

Mass Spectrometric Identification of *Propionibacterium* Isolates Requires Database Enrichment

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ABSTRACT

Propionibacterium species are mostly environmental bacteria, some being commensal of mammals including humans, and sometimes pathogenic. These bacteria are poorly identified using routine laboratory methods. Recently, Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) has emerged as a rapid and efficient method to identify bacterial species. We evaluated the use of MALDI-TOF-MS for identification of all validated *Propionibacterium* species. Only four of the 15 tested reference strains (26.7%) were correctly identified at the species level, and *P. acnes*, the most common human pathogenic species was not identified. When applying MALDI-TOF-MS to 48 *P. acnes* strains, only 18.7% were correctly identified, suggesting an intraspecific variability of proteic profiles among *Propionibacterium* strains. However, by enriching the Bruker database with spectra from five of these strains and re-testing the other 43 strains against this new database, 93.0% were correctly identified. Our study demonstrates that MALDI-TOF-MS may be used for the identification of *Propionibacterium* isolates but requires a database enrichment in spectra from additional isolates.

Keywords: *Propionibacterium*; *Propionibacterium acnes*; MALDI-TOF; Mass Spectrometry

1. Introduction

Propionibacterium sp. are non spore-forming, Gram-positive, anaerobic or microaerophilic bacteria, first described in 1909. To date, fourteen species and 2 subspecies are validated

(<http://www.bacterio.cict.fr/p/propionibacterium.html>).

On the basis of their natural habitat, *i.e.*, environment or human flora, species are divided into two main groups. Environmental species include *Propionibacterium freundenreichii*, *P. acidipropionici*, *P. jensenii* and *P. thoeni* that are associated to dairy products, and *P. cyclohexanicum*, *P. microaerophilum* and *P. australiense* that were isolated from spoiled orange juice, olive mill wastewater and bovine lesions, respectively. The remaining seven species are part of the human commensal flora. As such, *Propionibacterium* sp. were described as bacteria of no or low pathogenicity for many years. Currently, they are often considered as contaminant bacteria of blood and body fluid cultures but are able to cause serious human infections. The most common pathogenic species for humans is *P. acnes*, which belongs to the normal flora of the skin, oral cavity, large intestine, con-

junctiva, and external ear [1]. It is primarily recognized for its role in acne vulgaris [2]. However, *P. acnes* was also involved in other human infections, mainly as an opportunistic pathogen in immunocompromised patients and in post-operative infections, notably in presence of prosthetic material [3]. In particular, *P. acnes* was found to be the causative agent of spondylodiscitis, central nervous system infections, endocarditis, endophthalmitis, bone and joint infections [4] and SAPHO syndrome (synovitis, acne, pustulosis, hyperostosis, and osteitis) [5]. *P. avidum* was only described in splenic abscess, perianal abscess, sacroileitis, osteomyelitis, and more recently in breast abscess [6]. *P. granulosum* was demonstrated to cause bacteremia, endocarditis and endophthalmitis [7,8]. To date, *P. propionicum* was isolated from lacrymal apparatus infections [9], and *P. acidifaciens* from the human oral cavity [10]. In addition, two former *Propionibacterium* species, *P. innocuum* and *P. lymphophilum*, isolated from the human skin [11] or associated with certain types of lymphadenopathies [12], were reclassified into the *Propioniferax* and *Propionimicrobium* genera, respectively [13,14].

Propionibacterium sp. grow anaerobically, on 5% sheep blood agar and produce β -haemolytic colonies after a minimum of 48 hours of incubation. Phenotypic

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identification of these bacteria is time-consuming and unreliable at the species level [15]. In contrast, molecular methods provide a reliable but time-consuming and expensive species identification [16] that may not be adapted to routine bacterial identification in most laboratories.

In recent years, matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has been increasingly used for the identification of bacterial isolates on the basis of their peptidic spectra [17], and more recently for the direct identification of bacteria in clinical specimens [18]. MALDI-TOF MS was applied with success to identify *Arthrobacter* species [19], *Bacteroides* sp. [20], *Bartonella* [21], *Burkholderia cepacia* [22], *Campylobacter* species [23], *Clostridium* species [24], *Coxiella burnetii* [25], *Erwinia* species [26], *Escherichia coli* [27], *Francisella tularensis* [28], *Helicobacter pylori* [29], *Legionella* species [30], *Listeria* species [31], *Neisseria* species [32], *Salmonella* species [33], staphylococci [34], *Vibrio* species [35], *viridans* streptococci [36], *Yersinia enterocolitica* [27], nonfermenting bacteria [37,38], and oral anaerobic bacteria [39]. Moreover, recent studies emphasized that MALDI-TOF MS identification was an efficient and cost-effective method for rapid and routine identification of bacterial isolates [17,40-42]. To date, the Bruker database contains spectra from 3769 bacteria including 35 spectra for *Propionibacterium* spp.

In the present study, in order to determine whether

MALDI-TOF MS could be used as a routine tool for identifying clinical isolates of *Propionibacterium* sp., we first determined the peptidic spectrum of a representative strain of all validated species that have been cultured. Then, we attempted mass spectrometric identification of a collection of *P. acnes* clinical isolates, the most frequently pathogenic *Propionibacterium* species.

2. Materials and Methods

2.1. *Propionibacterium* Strains

Type strains from each of the 12 validated *Propionibacterium* species as well as *P. innocuum* and *P. lymphophilum*, were included in our study (**Table 1**). For *P. freundenreichii*, we included a type strain from each of the two validated subspecies, i.e., subsp. *freundenreichii* and *shermanii*. All strains were grown on Columbia blood agar containing 5% whole sheep blood (BioMerieux, Marcy l'Étoile, France) at 37°C in anaerobic atmosphere.

In addition, in order to estimate the validity of MALDI-TOF MS for *P. acnes* identification, the main pathogenic *Propionibacterium* species, 48 additional *P. acnes* isolates from clinical samples were tested by MS. *P. acnes* isolates were cultivated on Columbia blood agar at 37°C in anaerobic atmosphere as described above from blood (12), cardiac valve biopsies (2), surgically-removed pace-makers (3), a lung biopsy (1), lymph node biopsies (3), bone biopsies (3), cerebrospinal fluid (1), skin biopsy samples (23). The identification of these strains was

Table 1. *Propionibacterium*, *Propionifera* and *Propionimicrobium* reference strains used in this study.

Species	Reference in culture collections*	Best MALDI-TOF log scores against the Bruker database	
		Score	Species
<i>P. acidificiens</i>	DSM 21887	1.3	<i>Lactobacillus curvatus</i>
<i>P. acidipropionici</i>	CIP 103025	2.1	<i>P. acidipropionici</i>
<i>P. acnes</i>	CIP 53117	1.6	<i>P. acnes</i>
<i>P. australiense</i>	DSM 15818	1.7	<i>P. australiense</i>
<i>P. avidum</i>	CIP 103261	2.0	<i>P. avidum</i>
<i>P. cyclohexanicum</i>	CIP 105414	1.8	<i>P. cyclohexanicum</i>
<i>P. freundenreichii</i> subsp. <i>freundenreichii</i>	CIP 103026	1.2	<i>P. freundenreichii</i> subsp. <i>freundenreichii</i>
<i>P. freundenreichii</i> subsp. <i>shermanii</i>	CIP 103027	1.8	<i>P. freundenreichii</i> subsp. <i>shermanii</i>
<i>P. granulosum</i>	CIP 103262	1.9	<i>P. granulosum</i>
<i>P. innocuum</i>	DSM 8251	1.4	<i>Staphylococcus saprophyticus</i>
<i>P. jensenii</i>	CIP 103028	1.8	<i>P. jensenii</i>
<i>P. lymphophilum</i>	CIP 103263	1.9	<i>P. lymphophilum</i>
<i>P. microaerophilum</i>	DSM 13435	1.3	<i>P. microaerophilum</i>
<i>P. propionicum</i>	CIP 101941	1.3	<i>Thaera chlorobenzica</i>
<i>P. thoenii</i>	CIP 103029	1.8	<i>P. thoenii</i>

*CIP = Collection de l'Institut Pasteur; DSM = Deutsche Sammlung von Mikroorganismen.

confirmed by PCR amplification and sequencing of the 16S RNA, as previously described [43]. MS identification was performed as a blind test after anonymization of the isolates.

2.2. MALDI-TOF-MS

For each strain, four isolated colonies were harvested in 20 μ L of sterile water. One μ L of each mixture was deposited on a target plate (Bruker Daltonics, Bremen, Germany) in a separate well, and allowed to dry at room temperature. Two μ L of matrix solution made of alpha-cyano-4-hydroxycinnamic acid (Sigma) saturated with 2.5% trifluoroacetic acid and 50% acetonitrile, was then added and allowed to co-crystallize with the sample. Samples were processed using the Autoflex II MALDI-TOF-MS spectrometer (Bruker Daltonics) with the Flex Control software (Bruker Daltonics). Positive ions were extracted with an accelerating voltage of 20 kV in linear mode. Each spectrum was the sum of the ions obtained from 200 laser shots performed in five different regions of the same well. The spectra have been analyzed in an m/z range of 2000 to 20,000. The analysis was performed with the Flex Analysis and BioTyper softwares and calibrated with protein calibration standard I (Bruker Daltonics). The data obtained with the four replicates were added to minimize random effect. The presence and absence of peaks were considered as fingerprints for a particular isolate. A maximum of 100 peaks with a signal-to-noise (S/N) ratio of 3 were selected in the range of 3000 - 15,000 Da. Afterwards the main spectra were generated as a reference using all spectra given for a single microorganism. In general, 75 peaks were picked automatically, which occurred in at least 25% of the spectra and with a mass deviation of 200 ppm. A reference spectrum was determined for each species. The profiles were analyzed and compared using the MaldiBioTyper 3.0 software (Bruker Daltonik GmbH). The identification process starts with preprocessing of unknown

spectra and generation of peak lists. Unknown spectra were identified by the MALDI Biotyper software by comparison with reference spectra. Peaks of unknown spectra were compared and aligned with peaks of reference spectra. In the first step, unknown spectra are calibrated to the reference spectra to reach the highest grade of homology. After calibration each matching peak of the unknown spectrum receives a dedicated point value. The peak of the unknown spectrum will receive the same score value like the corresponding peak of the reference spectra. From the cumulative score value of the unknown spectrum and the maximum score of reference spectra a final score value for the unknown spectrum will be calculated. For the blind test, the 48 tested *P. acnes* strains were compared to the URMS (Unité des Rickettsies Mass Spectrometry) database that contained the complete Bruker database added with reference spectra from the 14 tested validated species (**Table 1**). Subsequently, in order to evaluate whether an enrichment of the database in *P. acnes* spectra would improve MALDI-TOF identification of isolates from this species, we added to the URMS database reference spectra from five of the 48 strains (with best scores against *P. acnes* of 1.5, 1.6, 1.8, 2.0 and 2.1, respectively), and re-tested the remaining 43 isolates against the new database (**Table 2**). Identification at the species level was obtained when a tested strain exhibited highest log scores ≥ 1.9 against the correct species for at least 2 replicates, and genus identification was confirmed when a tested strain exhibited highest log scores ≥ 1.7 but <1.9 against any *Propionibacterium* species. The complete set of reference spectra obtained in the present study is available in the URMS database online (<http://www.ifr48.com>).

3. Results

3.1. Type Strains

MALDI-TOF-MS spectra were obtained from all 15 strains from the 12 validated *Propionibacterium* species

Table 2. Forty-eight *Propionibacterium acnes* strains from clinical specimens used in this study.

Strains	Best MALDI-TOF log scores against the Bruker database		Best MALDI-TOF log scores against our database	
	Score	Species	Score	Species
3,303,341	1.8	<i>P. acnes</i>	2.2	<i>P. acnes</i>
4,400,128	2.1	<i>P. acnes</i>	ND*	
4,400,216	1.5	<i>Agromyces brachium</i>	2.1	<i>P. acnes</i>
4,400,228	1.8	<i>P. acnes</i>	1.9	<i>P. acnes</i>
4,400,388	1.4	<i>P. acnes</i>	1.8	<i>P. acnes</i>
4,400,738	1.5	<i>P. acnes</i>	2.5	<i>P. acnes</i>
4,401,670	2.0	<i>P. acnes</i>	2.3	<i>P. acnes</i>
4,401,894	1.5	<i>P. acnes</i>	2.4	<i>P. acnes</i>

Continued

4,402,010	1.9	<i>P. acnes</i>	2.5	<i>P. acnes</i>
4,402,068	1.6	<i>P. acnes</i>	1.7	<i>P. acnes</i>
4,402,728	1.5	<i>P. acnes</i>	2.4	<i>P. acnes</i>
4,402,815	1.8	<i>P. acnes</i>	1.9	<i>P. acnes</i>
4,403,079	1.5	<i>P. acnes</i>	2.4	<i>P. acnes</i>
4,403,202	1.8	<i>P. acnes</i>	2.3	<i>P. acnes</i>
5,400,539	1.6	<i>P. acnes</i>	2.2	<i>P. acnes</i>
5,400,600	1.8	<i>P. acnes</i>	2.5	<i>P. acnes</i>
5,401,116	1.7	<i>P. acnes</i>	2.4	<i>P. acnes</i>
5,401,386	1.6	<i>P. acnes</i>	2.1	<i>P. acnes</i>
5,401,633	1.9	<i>P. acnes</i>	2.3	<i>P. acnes</i>
5,401,785	1.8	<i>P. acnes</i>	2.7	<i>P. acnes</i>
5,402,218	1.6	<i>P. acnes</i>	2.6	<i>P. acnes</i>
5,402,337	1.5	<i>P. acnes</i>	2.6	<i>P. acnes</i>
5,402,846	1.7	<i>P. acnes</i>	2.4	<i>P. acnes</i>
6,210,647	1.9	<i>P. acnes</i>	2.3	<i>P. acnes</i>
6,400,877	1.7	<i>P. acnes</i>	2.2	<i>P. acnes</i>
6,401,319	1.5	<i>P. acnes</i>	2.7	<i>P. acnes</i>
6,401,672	1.6	<i>P. acnes</i>	2.2	<i>P. acnes</i>
6,402,157	1.6	<i>P. acnes</i>	2.5	<i>P. acnes</i>
6,402,313	1.7	<i>P. acnes</i>	2.5	<i>P. acnes</i>
6,402,537	1.9	<i>P. acnes</i>	2.5	<i>P. acnes</i>
7,144,024	1.7	<i>P. acnes</i>	2.5	<i>P. acnes</i>
7,201,011	1.5	<i>P. acnes</i>	2.5	<i>P. acnes</i>
7,201,012	1.6	<i>P. acnes</i>	2.6	<i>P. acnes</i>
7,201,013	1.8	<i>P. acnes</i>	2.8	<i>P. acnes</i>
7,400,385	1.5	<i>P. acnes</i>	2.4	<i>P. acnes</i>
7,400,791	1.6	<i>P. acnes</i>	2.1	<i>P. acnes</i>
7,401,007	1.7	<i>P. acnes</i>	2.5	<i>P. acnes</i>
7,401,696	1.8	<i>P. acnes</i>	2.2	<i>P. acnes</i>
7,402,515	1.6	<i>P. acnes</i>	2.2	<i>P. acnes</i>
7,402,660	1.7	<i>P. acnes</i>	1.9	<i>P. acnes</i>
7,402,869	1.5	<i>P. acnes</i>	2.5	<i>P. acnes</i>
8,230,904	1.6	<i>P. acnes</i>	1.8	<i>P. acnes</i>
8,242,878	1.5	<i>P. acnes</i>	ND*	
8,400,154	1.9	<i>P. acnes</i>	2.6	<i>P. acnes</i>
8,400,396	2.0	<i>P. acnes</i>	2.6	<i>P. acnes</i>
8,400,790	1.6	<i>P. acnes</i>	ND*	
8,402,959	1.8	<i>P. acnes</i>	ND*	
8,403,771	2.0	<i>P. acnes</i>	ND*	

*ND = Not done, because these five strains were used to enrich the database prior to re-testing of the other 43 strains.

and *P. innocuum* and *P. lymphophilum*. All tested species and subspecies exhibited distinct proteic profiles (**Figure 1**). When compared to 3670 bacteria within the Bruker database, only four/15 representative *Propionibacterium* strains (26.7%) were correctly identified at the species level despite the presence in the database of spectra from 12 species (scores ≥ 1.9 for *P. acidipropionici*, *P. avidum*, *P. granulosum* and *P. lymphophilum*) (**Table 1**). Another five strains (33.3%) were correctly identified at the genus level only (scores ≥ 1.7 and < 1.9 for *P. australiense*, *P. cyclohexanicum*, *P. freudenreichii* subsp. *shermanii*, *P. jensenii* and *P. thoenii*) although their highest scores were against the correct species. The remaining six strains (40%) were not identified (scores < 1.7), including *P. acidificiens*, *P. innocuum* and *P. propionicum* for which no spectra were available in the Bruker database, and *P. acnes*, *P. freudenreichii* subsp. *freudenreichii*, and *P. microaerophilum* which exhibited highest scores against their respective species. All obtained spectra were added to the URMS database.

3.2. Clinical Isolates

MALDI-TOF MS spectra were obtained for the 48 blind-tested clinical strains. When compared to the Bruker database, only 9/48 (18.7%) *P. acnes* clinical strains were correctly identified at the species with scores ≥ 1.9 , level, and another 15/48 (31.2%) were correctly identified at the genus level with scores ≥ 1.7 but < 1.9 (**Table 2**). Another *P. acnes* strain was misidentified as *Agromyces brachium* with a score of 1.5. All 23 remaining *P. acnes*

strains (47.9%) exhibited highest scores < 1.7 against *P. acnes* for at least 2/4 spectra.

When tested against the URMS database enriched with spectra from 5 *P. acnes* clinical strains, 40/43 *P. acnes* clinical isolates (93.0%) were correctly identified at the species level with scores ≥ 1.9 (**Table 2**). The remaining 3 strains exhibited scores ≥ 1.7 against *P. acnes* as best hit, but < 1.9 .

4. Discussion

Long considered as culture contaminants, *Propionibacterium* species have recently been recognized as human pathogens in a variety of diseases. In particular, the incidence of opportunistic and postoperative bone infections caused by *P. acnes* has increased [44]. However, the routine identification of these bacteria is time-consuming and unreliable using phenotypic methods, or expensive using molecular methods, which stimulated the development of efficient identification tools that would enable rapid and adequate management of patients. Over recent years, MALDI-TOF mass spectrometry has emerged as a rapid (less than an hour) and reliable tool for the identification of bacterial strains [17,40,42,45] and has successfully been applied to a variety of genera [19-39]. In an analysis of out of 1660 bacterial isolates by MALDI-TOF, Seng *et al.* tested 60 *Propionibacterium* sp. against the Bruker database (Bruker) and obtained an identification of only 45% of them at the genus level [17]. Such a low identification rate may be due to various factors, including: 1) the absence of reference spectra for a given

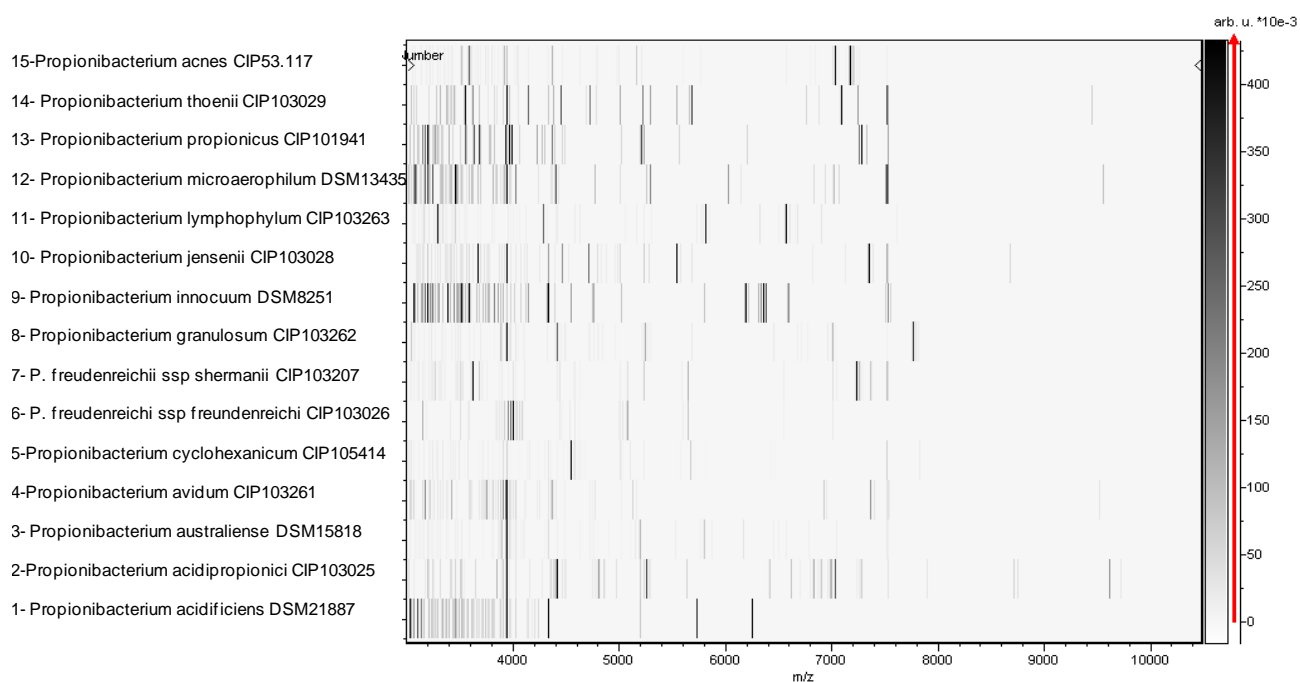


Figure 1. Virtual gel view of MS reference spectra from validated *Propionibacterium* species.

species; and 2) an insufficient number of reference spectra for a given species [46].

At the time the study was performed, the Bruker database contained reference spectra from 3769 bacterial isolates, including 35 spectra from validated *Propionibacterium*, *Propioniferax* and *Propionimicrobium* species. By comparison with the 12 validated *Propionibacterium* species, the *P. acidificiens* and *P. propionicum* spectra were missing from the Bruker database, as was that from *P. innocuum*, which explains why the isolates from these three species that we tested were not identified at the species level (**Table 1**), in contrast with other isolates. However, only four strains (26%) were correctly identified at the species level with a significant score, suggesting an intra-species proteomic variability. In addition, despite the use of a reference strain, no reliable identification was obtained for *P. acnes*, neither at the species nor genus level. Such a low efficiency of MALDI-TOF to identify *Propionibacterium* strains was confirmed by our blind test of 48 *P. acnes* strains, where only 18.7% were correctly identified at the species level, 50% not even being identified at the genus level (**Table 2**). Subsequently, in order to estimate whether such a poor sensitivity, particularly for *P. acnes*, the most frequent human pathogen among *Propionibacterium* species, could be improved by enriching the database in spectra, we added the spectra from 5 strains to our database. The result of this enrichment was that 40 of the 43 remaining clinical isolates were identified at the species level with scores ≥ 1.9 , thus increasing the sensitivity of MALDI-TOF for the identification of this species to 93.0%. The five strains used to enrich the database were selected so that their individual scores in the blind test ranged from 1.5 to 2.1. MALDI-TOF MS was known to be rapid and cost effective for bacterial identification. However, our study demonstrates that this method, to be efficiently used for the identification of *Propionibacterium* isolates, requires a database enrichment in isolates for the species of interest. The spectra obtained for all tested strains are freely available in our URMS database (<http://www.ifr48.com>).

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