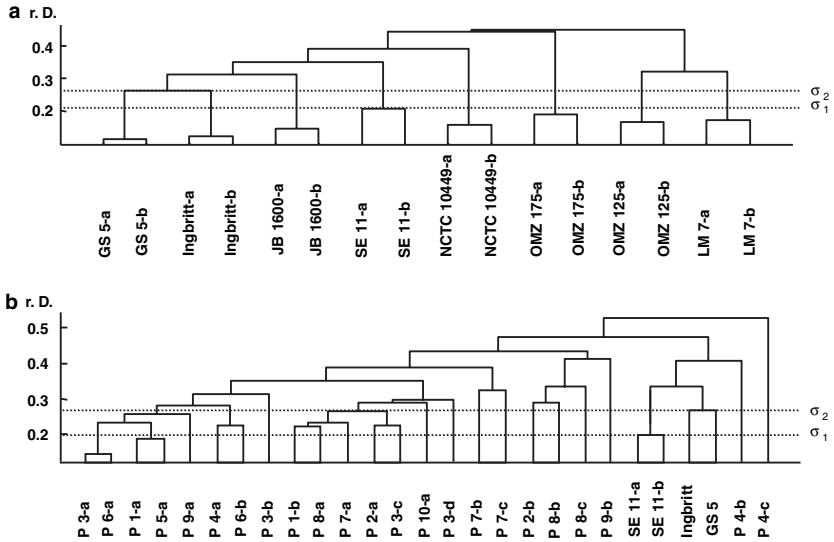

Fig. 4. Results of hierarchical clustering of spectra of *S. mutans*. a: Duplicates of spectra for reference strains. σ_1 indicates the maximum distance of duplicate spectra and σ_2 represents the minimum distance between spectra from different reference strains. b: Phenotypes of the probands. For each of the probands all spectra with mutual distances smaller than σ_1 were replaced by their centroid spectrum. The duplicate spectra of reference strain SE 11 represents the maximum distance observed for all duplicates and was therefore used to define σ_1. The spectra of reference strains Ingbritt and GS 5 showed the minimum distance among all analyzed reference strains and were therefore used to define σ_2.

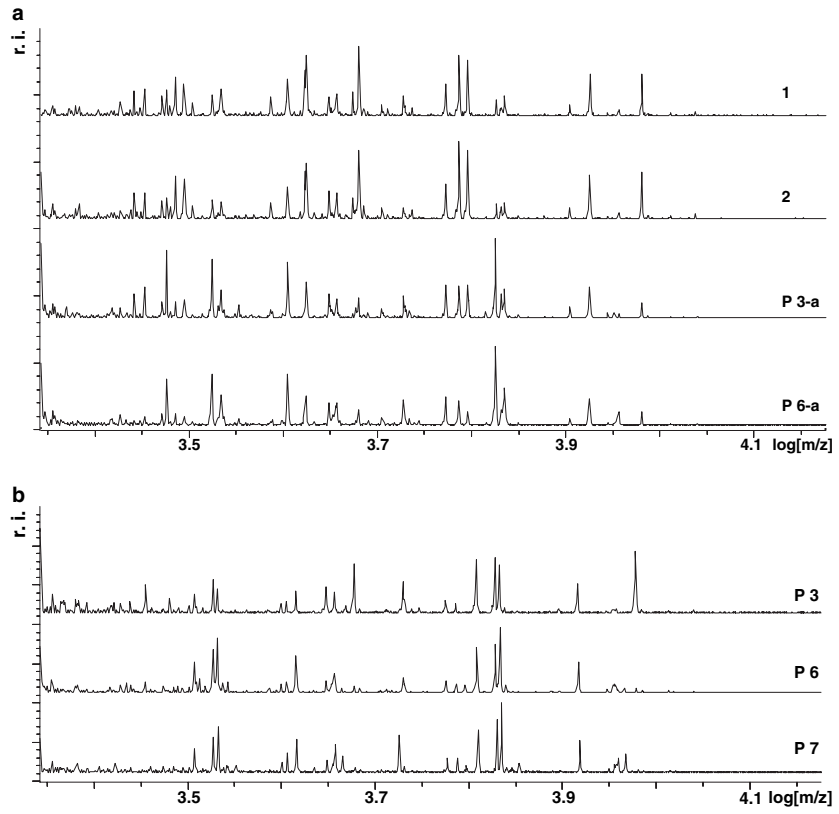

Fig. 5. Typical ICM mass spectra obtained for different strains of *S. mutans* (a) and *S. sobrinus* (b). a: Spectra 1 and 2: indistinguishable (distance < σ_1) individual ICM mass spectra obtained from proband 10, which contributed to centroid spectrum P 10-a; Spectra P 3-a and P 6-a: indistinguishable (distance < σ_1) individual ICM mass spectra obtained from probands P 3 and P 6, respectively. Spectra 1 and 2 differ unambiguously from spectra P 3-a and P 6-a by a distance > σ_2. b: Unambiguously distinguishable (distance > σ_2) individual ICM mass spectra obtained from probands 3, 6, and 7.

whose distances were between σ_1 and σ_2 were classified by hierarchical or quantum clustering. The robustness of the clustering procedures was cross validated by jackknife (leave-one-out) testing.

Results

The investigated strains of mutans streptococci and oral nonmutans streptococci listed in Table 1 yielded characteristic ICM mass spectra. Reproducible peaks were found up to a log[m/z] of 4.04 (11 kDa). Peaks with high intensities were found in the range of 2–10 kDa. The spectra from different species were strikingly dissimilar (Fig. 1a and 2). Spectra obtained from different strains of one species were noticeably similar (Fig. 1b). Remarkably, the ICM spectra of the reference strains AHT, E 49 (*Streptococcus criceti*), and V 100 (*S. mutans*) showed obvious dissimilarities to the spectra observed for other strains of the respective species (Fig. 1a, b). Moreover, when the spectra from different strains of one species, e.g. *S. mutans* OMZ 175 and NCTC 10449, are compared with the spectra from different species, e.g. *S. sobrinus* and *Streptococcus ratti,* it becomes obvious that a classification could not be made solely by visual inspection (Fig. 1a, b).

Therefore, after constructing a distance matrix of the spectra their similarities were analyzed by multivariate statistics using unsupervised clustering procedures as described in Material and methods. Remarkably, both hierarchical clustering (Fig. 3a) and quantum clustering (Fig. 3b) allowed an unambiguous and identical species assignment of all reference strains. Furthermore, the visual impression of misclassifications of AHT, E 49, and V 100 was verified. As shown in Fig. 2, AHT and E 49 fell into the clusters of *S. ratti,* whereas V 100 was found to be a *S. sobrinus* strain. DNA sequencing of the 16S rRNA genes confirmed the ICM-based re-classification of these three strains as well as the correct classification of all remaining reference strains.

From the 177 isolated strains that were classified as mutans streptococci by classical microbiology (see Material and methods) 159 were identified as *S. mutans and* 16 as *S. sobrinus* by cluster analysis of ICM data. Two isolates were clustered out and identified as *S. anginosus* by sequencing of their 16S rRNA genes (see Fig. 2 and 3a,b and Table 2).

The analysis of the mutans streptococci isolated from saliva on the subspecies level was based on the reproducibility of the spectra determined for the reference strains. Figure 4a shows the results of hierarchical clustering of spectra of *S. mutans* reference strains determined in duplicate. σ_1 indicates the maximum distance observed for duplicate spectra and σ_2 represents the minimum distance between spectra from different reference strains.

As a first step the isolates from each proband were analyzed separately. Using σ_1 as threshold parameter, the maximum number of different phenotypes per proband could be estimated by replacing indistinguishable individual spectra (e.g. Fig. 5a, spectra 1 and 2) by their centroid spectra. From one to six putatively different *S. mutans* phenotypes were found per proband. In the three probands carrying *S. sobrinus*, from one to four putatively different phenotypes were observed (Table 2). The spectra of the reference strains Ingbritt and GS 5 that were visually different and clearly separable by clustering analysis showed the minimum distance among all analyzed reference strains. This distance was therefore used to define σ_2. Using σ_2 as threshold parameter, the minimum number of unambiguously different phenotypes per proband could be estimated. From one to four different *S. mutans* phenotypes and one *S. sobrinus* phenotype (Fig. 5b) were found per proband (Table 2).

For analysis of the population of all isolates at the subspecies level, clustering analysis was performed using the individual spectra whereby those with mutual distances < σ_2 at the proband level were replaced by their centroid spectra. The results are shown in Fig. 4b.

Using σ_1 as the threshold parameter, the phenotypes of the samples from different probands, P 3-a and P 6-a (see Fig. 5a) as well as P 1-a and P 5-a, respectively, were indistinguishable. Using σ_2 as the threshold parameter at least 14 unambiguously different phenotypes, were observed. Typically, their spectra can be distinguished

still on a visual basis (compare spectra 1 and 2 vs. P 3-a and P 6-a in Fig. 5a). Distances between σ_1 and σ_2 were found for the spectra of three groups of isolates (P 3-a/P 6-a, P1-a/P 5-a, P 9-a; P 4-a, P 6-b and; P 1-b, P 8-a, P 7-a, P 2-a, P 3-c) (Fig. 4). The spectra cannot be differentiated visually in any of the groups. The respective phenotypes can be distinguished but they are more similar than those of the most similar reference strains (Ingbritt and GS 5).

The isolated *S. sobrinus* strains from three probands showed distances $> \sigma_2$ to each other and to the *S. sobrinus* reference strains. There was a greater difference between the two ICM mass spectra obtained from *S. anginosus* isolates (proband 10) than between the two most similar strains of *S. mutans* (Table 2).

Discussion

Reference strains of oral streptococci and isolated mutans streptococci strains from human saliva were analyzed by ICM. It was possible already by visual inspection of the master spectra to distinguish different but closely related species of mutans and other oral streptococci as well as different reference strains of one species. Closely similar spectra which can not be differentiated unambiguously by visual inspection could be distinguished and classified by multivariate statistics.

Under the applied conditions of spectra acquisition the mass peaks are thought to represent peptides and small proteins from all cell compartments (16, 29). The m/z-values are not sufficient to allow an assignment of individual mass peaks to specific molecules. Any identification of bacteria based on specific biomarkers should rely on known peptides. For mutans streptococci this approach is limited by the small number of database entries for specific proteins and peptides.

Irrespective of the identity of the molecules leading to individual mass peaks, the ICM spectra are complex fingerprints of the peptide composition of the cells. This has been proven by others for strains of typical laboratory species, like *Escherichia coli*, as well as for important pathogens (4, 5, 13, 29). In this paper a multivariate statistical analysis of the peak lists derived from the respective master spectra was performed to make a systematic classification of different mutans streptococci species. Two different methods, hierarchical and quantum clustering, were applied. Both produced identical results which matched the intuitive species assignment. However, the traditional classification of three strains (AHT, E 49, V 100) became conspicuous both on the basis of visual inspection and after the cluster analysis of the respective ICM mass spectra. Cluster analysis of ICM spectra suggested a reclassification of these strains to different species. This was confirmed by sequencing of the genes of 16S rRNA.

For the isolated mutans streptococci strains a reliable assignment to the respective species was possible by ICM. These results were confirmed by species specific PCRs for *S. sobrinus* and *S. mutans* or by 16S rDNA sequencing. The detection of two strains that were later identified as *S. anginosus* demonstrated the discriminatory power of the method.

In contrast to the straightforward differentiation of species of human pathogenic mutans streptococci by ICM, a classification of the investigated strains at the subspecies level by intuitive comparison of spectral patterns turned out to be insufficient and could only be achieved by multivariate statistical analysis. In addition, the quality of the internal calibration of spectra was found to be critical. For each bacterial strain, six individual ICM mass spectra were superimposed on to a master spectrum. The use of such master spectra was found to be the decisive factor for a reliable separation of the different reference strains, as demonstrated for the analysis of duplicates of the *S. mutans* reference strains. Based on individual sum spectra the spectra of highly similar strains (OMZ 125 and LM 7, or GS 5 and Ingbritt, respectively) could not be separated. Since cultivation conditions and the age of cultures are known to influence phenotype and therefore the results of ICM (15), the investigated strains were repeatedly cultured under identical, strictly controlled conditions. This was the second important factor ensuring highly reproducible results and enabling a differentiation of the mutans streptococci isolates from the saliva samples on the subspecies level.

In summary, ICM as a phenotyping method offers an attractive alternative to well established methods for bacterial characterization, for example, biochemical analysis or gas liquid chromatography. It allows to reveal differences between strains that appear very similar by other methods. However, phenotyping methods like ICM can not directly identify taxonomic entities, like species or subspecies, without the assistance of DNA-based methods. The outstanding features of ICM are its rapidity due to the minor hands-on time for sample processing and the speed of spectra acquisition as well as its potential for high sample throughput by automation of both MALDI-TOF-MS mutans and data analysis. To determine the limitations of ICM and to check the interlaboratory reproducibility of the obtained results, protocols for sample pretreatment and spectra acquisition as well as procedures for data analysis will have to be standardized in the future.

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