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Anne Jolivet-Gougeon[1,2]*, Nicolas Helsens[1], Elise Renard[1], Zohreh Tamanai-Shacoori[1], and Martine Bonnaure-Mallet[1,2]

[1] Université de Rennes, INSERM U 1241, INRA, Nutrition Metabolisms and Cancer, 2 avenue du Professeur Léon Bernard, 35043 Rennes, France

.

[2] CHU Pontchaillou, 2 rue Henri Le Guilloux - 35033 RENNES Cedex 9, France

***Corresponding author**

Anne Jolivet-Gougeon, Université de Rennes, INSERM NuMeCan/CIMIAD, 2, avenue du Professeur Léon Bernard, 35043 Rennes, France

Phone: (33) 2 23 23 49 05 – Fax: (33) 2 23 23 49 13

E-mail: anne.gougeon@univ-rennes1.fr

Abstract

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was evaluated for rapid identification of *cfxA* PCR positive and negative *Capnocytophaga* strains. Colonies were grown on blood agar, incubated anaerobically at 37°C for 48 h, and were then evaluated by MALDI-TOF MS and 16S rRNA gene sequencing. Both methods identified all colonies to the genus level. The MALDI-TOF MS method gave the same result, at the species level, as 16S rRNA gene sequencing for 41/53 *Capnocytophaga sp.* strains (77.4%), but the limit of this technique was the absence of some species (*C. leadbetteri*, *C.* AHN) in the Biotyper-Bruker® database used in this study. Distinction between the cefotaxime resistant and susceptible strains was unsuccessful using the MALDI-TOF MS method. This technique had low discriminatory power to rapidly detect beta-lactamase-producing *Capnocytophaga* strains in clinical samples. However, the results from a score-oriented dendrogram confirmed MALDI-TOF MS is a rapid, inexpensive, and reliable method for *Capnocytophaga* species identification. Enrichment of the reference database used (Biotyper®) will improve future results.

Key words: MALDI-TOF Mass Spectrometry; *Capnocytophaga; cfxA;* 16S rRNA; PCR; dendrogram

1. Introduction

Capnocytophaga are part of the normal bacterial flora of the oral cavity of humans and animals. Generally, they are considered commensal bacteria but, in immunocompetent or immunocompromised patients, they can be responsible for systemic diseases [1]. These bacteria belong to the family *Flavobacteriaceae*, order Flavobacteriales, class Flavobacteria, Bacteroidetes phylum, and Bacteria domain. This genus includes 10 different commensal species. There are six isolated from the oral cavity of humans: *Capnocytophaga ochracea, C. gingivalis, C. granulosa, C. haemolytica, C. sputigena, C. leadbetteri,* and four isolated from the oral cavity of animals: *C. canimorsus, C. cynodegmi, C. stomatis*, and *C. canis*. The classification of many described strains (AHN) remains uncertain.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is based on the protein composition of the microbial cell, particularly ribosomal protein contents, and can discriminate clinical and environmental species. This technique is effective to identify anaerobic bacteria in the routine clinical microbiology laboratory setting [2-4]. Some clinical case reports used this technique to correctly identify *Capnocytophaga* species at the genus level [5, 6].

The MALDI-TOF MS has also been used to detect several beta-lactamases, such as extended-spectrum β-lactamase (ESBL) [7, 8] and carbapenemases [2, 9, 10]. Only a few studies screened beta-lactamases in anaerobes, such as the class B metallo-beta-lactamase encoded by *cfiA* in *Bacteroides fragilis* [2] or carbapenemases [9, 11]. The prevalence of beta-lactamase producing *Capnocytophaga sp.* is increasing in the human oral cavity, as demonstrated by the high prevalence of *cfxA*-positive PCR strains [12-14], which is the major cause of third generation cephalosporin resistance in *Capnocytophaga* sp. The *cfxA* gene, conferring constant resistance to third generation cephalosporins, especially cefotaxime [15], has been described on plasmids and/or on chromosomes harbouring mobile genetic elements. This ability to gene mobilisation can be responsible for resistance gene dissemination and transfer [12]. When a beta-lactamase-producing *Capnocytophaga* strain is detected in a clinical sample, the empirical antimicrobial treatment administered to the patient should often be rapidly modified. However, the detection of the *cfxA* gene is not sufficient to assert the resistance. In previous work, we showed that the expression of this gene is not always constant: if 1st and 2nd generation cephalosporins are most often affected by the CfxA beta-lactamase, the phenotypic expression of resistance for cephalosporins of 3rd generation is reported variable according to the strains [16]. Expression of *cfxA* genes (*cfxA* mRNA levels) was quantified by using quantitative PCR, in *cfxA* PCR-positive isolates differentiated by their beta-lactam resistance profiles. One isolate remained susceptible to beta-lactams (despite a positive *cfxA* PCR), and the *cfxA* gene was not expressed (C_t value identical to distilled water used as negative control). But the normalized C_t values for the third-generation cephalosporins were not directly related to MIC levels. This expression could also be influenced by replacing the sequence in the *cfxA* gene, and enhanced expression related to the proximity of mobile elements such as *mobA* [16]. A simple and rapid method for detecting the presence of the CfxA beta-lactamase would therefore be useful for better interpreting the PCR results. Wybo *et al.* [2] managed to differentiate *cfiA*-positive from *cfiA*-negative isolates in a routine laboratory setting and so pinpoint *B. fragilis* strains potentially resistant to carbapenems. In the same way, we hoped to discriminate negative and positive *cfxA* strains: culture of bacteria with cefotaxime was also tested to confirm the experiment.

The aim of this study was to use MALDI-TOF MS for rapid species identification of *Capnocytophaga.* The study also tested the discriminatory power of this technique to differentiate the cefotaxime resistant from cefotaxime susceptible *Capnocytophaga* strains.

2. Materials And Methods

2.1 Strains used

Reference strains (n=5) were used for this study: *C. sputigena* ATCC 33595, *C. sputigena* ATCC 33612, *C. gingivalis* ATCC 33624, *C. ochracea* ATCC 27872, and *C. granulosa* ATCC 51502. Clinical strains of *Capnocytophaga* sp. (n=48) were isolated from routine cultures, at the Teaching Hospital of Rennes (France). Clinical strains were isolated from oral swabs (n=30) and sputum samples (n=8) on TBBP (Trypticase-blood-bacitracin-polymyxin B) agar [17], and from surgical bone samples (n=4), abscesses (n=4), and blood samples (n=2) on horse blood agar (Oxoid). All samples were conserved in frozen stock cultures (Cryobanks, MAST diagnostics), and cultured on horse blood agar in an anaerobic chamber for 24 to 48 h at 37°C for further experiments.

2.2 Susceptibility testing

The MICs were determined by Etest methodology (Etest ®, BioMérieux, France), and interpreted according to CLSI recommendations for anaerobes [18], since no breakpoint for third generation cephalosporins (as cefotaxime) is currently available for anaerobes, according to EUCAST recommendations [19]. The CLSI breakpoints of cefotaxime for susceptible, intermediate, and resistant strains were ≤16 mg/L, 32 mg/L, and ≥64 mg/L, respectively.

2.3 PCR amplification

Amplification of the *cfxA* gene by PCR was performed as previously described [20]. Briefly, the primers used were: cfxA1 (5'-CTTTGTCGGCAAATAAAGAT-3') and cfxA4 (5'-TGAACGAGGAATGAGTGTGG-3'), generating a 580bp fragment and cfxA5 (5'-TGGTTAATGTCGCTCAAACA-3') and cfxA6 (5'-TCAAAGCAAGTGCAGTTTAAGA-3'), generating a 507bp fragment. A 966-bp consensus sequence of *cfxA3* was then determined. The amplification conditions were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min (35 cycles).

Sequencing of the 16S rRNA gene was used as a reference method for *Capnocytophaga* species identification, based on previous work [21]. Real-time PCR was performed with Sybr green to target the 5′ part of the 16S rRNA gene (forward primer 27F, 5′-AGA GTT TGA TCM TGG CTC AG-3′; reverse primer 685R3, 5′-TCT RCG CAT TYC ACC GCT AC-3′; 658-bp amplification product; GenBank accession number NR 024570). The corresponding amplicons were sequenced in both strands and assembled, and the consensus sequences were compared with those in the Bioinformatics Bacteria Identification (BIBI) and BLAST databases. The rates of concordance between 16S rRNA gene PCR and bacteriological results were based on results at the genus (≥96% similarity) and species (≥98% similarity) levels. Positive and negative controls were added in each series [21]. To control DNA extraction and confirm the absence of PCR inhibitors, a fragment of the human beta-globin gene was amplified for each negative sample.

2.4 Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS (Biotyper, Bruker®) analyses were performed in positive linear mode in the range of 2000–20,000 masses-to-charge ratio (m/z), and used to identify the isolates to the level of species. Colonies were selected after culturing on horse blood agar in an anaerobic chamber for 24 to 48 h, in the absence of antibiotic or around to the cefotaxime disk (BioRad, 30 µg). Colony spotting was performed in triplicate and spectra were obtained and analyzed with MALDI Biotyper 2.0 software and references (Bruker® Daltonik, GmbH, Bremen, Germany), according to the manufacturer's instructions. For the direct colony method, bacteria were applied as a thin film onto a 96-spot steel plate (Bruker® Daltonics) and allowed to dry at room temperature. Subsequently, 1.2 µl of MALDI matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid [HCCA; Bruker® Daltonics] in 50% acetonitrile and 2.5% trifluoroacetic acid) was applied to the colony and allowed to dry before testing. For the extraction method, 1 to 2 colonies were extracted as already described [22]. Each series of measurements was preceded by calibration with a bacterial test standard (BTS 255343; Bruker Daltonik), to calibrate the instrument and validate the run. Negative controls were a matrix deposit alone and Brucella broth (supplemented with 5% blood, vitamin K1 and cefotaxime (0.5mg/L)). For identification, scores and reproducibility were considered according to the manufacturer's instructions (Bruker®). Results of the pattern matching process were expressed as log (score) values ranging from 0 to 3. Values from 0-1.6999 indicated not suitable identification, >1.7 indicated relationships on the genus level, and >2.0 indicated relationships on the species level (score: 2.3 to 3: high probability of identification to the species level, 2 to 2.2999: high probability of identification to the genus level and probable identification to the species level, 1.7-1.999: probable identification to the genus level). The interpretation considered two independent parameters: the value of the homology score and the reproducibility of the identification obtained (from 10 measurements carried out after laser impacts where the same bacterial species must be found at least three times with the highest scores).

To test if MALDI-TOF MS was able to differentiate between *cfxA*-negative and *cfxA*-positive isolates, bacterial pellets were also extracted after incubation of each isolate in the presence of cefotaxime (0.5 mg/L) in Brucella broth supplemented with 5% blood and vitamin K1 (1 mg/L) under anaerobic conditions for 6 h. Identification was performed, as indicated above, from a deposit of a drop of pellet left to dry and score-oriented dendrogram of matrix-assisted laser desorption ionization time-of-flight mass spectrometry profiles were compared.

The MALDI-TOF MS spectral data resulting from the analysis of 53 isolates were processed using the Bruker database software to identify species-specific masses that were then used to generate dendrograms. A class dendrogram of all the study isolates was constructed (with the correlation distance measured by the average linkage algorithm; Euclidean distances) of the Biotyper 2.0 software (Bruker Daltonics) [23]. The MALDI-TOF spectra similarity dendrogram between *cfxA* positive and negative strains, based on the mass spectral patterns, was used to study dispersion of spectra [24].

3. Results

3.1 Identifications of strains by MALDI-TOF MS and 16S *rRNA* gene sequencing

The MALDI-TOF MS and 16S *rRNA* gene sequencing of the clinical and reference isolates clearly identified all strains as *Capnocytophaga* (100% agreement) at the genus level, and 41/53 (77.4%) were identified as the same at the species level (Table 1), with a score >1.8 and a reproducibility >4 (minimum of 3/10 replicates had MALDI confidence scores >1.8). Both methods identified the *C. sputigena* (n=31) and *C. ochracea* (n=3) strains. The four *Capnocytophaga* AHN (AHN9576/AHN9798/AHN8471/ChDc) strains identified by 16S *rRNA* sequencing were characterized as *C. sputigena* by MALDI-TOF MS. The six *C. leadbetteri* strains recognized by 16S *rRNA* sequencing were identified as *Capnocytophaga. sp.* by MALDI-TOF MS. One strain classified as *Capnocytophaga geno sp., C. granulosa* and another as *C. gingivalis* by 16S *rRNA* sequencing were identified as *C. sputigena, C. gingivalis* and *C. granulosa*, respectively, by MALDI-TOF MS. The MALDI-TOF MS technique had limited ability to discriminate between *C. gingivalis* and *C. granulosa* (two misidentified strains), but these species were similar, as shown on the dendrogram of all *Capnocytophaga* species identified using MALDI-TOF MS (Figure 1).

3.2 Susceptibility testing

From the total clinical *Capnocytophaga* sp. (n=48), 35 strains were cefotaxime-susceptible, three were intermediate, and 10 were resistant, according to CLSI criteria [17]. The MICs of cefotaxime were: MIC_{50} = 1.5 and MIC_{90} ≥256 mg/L (range: 0.016->256 mg/L). Among these *Capnocytophaga* sp. isolates, the *cfxA* gene was amplified by PCR in 42 (87.5%) all resistant to cefalotin (data not shown): 13 strains were resistant/intermediate and 29 remained phenotypically susceptible to cefotaxime despite a positive *cfxA* PCR. Five of the latter (four from saliva and one from osteo-articular fluid) *cfxA*+, susceptible to cefotaxime with MIC range: 0.016-0.5 mg/L were found to be gene misexpressed and/or beta-lactamase inactivated, as previously suggested. The five reference strains were *cfxA* PCR negative and susceptible to beta-lactams.

3.3 MALDI-TOF spectra similarity dendrogram

The MALDI-TOF spectra similarity dendrogram, in which the strains were dispersed irrespective of their mass spectral patterns, was unsuccessful in differentiating susceptible and resistant strains (data not shown).

4. Discussion

In a previous study, Conrads *et al.* [25] used oligonucleotide probes and demonstrated that the dendrogram of 16S *rRNA* sequences of the *Capnocytophaga* reference strains showed a very close relationship, especially *C. granulosa* and *C. gingivalis* (97.7% similarity) and *C. ochracea* and *C. sputigena* (95.0% similarity). Previously, MALDI-TOF MS was used with success to identify strict anaerobes, such as *Bacteroides* [26] or *Prevotella* [27], with clinical isolates of *Prevotella* sp. identified to the species (62.7%) and genus (73.5%) levels. Recently, Magnette *et al.* [28] showed that MALDI-TOF MS provided a reliable identification to the species level for 100% of 45 blind-coded *Capnocytophaga canimorsus* and *C. cynodegmi,* but only following the establishment of a complementary homemade reference database. Using MALDI-TOF MS in this study, we found improved *Capnocytophaga* genus level identification (100%) and a good human oral *Capnocytophaga* species level identification (77.4%). The six *C. leadbetteri* and four *C.* AHN strains (recognized by 16S *rRNA* sequencing) were identified by MALDI-TOF MS as *C. spp.* and *C. sputigena*, respectively. This result was not surprising because *C. leadbetteri* and *C.* AHN do not exist in the Biotyper-Bruker® database used, as was previously observed [4]. In addition, the MALDI-TOF MS method could not completely discriminate between *C. gingivalis* and *C. granulosa*, a finding that is consistent with previous reports using multilocus enzyme electrophoretic analysis and serotyping of immunoglobulin A1 proteases [29]. Thus, our results suggest that MALDI-TOF MS can be used for rapid and reliable identification of most clinical *Capnocytophaga* strains.

We also investigated if MALDI-TOF MS could be used to detect the presence of the beta-lactamase CfxA (mainly responsible for cefotaxime resistance) or one of its metabolites in *Capnocytophaga* strains. The MALDI-TOF MS method failed to detect *cfxA* positive *Capnocytophaga* strains (compared to *cfxA* negative strains), cultured in the presence or absence of cefotaxime. Most previous studies used inhibitors of beta-lactamases (ESBL or carbapenemases) to reveal by MALDI-TOF MS the intermediates of beta-lactamases [30] or enzyme inhibition [31]. We did not do this because the intrinsic activity of all beta-lactamase inhibitors was demonstrated in *Capnocytophaga* species [32]. Wybo *et al.* [2] demonstrated that MALDI-TOF MS could be used to differentiate between *cfiA*-positive and *cfiA*-negative *Bacteroides* strains, cultured on fastidious anaerobic agar at 35°C in an anaerobic chamber for 24 to 48 h, without use of antibiotic or beta-lactamase inhibitors. This result was confirmed by Fenyvesi *et al.* [33], who characterized 5/60 *cfiA* positive strains using this method. In our study, we could not discriminate between cefotaxime susceptible (*cfxA*-positive) and resistant (*cfxA*-negative) strains using the MALDI-TOF MS method and MALDI Biotyper 2.0 software, as recommended by Wybo *et al.* [2].

The limits of this technique for beta-lactamase detection have already been highlighted. Trevino *et al.* [11] used MALDI-TOF MS for rapid characterization of carbapenemase-producing *Enterobacteriaceae* and genetic diversity among carbapenemase-producing strains. Trevino *et al.* [11] also showed that fingerprinting analyses by automated repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) (DiversiLab system) showed higher discriminatory power than MALDI-TOF MS for clonal strain typing. With this last method, distinct mass spectra were obtained only within a mass range of 2,000 to 20,000 Da using whole cell MALDI-TOF MS [34], and the mass range of the CfxA beta-lactamase was estimated to 35,275 Da and CfiA to be 27,260 Da. By MALDI-TOF MS, only degradation products of the beta-lactam cefotaxime might have been detected, but no difference in profiles was observed when *Capnocytophaga* strains were cultured with and without antibiotic. Moreover, the expression of CfxA enzyme has been described as variable with different MICs reported. Wybo *et al.* [2] suggested that the *cfiA* gene was not always activated, and Soki *et al.* [35] hypothesized that the *cfiA* gene was activated by its own promotor. The differentiation of *cfiA*-negative and *cfiA*-positive *Bacteroides fragilis* [2] was probably due to a selection bias in identifying strains, because the *cfiA* gene, encoding the unique carbapenemase found in *Bacteroides,* was restricted to division II *Bacteroides fragilis* strains [36].

5. Conclusion

In conclusion, MALDI-TOF MS can be used for rapid and low cost oral human *Capnocytophaga* genus identification. But the limits of this technique was the absence of some species (*C. leadbetteri*, *C.* AHN) in the Biotyper-Bruker® database used in this study, and its inability to completely discriminate between *C. gingivalis* and *C. granulosa*. This method was not also suitable for quickly detecting beta-lactamase producing *Capnocytophaga* strains.

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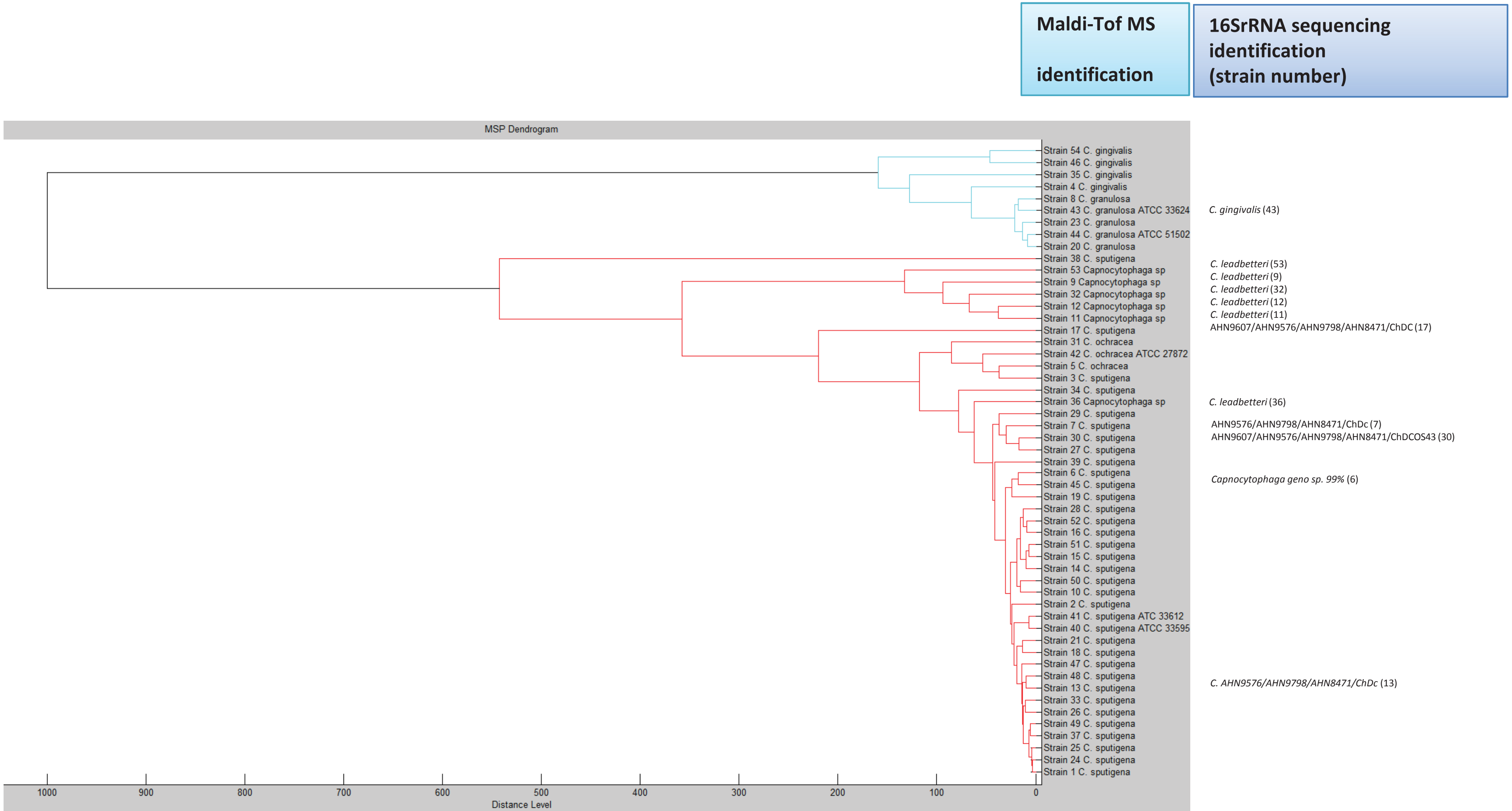
FIGURE LEGEND

Figure 1 Score-oriented dendrogram of matrix-assisted laser desorption ionization time-of-flight mass spectrometry profiles generated by the default setting in MALDI Biotyper software version 2.0. for 53 *Capnocytophaga* strains cultured on blood agar for 48 h under anaerobic conditions. Reference strains were *C. sputigena* ATCC 33595 (strain 40), *C. sputigena* ATCC 33612 (strain 41), *C. ochracea* ATCC 27872 (strain 42), *C. gingivalis* ATCC 33624 (strain 43), and *C. granulosa* ATCC 51502 (strain 44). The misidentified (compared with the 16S rRNA gene sequencing identification technique) species were: *C. granulosa/C. gingivalis* (n=1), *C. spp./C. leadbetteri* (n=6), *C. sputigena/C.* AHN (n=4), and *C. sputigena/C sp.* (n=1). Two distinct clades appeared on dendrogram using MALDI-TOF corresponding to: clade at the top: clade for *C. gingivalis/granulosa,* and clade down: clade for other *Capnocytophaga* spp.

Table 1 The number of isolates identified at the indicated level by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) compared with 16S rRNA gene sequencing results for 53 *Capnocytophaga* strains.

	Identification by 16S rRNA gene sequencing	**No. of isolates identified at the indicated level by MALDI-TOF MS using the Bruker® Reference Library 3.2.0 database**		
	Total No. of isolates	Species	Genus	Absence of identification
C. sputigena	31	31	0	0
C. ochracea	3	3	0	0
C. gingivalis	3	2	1	0
C. granulosa	5	4	1	0
C. leadbetteri	6	0	6	0
AHN9576/AHN9798/AHN8471/ChDc	4	0	4	0
Capnocytophaga sp.	1	0	1	0
Total	53	40	13	0


Maldi-Tof MS identification
16SrRNA sequencing identification (strain number)
MSP Dendrogram
Strain 54 C. gingivalis
Strain 46 C. gingivalis
Strain 35 C. gingivalis
Strain 4 C. gingivalis
Strain 8 C. granulosa
Strain 43 C. granulosa ATCC 33624
Strain 23 C. granulosa
Strain 44 C. granulosa ATCC 51502
Strain 20 C. granulosa
Strain 38 C. sputigena
Strain 53 Capnocytophaga sp
Strain 9 Capnocytophaga sp
Strain 32 Capnocytophaga sp
Strain 12 Capnocytophaga sp
Strain 11 Capnocytophaga sp
Strain 17 C. sputigena
Strain 31 C. ochracea
Strain 42 C. ochracea ATCC 27872
Strain 5 C. ochracea
Strain 3 C. sputigena
Strain 34 C. sputigena
Strain 36 Capnocytophaga sp
Strain 29 C. sputigena
Strain 7 C. sputigena
Strain 30 C. sputigena
Strain 27 C. sputigena
Strain 39 C. sputigena
Strain 6 C. sputigena
Strain 45 C. sputigena
Strain 19 C. sputigena
Strain 28 C. sputigena
Strain 52 C. sputigena
Strain 16 C. sputigena
Strain 51 C. sputigena
Strain 15 C. sputigena
Strain 14 C. sputigena
Strain 50 C. sputigena
Strain 10 C. sputigena
Strain 2 C. sputigena
Strain 41 C. sputigena ATC 33612
Strain 40 C. sputigena ATCC 33595
Strain 21 C. sputigena
Strain 18 C. sputigena
Strain 47 C. sputigena
Strain 48 C. sputigena
Strain 13 C. sputigena
Strain 33 C. sputigena
Strain 26 C. sputigena
Strain 49 C. sputigena
Strain 37 C. sputigena
Strain 25 C. sputigena
Strain 24 C. sputigena
Strain 1 C. sputigena
1000
900
800
700
600
500
400
300
200
100
0
Distance Level
C. gingivalis (43)
C. leadbetteri (53)
C. leadbetteri (9)
C. leadbetteri (32)
C. leadbetteri (12)
C. leadbetteri (11)
AHN9607/AHN9576/AHN9798/AHN8471/ChDC (17)
C. leadbetteri (36)
AHN9576/AHN9798/AHN8471/ChDc (7)
AHN9607/AHN9576/AHN9798/AHN8471/ChDCOS43 (30)
Capnocytophaga geno sp. 99% (6)
C. AHN9576/AHN9798/AHN8471/ChDc (13)