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Species identification of clinical isolates of *Bacteroides* by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry

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Abstract

Bacteroides fragilis and related species are important human pathogens involved in mixed infections of different origins. The *B. fragilis* group isolates are phenotypically very similar, grow more slowly than aerobic bacteria and, accordingly, are frequently misidentifed with classical or automated phenotypical identification methods. Recent taxonomic changes and new species accepted as members of the *Bacteroides* genus are not included in the different databases of commercially available identification kits. The use of matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was therefore evaluated for the species identification of 277 clinical isolates of the *Bacteroides* genus. Species identification was carried out with MALDI Bruker Daltonik Biotyper software (Bruker Daltonik GmbH, Bremen, Germany) by comparing the mass spectrum of each strain with the mass spectra of the 3260 reference strains currently available. The results of conventional phenotypical identification of the isolates were used as a reference. 16S rRNA gene sequencing was performed for a selection of the strains that gave discrepant results and for all those inconclusively identified by MALDI-TOF MS; 270 isolates (97.5%) were unequivocally identified [log(score) ≥2.0] by comparison with the reference strains present in the MALDI Biotyper database. Of the 23 isolates for which the MALDI-TOF MS species identification differed from the conventional phenotypical identification, 11 were sequenced. The sequencing data confirmed the MALDI-TOF MS result in ten cases and, for the remaining isolate, the sequencing data did not lead to the determination of the species, but only to that of the genus (*Bacteroides* sp.). The discriminating power and identification accuracy of MALDI-TOF MS proved to be superior to that of biochemical testing for *Bacteroides thetaiotaomicron*, *Bacteroides ovatus* and *Bacteroides uniformis*.

Keywords: Bacteroides, clinical isolates, identification, MALDI-TOF MS, sequencing
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Introduction

Bacteroides fragilis and related species are important components of the commensal flora in the lower intestinal tract of mammals, but are also important and frequently isolated as opportunistic anaerobic pathogens causing severe infections, including intra-abdominal, pelvic, lung and brain abscesses, peritonitis and sepsis [1]. Bacteraemia due to *B. fragilis* group isolates contributes appreciably to morbidity and mortality [1–3]. Infections caused by *Bacteroides* strains, alone or in mixed cultures, are especially significant because they exhibit resistance mechanisms to almost all groups of antibiotics and the level of resistance to some antibiotics may be extremely high [4]. Resistance to the most potent antibiotics, such as carbapenems, *beta*-lactam and *beta*-lactamase inhibitor combinations and fourth-generation fluoroquinolones, has slowly increased in Europe over the past 15 years [5,6]. Correct identification at the species level is necessary because the resistance to different anti-anaerobic drugs may differ according to the species [7]. Presumptive identification may be performed with antibiotic discs and spot tests (Wadsworth method) [8]; for example, at the genus level (*Bacteroides*

ides fragilis group). Definitive species-level identification requires a battery of biochemical tests, low molecular weight fatty acid profiling and, occasionally, molecular genetic methods, such as 16S rRNA gene sequencing. Routine laboratories can identify *Bacteroides* isolates using different substrates in a conventional manner or via commercially available identification kits that require a shorter incubation period in an aerobic environment (i.e. detection of preformed enzymes) or a longer incubation in an anaerobic environment (i.e. detection of inducible enzymes). However, these phenotypic methods do not always clearly distinguish closely related species and definitive identification requires a lengthy incubation after isolation of these bacteria from clinical specimens.

The taxonomy of *Bacteroides* has undergone significant changes in recent years [9]. The presumed genus *Bacteroides* was found to contain species from several genera. Most of the species previously included among the *Bacteroides* were placed into two new genera, *Porphyromonas* and *Prevotella* [9], but other genera have subsequently been described for further species that do not belong in these three major groups (e.g. *Anaerorhabdus*, *Dichelobacter*, *Dialister*, *Fibrobacter*, *Megamonas*, *Mitsuokella*, *Rikenella*, *Sebaldella*, *Tannerella*, *Tissierella* and *Alistipes*) [8]. The new *Bacteroides* spp. *Bacteroides nordii* and *Bacteroides salyersae*, found in clinical specimens [10], can be misidentified as *Bacteroides stercoris* and *Bacteroides uniformis* with routine biochemical tests. The recently described *Bacteroides goldsteinii* was phylogenetically close to *Bacteroides distasonis* and *Bacteroides merdae* as classified by 16S rRNA gene sequencing [11]. However, these latter three species were recently reclassified as *Parabacteroides*, *Parabacteroides distasonis*, *Parabacteroides merdae* and *Parabacteroides goldsteinii*; they are phenotypically similar to members of the *Bacteroides* genus (*senso stricto*) but are distinct phylogenetically [12].

Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been shown to constitute a useful and simple method for the rapid identification of microorganisms associated with infectious diseases and also for discriminating between different subtypes of pathogens [13–15]. The present study aimed to set up a database for the most frequently isolated anaerobic *Bacteroides* spp. and to test the applicability of MALDI-TOF MS profiling for the identification of *Bacteroides* spp.

Materials and Methods

Isolates

The 277 clinical isolates analysed in the present study were collected during a Europe-wide antibiotic resistance surveillance of *Bacteroides* spp, the results of which will be published soon.

Their identification was performed in the collecting laboratories with different phenotypical methods, such as classical biochemical tests, rapid ID 32A (ATB) and API20 ANA (bioMérieux SA, Marcy-l'Etoile, France). In some cases, only genus-level identification was available. Because identification at the species level may be important for the evaluation of differences in antibiotic resistance of *Bacteroides* strains, the central laboratory (Szeged) re-identified all strains received for the study. Part of the present work involved parallel classical biochemical tests [8] and analysis with MALDI Biotyper software. All strains were cultured on Columbia agar base (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) cattle blood, haemin (1 mg/L) and vitamin K_1 (5 mg/L) for 24–48 h at 37°C in an anaerobic chamber (Bactron from Sheldon Manufacturing Inc., Cornelius, OR, USA). Cryobank vials (Mast Diagnostics, Reinfeld, Germany) were used for long-term strain storage. ATCC 25285 *B. fragilis*, DSM2151 *B. fragilis* and ATCC 29742 *Bacteroides thetaiotaomicron* were used as control strains.

Sample preparation for MALDI-TOF MS measurement

One colony of each bacterial strain that had been subcultured for 24 h was transferred into an Eppendorf vial and carefully suspended in 300 μL of bidistilled water. Ethanol (900 μL) was added to the suspension and mixed well. At this stage, the stabilized samples were sent from the Szeged reference laboratory to the Bruker laboratory in Leipzig. There, the samples were centrifuged (13 000 ***g*** for 2 min), the supernatants were removed and the pellets were dried at room temperature. Each bacterial pellet was re-suspended in 50 μL of 70% aqueous formic acid and 50 μL of acetonitrile. After centrifugation (13 000 ***g*** for 2 min), 1 μL of the supernatant was spotted onto a sample position on a ground-steel MALDI target plate and dried at room temperature. Subsequently, a further 2-μL aliquot of MALDI matrix (a saturated solution of HCCA (α-cyano-4-hydroxycinnamic acid) in 50% acetonitrile/2.5% trifluoro-acetic acid) was added to the spot, which was again dried. The MALDI target plate was subsequently introduced into the MALDI-TOF mass spectrometer for automated measurement and data interpretation.

Instrumentation and data acquisition

Samples were analysed with a microflex LT or ultraflex TOF/TOF MALDI-TOF instrument (Bruker Daltonik GmbH, Bremen, Germany). Spectra were acquired in the linear, positive ion mode with a laser frequency of 20 Hz (microflex) or 200 Hz (ultraflex). Parameter settings for microflex

LT were: IS1 20 kV, IS2 18.5 kV, lens 8.5 kV, PIE 250 ns, no gating; and for ultraflex: IS1 20 kV, IS2 18.75 kV, lens 5.75 kV, PIE 350 ns, gating 1250 Da.

Data processing with MALDI Biotyper software

Recorded mass spectra were processed with the MALDI Biotyper 2.0 software package, using the standard settings. Spectra were smoothed and baseline corrected, and peak masses were determined and transferred to a list of up to 70 peak masses. For the construction of new database entries, several (up to 20) mass spectra were processed with the software functionality and standard settings. The spectral peak lists for a particular strain were transferred into a main spectrum (MSP) containing information on average peak masses, average peak intensities and peak frequencies.

Identification of clinical isolates

The peak lists derived from the bacterial MALDI-TOF profile mass spectra were compared with each entry of the MALDI Biotyper database, currently containing 3260 references, using the standard parameters of the pattern-matching algorithm. The MALDI Biotyper output is a log(score) in the range 0–3.0, computed by comparison of the peak list for an unknown isolate with the reference MSP in the database. A log(score) ≥1.7 is indicative of a close relationship (i.e. at the genus level) and a log(score) of ≥2.0 is the set threshold for a match at the species level.

16S rRNA gene sequencing for discrepant isolates

For amplification of the 16S rRNA gene fragment, the unique universal primers E8F and E533R [16] were used. Sequencing was achieved with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 and the ABI Prism 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The sequence data for the 16S rRNA genes were compared with those of the GenBank database using BLAST software (http://www.ncbi.nlm.nih.gov/blast/cgi). A homology level >98% was considered adequate for species identification.

Results

Two hundred and seventy-seven clinical *Bacteroides* isolates were selected from a European strain collection to be tested by a MALDI-TOF MS method for species identification, in comparison with a classical phenotypical species determination. Two hundred and seventy of the 277 isolates were unequivocally identified [log(score) ≥2.0] by comparison with the reference strains present in the MALDI Biotyper database (Table 1). For seven isolates, identification was inconclusive because of the lack of an appropriate reference in the database [log(score) <1.7]. All seven of these isolates were therefore selected for 16S rRNA gene sequencing. The sequencing data confirmed the biochemical identification of *P.* (*B.*) *distasonis* in three cases (Table 2). One of these strains was subsequently prepared in two parallels for MALDI-TOF MS and 20 spectra were acquired, transferred into the main spectrum and added to the database of the MALDI Biotyper database. The three other isolates, which could not be identified according to their spectra, and which belonged to P. (*B*) *distasonis* and *B. thetaiotaomicron* according to the conventional biochemical tests, gave a log(score) >2.5 for these species (Table 2). The other three strains proved to be *Bacteroides eggerthii*, *P.* (*B.*) *goldsteinii* and *B. intestinalis*. The spectra of two type strains of *B. fragilis* (*B. fragilis* ATCC 25285 grown in Szeged and *B. fragilis* DSM 2151 grown in Leipzig) were compared. Comparatively, identification led to a highly significant log(score) of 2.5, demonstrating the reproducibility of the method even if the culture conditions were not identical.

One potential advantage of the MALDI method is the rapid and simple differentiation of subtypes of different species. For the *B. fragilis* strains, several MS peaks may be used for subtyping (Fig. 1). The peak masses that may discriminate different *B. fragilis* isolates are 8776 and 8907 Da for the first group, 8788 and 8919 Da for the second group, and 8808 and 8941 Da for the third group of strains. The differentiating features were reproducible in multiple measurements. Newly accepted species, for example *B. salyersae* and *B. nordii*,

TABLE 1. Species determination of 277 clinical isolates of *Bacteroides* by MALDI-TOF MS

Species	Number of isolates		
	With an identification score ≥2.0 by MALDI-TOF MS	With discrepant species identification by biochemical tests	Selected for 16S rRNA gene[a] sequencing
B. fragilis	179	5	2
B. thetaiotaomicron	43	5	4
B. ovatus	15	6	1
B. vulgatus	20	1	1
B. uniformis	5	3	2
B. eggerthii	1	0	
B. nordii	4	3	1
B. salyersiae	1	0	
B. massiliensis	2	0	
Inconclusive identification by MALDI-TOF MS	7		7

[a]Only strains with a MALDI-TOF MS score of 2.0–2.5 and those inconclusively identified were selected for sequencing.

TABLE 2. **Results of 16S rRNA sequencing of selected strains which gave discrepant results by MALDI-TOF MS and biochemical testing and inconclusive identification with the MALDI Biotyper**

Sample number	Species determined by		
	Biochemical tests	MALDI-TOF MS	16S rRNA gene sequencing
1	*Bacteroides ovatus*	*Bacteroides fragilis*	*Bacteroides fragilis*
2	*Bacteroides uniformis*	*Bacteroides fragilis*	*Bacteroides fragilis*
3	*Bacteroides ovatus*	*Bacteroides thetaiotaomicron*	*Bacteroides thetaiotaomicron*
4	*Bacteroides uniformis*	*Bacteroides thetaiotaomicron*	*Bacteroides thetaiotaomicron*
5	*Bacteroides uniformis*	*Bacteroides thetaiotaomicron*	*Bacteroides thetaiotaomicron*
6	*Bacteroides ovatus*	*Bacteroides thetaiotaomicron*	*Bacteroides thetaiotaomicron*
7	*Bacteroides uniformis*	*Bacteroides ovatus*	*Bacteroides ovatus*
8	*Bacteroides thetaiotaomicron*	*Bacteroides vulgatus*	*Bacteroides* sp. (new?)
9	*Bacteroides ovatus*	*Bacteroides uniformis*	*Bacteroides uniformis*
10	*Bacteroides ovatus*	*Bacteroides uniformis*	*Bacteroides uniformis*
11	*Bacteroides ovatus*	*Bacteroides nordii*	*Bacteroides nordii*
12	*Bacteroides thetaiotaomicron*	II (log(score) >2.5)[a]	*Parabacteroides* (B.) *distasonis*
13	*Parabacteroides* (B.) *distasonis*	II	*Parabacteroides* (B.) *distasonis*
14	*Parabacteroides* (B.) *distasonis*	II (log(score) >2.5)	*Parabacteroides* (B.) *distasonis*
15	*Parabacteroides* (B.) *distasonis*	II (log(score) >2.5)	*Parabacteroides* (B.) *distasonis*
16	*Bacteroides thetaiotaomicron*	II	*Bacteroides eggerthii*
17	*Bacteroides merdae*	II	*Bacteroides goldsteinii*
18	*Bacteroides* sp.	II	*Bacteroides intestinalis*

II, inconclusive identification.
[a]Achieved log(score) by MALDI-TOF MS analysis after adding to the data base the MS of the sequenced *P.* (*B.*) *distasonis* (13).

could be identified by MALDI-TOF MS (Table 1). Clear differentiation was achieved for the different human pathogenic *Bacteroides* isolates (Fig. 2).

Of the 270 isolates unequivocally identified by MALDI-TOF MS, 23 gave a discrepant result using classical biochemical identification (Table 1). Eleven of the 23 isolates that had a log(score) of 2.0–2.5 by MALDI-TOF MS were selected for 16S rRNA gene sequencing (Table 2). The sequencing data confirmed the MALDI-TOF MS finding for ten isolates but, in one case, which was identified by MALDI-TOF MS as *B. vulgatus* with a log(score) 2.017 (sample number 8), the sequencing data did not lead to a species determination, but ended at the genus level (a new *Bacteroides* sp.) showing a relationship with *B. uniformis*, *B. eggerthii* and *B. thetaiotaomicron*. The discriminatory power and identification accuracy of MALDI-TOF MS was superior to biochemical testing in the cases of *B. thetaiotaomicron*, *B. ovatus* and *B. uniformis*.

Discussion

The important role of anaerobic bacteria in a variety of human infections has been accepted ever since the 1960s [1]. *B. fragilis* group strains are the most common agents in infections caused by anaerobic Gram-negative bacilli

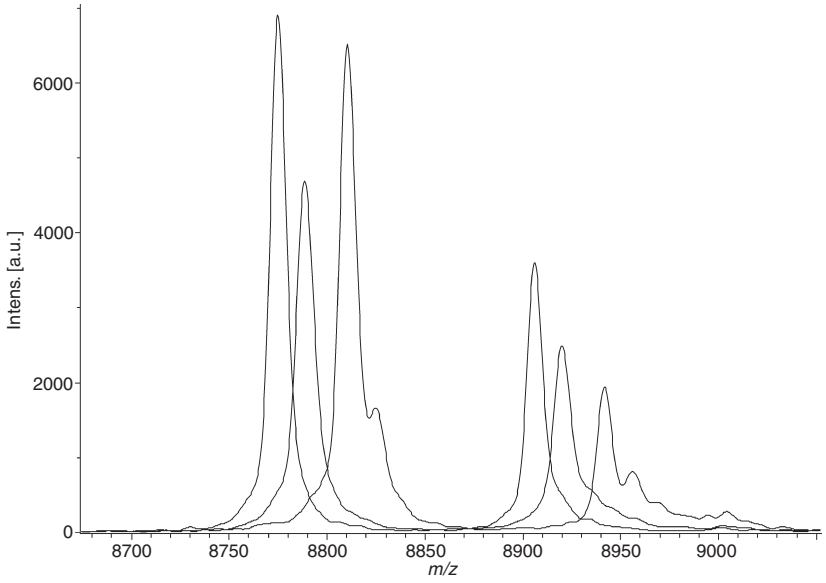

FIG. 1. Example of shifts distinguishing the *Bacteroides fragilis* strains in three subgroups.

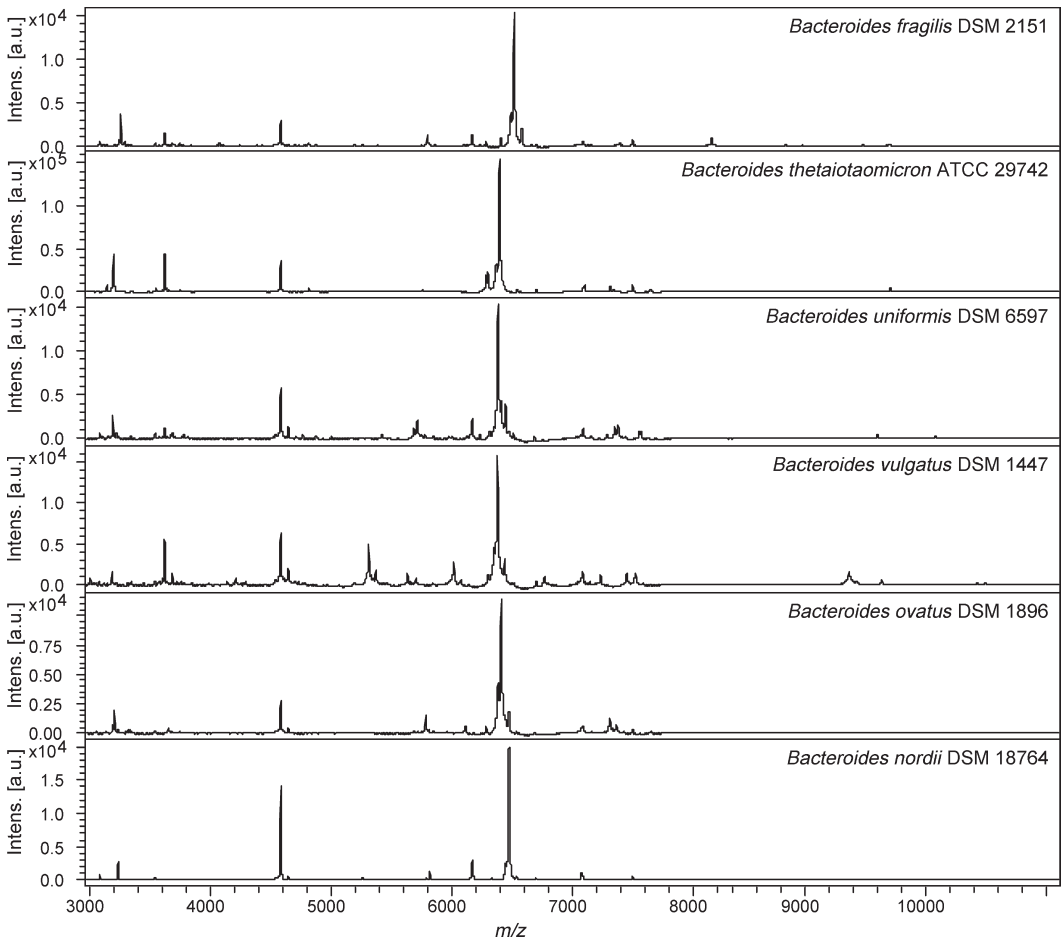

FIG. 2. Mass spectra of type strains of six different *Bacteroides* species.

[2,17–20]. The different species in the *B. fragilis* group are phenotypically very similar. The automated methods used routinely in laboratories for the identification of *Bacteroides* strains are not always sufficiently discriminating, and may be highly influenced by the level of anaerobiosis during incubation; in addition, their databases do not contain all accepted, newly-recognized species. Compared with conventional methods, rapid identification with preformed enzyme kits, without additional tests, gives a correct identification in only 78–79% of the *B. fragilis* group strains [21,22]. Direct sequencing of PCR-amplified bacterial 16S rDNA is a relatively new molecular method. It is faster than the conventional phenotypical method but is seldom used by clinical microbiology laboratories for species identification on a routine basis.

To overcome the problems of inconclusive species identification with phenotypical methods and the lack of sequencing facilities in many routine laboratories, MALDI-TOF MS was evaluated for identification of *Bacteroides* spp. This technique initially unequivocally identified 97.5% of the strains tested (270 of the 277). The rate increased to 98.6% when the mass spectrum of a clinical isolate proven by sequencing data to be *P.* (*B.*) *distasonis* was added to the MALDI Biotyper database. This database currently does not contain the mass spectra of all possible human pathogenic anaerobic bacteria; species not represented in the database include some of the *B. fragilis* group strains, or species earlier classified as *Bacteroides*, but later moved to other genera. However, the possibility of extending the database with the mass spectra of further reference strains, or sequenced clinical isolates, will improve the identification capacity of the method still further. In the present study, 8.5% of the strains (23/270), with a high MALDI log(score) for species identification gave a different species determination compared to conventional biochemical testing. The majority (n = 14) of these strains were identified biochemically as *B. ovatus*, *B. thetaiotaomicron* and *B. uniformis*. Using 16S rRNA gene sequencing for species identification of *B. thetaiotaomicron*, Teng *et al.* [23] found that some isolates of this species, previously identified phenotypically, proved to be *B. ovatus* or *B. distasonis* from the sequencing data. Conversely, *B. uniformis* and *B. caccae* were re-identified as *B. thetaiotaomicron*. In all cases where

sequencing was performed on such species in the present study, the sequencing data revealed that MALDI-TOF MS was superior to biochemical testing for species identification (Table 2). *B. nordii* is a newly accepted species of clinical importance. Altogether, four isolates were identified as *B. nordii* by MALDI-TOF MS. Of these four isolates, only one was correctly identified by the original laboratory using phenotypical methods, two were identified only as *Bacteroides* sp., and one as *B. ovatus*. The sequencing of this latter isolate demonstrates the superiority of MALDI-TOF MS relative to biochemical identification for the species determination of this infrequently isolated *Bacteroides* species.

MALDI-TOF MS has previously been shown to be suitable for the subtyping of different species [14]. The mass spectra of *Listeria* strains allow their identification at the species level and the differentiation of pathogenic *L. monocytogenes* strains at the level of clonal lineages [24]. For *B. fragilis* isolates, the subtyping potential is obvious, but must be explored in detail in future studies (Fig. 1).

A major problem in anaerobic bacteriology is the special incubation environment needed for isolation and, in most cases, for the biochemical identification of anaerobic bacteria, which demands a lengthier procedure than for aerobic pathogens. MALDI-TOF MS using the database of the MALDI Biotyper requires only 10 min from starting the preparation by picking a colony, to species identification.

Processing 96 samples takes less than 3 h [13] and the cost is minimal. The protocol for sample preparation is simple and can be used for other anaerobes such as *Fusobacterium* and *Prevotella* (data not shown). A collection of *Clostridium* strains pathogenic to animals and humans was recently investigated; 64 strains belonging to 31 different species were identified correctly by MALDI-TOF MS with good discriminatory power, even for *Clostridium* species, which are normally difficult to differentiate using traditional methods [15].

In summary, the MALDI-TOF MS method provided accurate and fast species identification for the most frequently isolated human pathogenic anaerobic bacteria (i.e. members of the genus *Bacteroides*), with good discriminatory power for closely related species. Extension of the database to include other anaerobic bacteria of clinical importance will enhance the value of this methodology in routine clinical microbiology laboratories for species identification of anaerobic bacteria.

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Transparency Declaration

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