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Original article

The optimization and validation of the Biotyper MALDI-TOF MS database for the identification of Gram-positive anaerobic cocci

A.C.M. Veloo [1, *], E.D. de Vries [1], H. Jean-Pierre [2, 3], U.S. Justesen [4], T. Morris [5], E. Urban [6], I. Wybo [7], A.J. van Winkelhoff [1, 8]on behalf of the ENRIA workgroup

[1] Department of Medical Microbiology, University of Groningen, University Medical Centre Groningen, The Netherlands
[2] Centre Hospitalier Universitaire de Montpellier, Hôpital Arnaud de Villeneuve, Laboratoire de Bactériologie, Montpellier, France
[3] Université Montpellier 1, UMR5119 ECOSYM, Equipe Pathogènes Hydriques Santé Environnements, UMR 5569 Hydrosciences, UFR Pharmacie, Montpellier, France
[4] Department of Clinical Microbiology, Odense University Hospital, Odense, Denmark
[5] UK Anaerobe Reference Unit, Public Health Wales Microbiology, Cardiff, UK
[6] Institute of Clinical Microbiology, University of Szeged, Hungary
[7] Department of Microbiology and Infection Control, Universitair Ziekenhuis Brussel, Brussels, Belgium
[8] Department of Dentistry and Oral Hygiene, University of Groningen, University Medical Centre Groningen, The Netherlands

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ABSTRACT

Gram-positive anaerobic cocci (GPAC) account for 24%–31% of the anaerobic bacteria isolated from human clinical specimens. At present, GPAC are under-represented in the Biotyper MALDI-TOF MS database. Profiles of new species have yet to be added. We present the optimization of the matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) database for the identification of GPAC. Main spectral profiles (MSPs) were created for 108 clinical GPAC isolates. Identity was confirmed using 16S rRNA gene sequencing. Species identification was considered to be reliable if the sequence similarity with its closest relative was ≥98.7%. The optimized database was validated using 140 clinical isolates. The 16S rRNA sequencing identity was compared with the MALDI-TOF MS result. MSPs were added from 17 species that were not yet represented in the MALDI-TOF MS database or were under-represented (fewer than five MSPs). This resulted in an increase from 53.6% (75/140) to 82.1% (115/140) of GPAC isolates that could be identified at the species level using MALDI-TOF MS. An improved log score was obtained for 51.4% (72/140) of the strains. For strains with a sequence similarity <98.7% with their closest relative ($n = 5$) or with an inconclusive sequence identity ($n = 4$), no identification was obtained by MALDI-TOF MS or in the latter case an identity with one of its relatives. For some species the MSP of the type strain was not part of the confined cluster of the corresponding clinical isolates. Also, not all species formed a homogeneous cluster. It emphasizes the necessity of adding sufficient MSPs of human clinical isolates. **A.C.M. Veloo, CMI 2016;22:793**

Introduction

The introduction of matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the identification of bacteria isolated from human specimens has led to a revolution in medical diagnostic microbiology laboratories [1]. This is especially relevant for bacteria for which the identification is time consuming and technically challenging. Identification of anaerobic bacteria by MALDI-TOF MS results in faster and more reliable results [2–4]. The two main groups of anaerobic bacteria encountered in human infections are the *Bacteroides fragilis* group (43%) and Gram-positive anaerobic cocci (GPAC, 24%–31%) [5,6]. The performance of MALDI-TOF MS for the identification of *B. fragilis* group species has already been validated with 94%–98% identified to the species level [7,8]. The validation of MALDI-TOF MS for the identification of GPAC has been limited until now, except for the most prevalent GPAC species (*Finegoldia magna*, *Parvimonas*

* Corresponding author. A.C.M. Veloo, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.
E-mail address: a.c.m.veloo@umcg.nl (A.C.M. Veloo).

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micra and *Peptoniphilus harei*). At present, GPAC profiles are underrepresented in the MALDI-TOF MS database and profiles of new species have yet to be added. Previous studies have shown that the addition of reference spectra of clinical isolates of anaerobic bacteria results in an increase in the number of correct identifications [9,10]. In this study we present the optimization of the Biotyper MALDI-TOF MS database for the identification of GPAC by including main spectral profiles (MSP) of 16S rRNA sequenced clinical isolates. The primary goals were to have at least five MSPs [11] present in the database for each species and also the addition of MSPs of species not yet represented in the Biotyper MALDI-TOF MS database.

Material and methods

Bacterial strains

A selection of 108 clinical isolates of GPAC, including six type strains, were collected by laboratories associated with the European Network for the Rapid Identification of Anaerobes (ENRIA) and sent to the University Medical Centre, Groningen (Table 1). Upon arrival strains were cultured on Brucella Blood Agar (Mediaproducts, Groningen, the Netherlands) supplemented with haemin and vitamin K1 and incubated at 37°C in an anaerobic atmosphere (80% N_2, 10% CO_2, 10% H_2) for 48 h. After repeated subculturing on Brucella Blood Agar, strains were stored on microbank™ beads (Pro-Lab Diagnostics, Bromborough, UK) at −80°C and ethanol suspensions were made as described previously [12], which were stored at −20°C until use.

Table 1
An overview of main spectral profiles (MSPs) of Gram-positive anaerobic cocci (GPAC) species already present in the MALDI-TOF MS database and of MSPs added to the database

Species	No. of MSPs		
	Present	Added	Total
Peptostreptococcus stomatis	0	5	5
Peptostreptococcus anaerobius	4	3	7
Peptostreptococcus canis	0	2	2
Peptococcus niger	1	5	6
Finegoldia magna	11	0	11
Murdochiella asaccharolytica	0	3	3
Parvimonas micra	7	0	7
Anaerococcus murdochii	1	8	9
Anaerococcus degenerii [a]	0	2[b]	2
Anaerococcus lactolyticus	1	4	5
Anaerococcus tetradius	2	4	6
Anaerococcus prevotii	2	2	4
Anaerococcus vaginalis	1	12	13
Anaerococcus hydrogenalis	3	0	3
Anaerococcus senegalensis [a]	0	1	1
Anaerococcus obesiensis [a]	0	1	1
Anaerococcus octavius	2	0	2
Anaerococcus provenciensis [a]	0	2	2
Anaerococcus nagyae [a]	0	3[b]	3
Peptoniphilus grossensis [a]	0	6	6
Peptoniphilus tyrelliae	0	1[b]	1
Peptoniphilus rhinitidis [a]	0	3	3
Peptoniphilus harei	4	7	11
Peptoniphilus gorbachii	1	5	6
Peptoniphilus timonensis [a]	0	1	1
Peptoniphilus olsenii	0	4[b]	4
Peptoniphilus lacrimalis	0	5	5
Peptoniphilus koenoeneniae	0	2[b]	2
Peptoniphilus duerdenii	0	6[b]	6
Peptoniphilus indolicus	2	0	2
Peptoniphilus asaccharolyticus	1	0	1
Peptoniphilus ivorii	1	3	4
Peptoniphilus coxii	0	8	8

GPAC, Gram-positive anaerobic cocci; MALDI-TOF, matrix-assisted laser desorption–ionization time-of-flight mass spectrometry; MSP, main spectral profiles.
[a] These species are not validly published.
[b] The type strains of these species were also included; *Anaerococcus degenerii* DSM29674, *Anaerococcus nagyae* DSM101193, *Peptoniphilus tyrelliae* CCUG59621, *Peptoniphilus olsenii* CCUG53342, *Peptoniphilus koenoeneniae* CCUG56067 and *Peptoniphilus duerdenii* CCUG56065.

Identification using 16S rRNA sequencing

All strains were identified with 16S rRNA gene sequencing. DNA of the strains was isolated as described previously [13]. The PCR was performed using universal primers targeting the 16S rRNA gene [14]. The identity of the strains was determined by comparing either the sequence obtained with the forward primer and/or reverse primer or in the case of consensus the complete sequence of the 16S rRNA gene with sequences available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) using BLASTn (https://blast.ncbi.nlm.nih.gov). Identification at the species level was considered reliable if the sequence similarity between the unknown strain and its closest relative was ≥98.7% [15]. Only strains that could be identified at the species level were included in the MALDI-TOF MS database.

Identification using MALDI-TOF MS

Strains were identified using MALDI-TOF MS as described previously [12]. Briefly, after 48 h of incubation, fresh colonies were spotted twice on to a stainless steel target using a toothpick. One spot was overlaid with 1 μL HCCA matrix (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid) and left to dry at ambient temperature. An on-target extraction was performed on the second spot by overlaying the bacteria with 1 μL 70% formic acid. After drying at ambient temperature the spot was immediately covered with 1 μL HCCA matrix. Measurements were performed using the Microflex (Bruker Daltonik GmbH, Bremen, Germany). Spectra were obtained by summing shot steps of 40, with a minimum laser power of 30% and a maximum laser power of 40%, until 240 satisfactory shots were obtained.

Main spectral profiles

A full extraction was performed on the bacterial ethanol suspensions as described previously [12]. Briefly, the suspension was centrifuged at 13 000 ***g*** for 2 min and the supernatant was disregarded. The centrifugation step was repeated and the remaining supernatant was carefully removed by pipetting. The pellet was resuspended in 30 μL 70% formic acid and an equal volume of acetonitrile. After centrifugation at 13,000 ***g*** for 2 min, 1 μL of the supernatant was spotted on the stainless steel target 12 times. Immediately after drying at ambient temperature, 1 μL HCCA matrix was added to the spot and left to dry at ambient temperature. From each spot, three spectra were obtained using the Microflex. Before each measurement, the MALDI-TOF MS system was calibrated using a bacterial test standard (Bruker Daltonik). For each spectrum, 240 satisfactory shots were summed in shot steps of 40, with a minimum laser power of 30% and a maximum laser power of 40%.

Obtained spectra were manually evaluated in FLEXANALYSIS 3.3.80.0. For each spectrum the appropriate method was chosen, MBT_standard, smoothing and baseline subtraction were performed. The set of spectra derived from one strain were checked for peak shifts, which should not exceed 500 ppm (the peak shift should not exceed 2 Da for a peak at 4000 Da). Flat liners, outliers and spectra that differ remarkably from the rest were removed from the data set. An MSP from the remaining spectra, comprising

at least 20 spectra of good quality, was calculated using Biotyper 3.0. Dendrograms of the created MSPs and the MSPs already present in the MALDI-TOF MS database were calculated in Biotyper 3.0.

Validation

The performance of the optimized database was validated using a total of 140 clinical isolates of GPAC, which were consecutively isolated from a variety of human clinical specimens. To evaluate also the MSPs of species less commonly encountered in clinical specimens, a minority of the strains ($n = 11$) were from a different period. The identity of strains was established using 16S rRNA gene sequencing as described above and compared with the identity obtained using MALDI-TOF MS.

Only strains belonging to the genera mostly encountered in human infections—*Peptoniphilus*, *Anaerococcus*, *Peptostreptococcus*, *Parvimonas*, *Finegoldia*, *Peptococcus* and *Murdochiella*—were used. Spectra obtained were compared with the current Bruker database (db5627) and with the optimized Bruker database augmented with GPAC MSPs of the ENRIA strains. Obtained log scores were interpreted as recommended by the manufacturer. Log scores $\geq$2 as a high confidence identification, log score >1.7 and <2 as a low confidence identification and log score $\leq$1.7 as no reliable identification.

Results

Optimization of the MALDI-TOF MS database

MSPs of strains that were not represented by five or more profiles and MSPs of species that were not present in the Bruker database were created and added (Table 1). The obtained dendrograms are shown in Figs. 1 and 2. The species in the genera *Peptococcus*, *Murdochiella* and *Peptostreptococcus* each form a confined cluster, without obvious intra-species variation. For some species of the genus *Anaerococcus* intra-species variation is observed. This phenomenon was observed for *Anaerococcus murdochii*, *Anaerococcus lactolyticus*, *Anaerococcus prevotii* and *Anaerococcus tetradius*. The MSPs of the type strains of *Anaerococcus vaginalis*, *Anaerococcus lactolyticus* and *P. harei*, which were already present in the Bruker database, are not part of the confined cluster of MSPs of the clinical isolates of these three species. This phenomenon was not observed for all species of which MSPs were added to the database.

Validation

The results of the validation of the optimized database are summarized in Table 2. The MSPs of *P. micra* and *F. magna* were not added since these species were already represented in the current database with five or more profiles. One *F. magna* strain repeatedly failed to produce any peaks. *Peptostreptococcus stomatis*, *Murdochiella asaccharolytica*, *Peptoniphilus lacrimalis*, *Peptoniphilus gorbachii*, *Peptoniphilus grossensis*, *Peptoniphilus duerdenii*, *Peptoniphilus olsenii*, *Peptoniphilus coxii*, *A. vaginalis* and *Anaerococcus senegalensis* all provided useful MSPs and clinical isolates could be identified with high confidence. Even though one MSP was added of *Anaerococcus obesiensis*, this species could not be identified using the optimized database. *Anaerococcus hydrogenalis* could only be identified with low confidence, even though three MSPs were already present in the database. In general, the log score of clinical isolates belonging to species of which MSPs were added increased. This was especially observed within the species *P. harei*, *A. vaginalis*, *Peptostreptococcus anaerobius*, *Peptoniphilus ivorii*, *A. lactolyticus* and *A. tetradius*. After optimization of the Bruker database, the proportion of species identified with high confidence

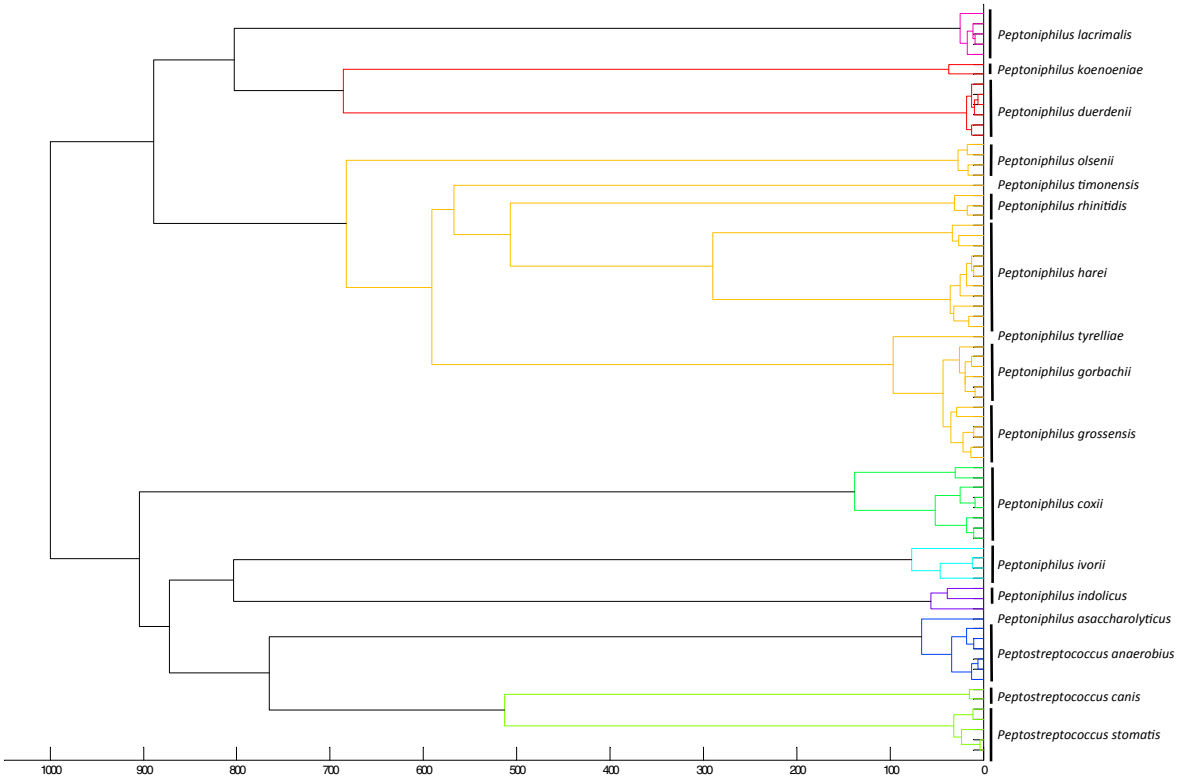

Fig. 1. Dendrogram of main spectral profiles created of strains belonging to the genera *Peptoniphilus* and *Peptostreptococcus*.

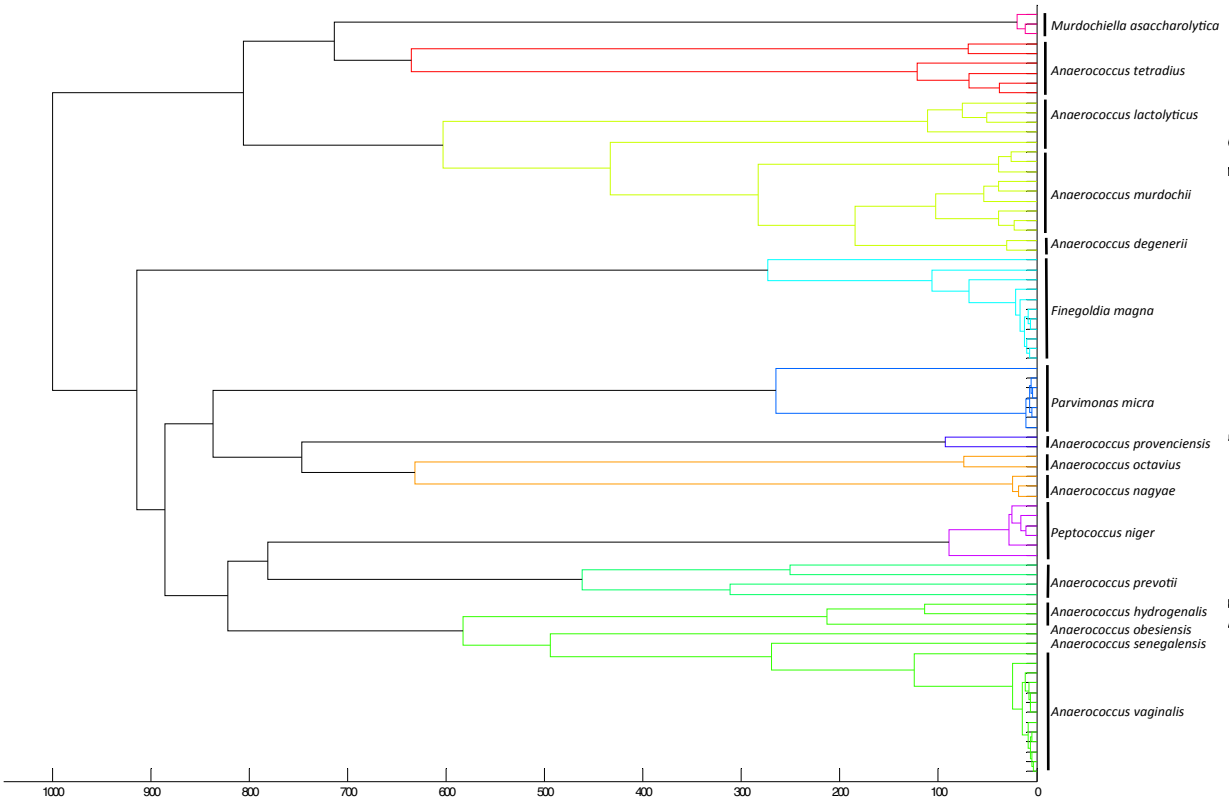

Fig. 2. Dendrogram of main spectral profiles created of strains belonging to the genera *Anaerococcus*, *Murdochiella*, *Peptococcus*, *Finegoldia* and *Parvimonas*.

Table 2
MALDI-TOF MS identification results of GPAC clinical isolates ($n = 140$) compared with the Bruker database and with the Bruker database supplemented with MSPs of well-characterized GPAC species

Species ID	Bruker database			Bruker database + ENRIA GPAC database			
	Score ≥2	Score >1.7 to <2	Score ≤1.7	Score ≥2	Score >1.7 to <2	Score ≤1.7	No. of strains of which the log score increased
Peptoniphilus harei ($n = 28$)	19	8	1	28	0	0	26
Finegoldia magna ($n = 25$)[a]	16	8	1	16	8	1	0
Parvimonas micra ($n = 27$)[a]	25	2	0	25	2	0	0
Anaerococcus vaginalis ($n = 8$)	0	2	6	8	0	0	8
Anaerococcus murdochii ($n = 5$)	4	1[e]	0	5	0	0	1
Anaerococcus hydrogenalis ($n = 2$)	0	0	2	0	2[e]	0	2
Anaerococcus obesiensis ($n = 2$)[b]	0	0	2	0	0	2	0
Peptostreptococcus anaerobius ($n = 8$)	7	1	0	8	0	0	8
Peptostreptococcus stomatis ($n = 2$)[b]	0	0	2	1	1	0	2
Murdochiella asaccharolytica ($n = 3$)[b]	0	0	3	3	0	0	3
Peptoniphilus coxii ($n = 6$)[b]	0	0	6	5	1	0	6
Peptoniphilus lacrimalis ($n = 3$)[b]	0	0	3	3	0	0	3
Peptoniphilus grossensis ($n = 2$) [b]	0	1	1	1	1	0	2
Peptoniphilus lactolyticus ($n = 2$)	1	0	1	2	0	0	1
Peptoniphilus gorbachii ($n = 2$)[b]	1	0	1	2	0	0	2
Peptoniphilus duerdenii ($n = 2$)[b]	0	0	2	2	0	0	2
Different GPAC species ($n = 4$)[c]	0	0	4	4	0	0	4
GPAC ($n = 9$)[d]	2	0	7	2	1	6	2
Total (n)	75	23	42	115	16	9	72
% of all strains	53.6%	16.4%	30.0%	82.1%	11.4%	6.4%	51.4%

GPAC, Gram-positive anaerobic cocci; MALDI-TOF MS, matrix-assisted laser desorption–ionization time-of-flight mass spectrometry; MSP, main spectral profile.

[a] No additional reference spectra were added to the MALDI-TOF MS database.

[b] Species was not present in the MALDI-TOF MS database.

[c] This group consisted of *Peptoniphilus olsenii*, *Anaerococcus senegalensis*, *Anaerococcus degenerii* and ***Anaerococcus tetradius***. Species indicated in bold were already represented in the original Bruker database.

[d] Strains could not be identified at the species level using 16S rRNA gene sequencing. It contains possible new species and strains of which the sequence could not differentiate between two species. Two strains identified by sequencing as *Anaerococcus murdochii/degenerii* and *Anaerococcus vaginalis/obesiensis* were identified by MALDI-TOF MS as *Anaerococcus murdochii* (log score >2) and *Anaerococcus vaginalis* (log score >2), respectively.

[e] The obtained log score indicated the correct genus, but the wrong species name.

(log score ≥2) increased from 53.6% (75/140) to 82.1% (115/140) and the number of strains that could not be identified (log score <1.7) decreased from 30.0% (42/140) to 6.4% (9/140). Almost half of the strains (51.4%, 72/140) yielded a higher log score.

Nine clinical isolates could not be identified by 16S rRNA gene sequencing. This was either because the sequence similarity with two different close relatives was highly similar. Therefore, no differentiation could be made between the two species. This was the case for four strains of which for two a MALDI-TOF MS identification was obtained with a log score ≥2 with one of its closest relatives. Five strains yielded a sequence similarity <98.7% with their closest relative and could not be identified using MALDI-TOF MS. These strains may represent new species.

Discussion

The aim of the ENRIA project is to optimize and validate the Biotyper MALDI-TOF MS database for the identification of anaerobic bacteria. The GPAC are a clinically important group of anaerobic bacteria comprising several genera. They are a phylogenetically heterogeneous group of organisms [16]. The MALDI-TOF MS spectra used for identification represent the mass range of 2–20 kDa and consist predominantly of ribosomal and housekeeping proteins that have taxonomic relevance [17]. The heterogeneity encountered in phylogenetic analyses based on 16S rRNA is therefore also expected while creating MSPs for database optimization.

It was observed that certain species in the genus *Anaerococcus* do not form a confined cluster. Also multiple clusters are observed for the species *A. murdochii* and *A. lactolyticus*. Phylogenetic analyses showed that *Anaerococcus degenerii* is closely related to *A. murdochii* and *A. lactolyticus* [18]. *F. magna* tends to give log scores <2, probably because it possesses a very hard cell wall that hampers the release of proteins. As it is the only species present in the genus *Finegoldia*, accepting a log score >1.8 for high confidence identification is justified.

For some GPAC species the type strain yielded a different MSP compared with the clinical isolates of that species. This was also observed for *P. harei* in a previous study by Veloo *et al.* [9], in which a different MALDI-TOF MS system and database were optimized for the identification of GPAC. In the present study this observation was confirmed for this and other species. This emphasizes the necessity of adding new MSPs of well-characterized clinical isolates to the database of the MALDI-TOF MS system. The fact that *A. obesiensis* and *A. hydrogenalis* cannot be identified even though one and two MSPs are present, demonstrates the necessity to have sufficient MSPs present in the database. Due to the addition of GPAC MSPs of 108 clinical isolates, the rate of reliable species identification increased from 53.6% (75/140) to 82.1% (115/140). For 51.4% (72/140) of the tested strains a higher log score was obtained after addition of the new MSPs. This is, in part, due to the fact that MSPs of 17 species that were not yet represented in the database were added, and partly due to the addition of MSPs of species that were under-represented in the database. In the optimized database, 33 GPAC species are represented, of which 18 species are still under-represented (fewer than five MSPs) [11].

Four strains with a 16S rRNA sequence similarity insufficient to differentiate between species were either identified as one of its closest relatives or could not be identified at all. For example, one strain had similar sequence similarities with *A. vaginalis* and *A. obesiensis* and was identified with MALDI-TOF MS as *A. vaginalis*. Five strains with a sequence similarity <98.7% could not be identified at all. Three of these unidentified strains had similar sequence results (data not shown) and may represent a new species. One strain has just been described as being a new species, *Anaerococcus nagyae* [19].

We conclude that the addition of further new MSPs of well-characterized clinical isolates, permits more GPAC isolates encountered in a medical microbiology laboratory to be identified to species level. This seems especially valuable for species with a high intra-species variation. The addition of species that were not present in the database enhances the ability of MALDI-TOF MS to comprehensively identify a broader range of clinical isolates, which may allow us to better clarify their clinical relevance.

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Transparency declaration

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The ENRIA workgroup

The ENRIA workgroup consists out of T. Morris (Cardiff, UK), H. Shah (London, England), H. Jean-Pierre (Montpellier, France), U.S. Justesen (Odense, Denmark), E. Nagy (Szeged, Hungary), E. Urban (Szeged, Hungary), M. Kostrzewa (Bremen, Germany), A. Veloo (Groningen, the Netherlands), A.J. van Winkelhoff (Groningen, the Netherlands), A.W. Friedrich (Groningen, the Netherlands).

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