MALDI-TOF Mass Spectrometry Is a Fast and Reliable Platform for Identification and Ecological Studies of Species from Family *Rhizobiaceae*

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Abstract

Family *Rhizobiaceae* includes fast growing bacteria currently arranged into three genera, *Rhizobium*, *Ensifer* and *Shinella*, that contain pathogenic, symbiotic and saprophytic species. The identification of these species is not possible on the basis of physiological or biochemical traits and should be based on sequencing of several genes. Therefore alternative methods are necessary for rapid and reliable identification of members from family *Rhizobiaceae*. In this work we evaluated the suitability of Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS) for this purpose. Firstly, we evaluated the capability of this methodology to differentiate among species of family *Rhizobiaceae* including those closely related and then we extended the database of MALDI Biotyper 2.0 including the type strains of 56 species from genera *Rhizobium*, *Ensifer* and *Shinella*. Second, we evaluated the identification potential of this methodology by using several strains isolated from different sources previously identified on the basis of their *rrs*, *recA* and *atpD* gene sequences. The 100% of these strains were correctly identified showing that MALDI-TOF MS is an excellent tool for identification of fast growing rhizobia applicable to large populations of isolates in ecological and taxonomic studies.

Introduction

The family *Rhizobiaceae* currently contains fast growing species of bacteria that may be saprophytic or able to establish beneficial or deleterious plant interactions. These species are currently arranged into three genera, *Rhizobium*, *Ensifer* and *Shinella* [1,2]. The former genera *Agrobacterium* and *Allohrizobium* are now included in genus *Rhizobium* [3] and *Sinorhizobium* is currently named *Ensifer* [4]. The identification of members of the family *Rhizobiaceae* is necessarily based on gene sequencing since there is not phenotypic information that allows the differentiation and identification of rhizobial species [3]. Therefore, although gene sequencing is the most reliable method for identification of rhizobia, it is still a tedious and time-consuming method to be applied to wide populations and therefore alternative methods are necessary for reliable identification of these bacteria shortening the time needed to achieve this process.

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) has been suggested as a fast and reliable method for bacterial identification, based on the characteristic protein profiles for each microorganism. Using this technology it has been estimated that up to 99% of strains tested are correctly identified when comparing with commercial phenotypic identification panels or *rrs* gene sequencing [5–8]. However MALDI-TOF MS has been basically applied to the identification of clinical isolates [9–16] so most of the species currently included on available databases are those of clinical interest. For example in the case of family *Rhizobiaceae* only the type strains of three species, *Rhizobium tropici*, *Rhizobium radiobacter* and *Rhizobium rubi*, and eight pathogenic non-type strains of *R. radiobacter*, *R. rhizogenes* and *Agrobacterium tumefaciens* (currently *R. radiobacter*) are included in Biotyper 2.0 database (Bruker Daltonics) used in this study.

Therefore the objectives of this work were: (i) the evaluation of MALDI-TOF MS technology for species differentiation within family *Rhizobiaceae*, (ii) the construction of a database that includes the type strains of currently accepted species within family *Rhizobiaceae* and (iii) the validation of the MALDI-TOF MS technology to identify rhizobial strains isolated from nodules and tumours previously identified by gene sequencing.

Materials and Methods

Bacterial strains and culture conditions

To build a reference database for MALDI-TOF MS-based rhizobial species identification, the type strains of 56 species belonging to the family *Rhizobiaceae* were used (Table 1). In addition 35 strains isolated from legume nodules or plant tumours
Table 1. Type strains of family *Rhizobiaceae* included in the extended database for MALDI-TOF MS-based species identification.

| Species                  | Strains included in database | Source of isolation | References |
|--------------------------|------------------------------|---------------------|------------|
| *Ensifer adhaerens*      | LMG 20216<sup>T</sup>        | soil                | [50]       |
| *Ensifer americanum*     | DSM 15007<sup>T</sup>        | T soil              | [36,51]    |
| *Ensifer arboris*        | LMG 14919<sup>T</sup>        | T Acacia nodules    | [36,52]    |
| *Ensifer fredii*         | USDA 205<sup>T</sup>, LMG 6217<sup>T</sup> | Glycine max nodules | [36,53,54] |
| *Ensifer garamanticus*   | LMG 24692<sup>T</sup>        | T Agyrolobium uniflorum nodules | [55] |
| *Ensifer kostiense*      | LMG 19227<sup>T</sup>        | T Acacia senegal nodules | [36,52] |
| *Ensifer kummerowiae*    | CCBAU 71714<sup>T</sup>      | T Kummerowia stipulacea nodules | [36,56] |
| *Ensifer medicae*        | USDA 1037<sup>T</sup>        | T Medicago truncatula nodules | [36,57] |
| *Ensifer melloti*        | ATCC 9930<sup>T</sup>        | T Medicago sativa nodules | [36,58] |
| *Ensifer morelense*      | LC04<sup>T</sup>             | T Leucaena leucocephala nodules | [36,59] |
| *Ensifer numidicus*      | LMG 24690<sup>T</sup>        | T Agyrolobium uniflorum nodules | [55] |
| *Ensifer saheli*         | LMG 7837<sup>T</sup>         | T Sesbania cannabina nodules | [36,58] |
| *Ensifer terangae*       | LMG 7834<sup>T</sup>         | T Acacia laeta nodules | [36,58] |
| *Rhizobium aggregatum*   | DSM 1111<sup>T</sup>         | T Surface lake water | [30,60] |
| *Rhizobium alamii*       | LMG 24466<sup>T</sup>        | T Plant rhizosphere | [61] |
| *Rhizobium alcalisoli*   | DSM 21826<sup>T</sup>        | T Caragana microphylla nodules | [62] |
| *Rhizobium borbori*      | LMG 23925<sup>T</sup>        | T Activated sludge  | [63] |
| *Rhizobium cellulosilyticum* | ALA10B2<sup>T</sup>       | T Populus alba sawdust | [64] |
| *Rhizobium daejeonense*  | DSM 17795<sup>T</sup>        | T Cyanide treatment bioreactor | [65] |
| *Rhizobium etli*         | CFN42<sup>T</sup>            | T Phaseolus vulgaris nodules | [32] |
| *Rhizobium fabae*        | LMG 23997<sup>T</sup>        | T Vicia faba nodules | [66] |
| *Rhizobium galegae*      | HAMBI 540<sup>T</sup>        | T Galega orientalis nodules | [67] |
| *Rhizobium gallicum*     | R602sp<sup>T</sup>           | T Phaseolus vulgaris nodules | [45] |
| *Rhizobium gigantiini*   | H152<sup>T</sup>             | T Phaseolus vulgaris nodules | [45] |
| *Rhizobium hainanense*   | i66<sup>T</sup>              | T Desmodium sinustum nodules | [68] |
| *Rhizobium huastense*    | SO2<sup>T</sup>              | T Sesbania herbacea nodules | [69] |
| *Rhizobium indigofae*    | CCBAU 71042<sup>T</sup>      | T Indigofera amblyantha nodules | [56] |
| *Rhizobium larsymoorei*  | LMG 21410<sup>T</sup>        | T Ficus benjamina aerial tumours | [3,70,71] |
| *Rhizobium leguminosarum*| USDA 2370<sup>T</sup>        | T Pisum sativum nodules | [29] |
| *Rhizobium leucense*     | CCBAU 71908<sup>T</sup>, LMG23187<sup>T</sup> | T Astragalus complanatus nodules | [72] |
| *Rhizobium lisianum*     | p1-7<sup>T</sup>             | T Phaseolus vulgaris nodules | [48] |
| *Rhizobium mesosinicum*  | LMG 24135<sup>T</sup>        | T Albizia julibrissin | [73] |
| *Rhizobium miluonense*   | LMG 24208<sup>T</sup>        | T Lespedeza chinensis nodules | [74] |
| *Rhizobium mongolense*   | USDA 1844<sup>T</sup>        | T Medicago ruthenica nodules | [75] |
| *Rhizobium multihospitium* | LMG 23946<sup>T</sup>    | T Halimodendron halodendron nodules | [76] |
| *Rhizobium oryzae*       | LMG 24253<sup>T</sup>        | T Wild rice surface sterilized roots | [77] |
| *Rhizobium phaseoli*     | ATCC 14482<sup>T</sup>       | T Phaseolus vulgaris nodules | [29,32] |
| *Rhizobium pisi*         | DSM 30132<sup>T</sup>        | T Pisum sativum nodules | [29] |
| *Rhizobium radiobacter*  | ATCC 19358<sup>T</sup>, NCB1 9042<sup>T</sup>, CIP 104325<sup>T</sup> | T Soil | [3,28] |
| *Rhizobium rhizogenes*   | ATCC 11325<sup>T</sup>       | T Hairy roots       | [3,28] |
| *Rhizobium rosseus*      | CCM 7583<sup>T</sup>         | T hexachlorocyclohexane (HCH) dump site | [30] |
| *Rhizobium rubi*         | ATCC 13335<sup>T</sup>, DSM 6772<sup>T</sup> | T Rubus tumours | [3,78] |
| *Rhizobium selenitireducens* | LMG 24075<sup>T</sup>    | T Cyanide treatment bioreactor | [79] |
| *Rhizobium sulfureus*    | IS 123<sup>T</sup>           | T Hedysarum coronarium nodules | [80] |
| *Rhizobium tibeticum*    | DSM 21102<sup>T</sup>        | T Trigonella archiducis-nicolai | [81] |
| *Rhizobium tropici*      | CIAT 899<sup>T</sup>         | T Leucaena leucocephala nodules | [82] |
| *Rhizobium tubenense*    | LMG 25225<sup>T</sup>        | T Oxypolis glabra nodules | [83] |
| *Rhizobium undicola*     | LMG 11875<sup>T</sup>        | T Neptunia natans nodules | [3,84] |
previously characterized by gene sequencing were used to validate MALDI-TOF MS as an identification tool for this group of bacteria (Table 2).

In order to establish the more adequate medium and growth conditions for fast-growing rhizobia analysis, selected strains were cultivated on TY [17] and YMA plates [18] and incubated at 28°C for 24 and 48 hours.

Sample preparation for MALDI-TOF MS

Cells of a whole colony were transferred from the plate to a 1.5 ml tube (Eppendorf, Germany) with a pipette tip and mixed thoroughly in 300 μl of water to resuspend the bacterial cells. Then, 900 μl of absolute ethanol was added and the mixture was centrifuged at 15,500 g for 2 min, and the supernatant was discarded. The pellet was air-dried at room temperature for 1 hour. Subsequently, 50 μl of formic acid (70% v/v) was added to the pellet and mixed thoroughly before the addition of 50 μl acetonitrile to the mixture. The mixture was centrifuged again at 15,500 g for 2 min. One microliter of the supernatant was placed onto a spot of the steel target and air-dried at room temperature. Each sample was overlaid with 1 μl of matrix solution and air-dried.

MALDI-TOF MS

Measurements were performed on an Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany) equipped with a 200-Hz smartbeam laser. Spectra were recorded in the linear, positive mode at a laser frequency of 200 Hz within a mass range from 2,000 to 20,000 Da. The IS1 voltage was 20 kV, the IS2 voltage was maintained at 18.6 kV, the lens voltage was 6 kV, and the extraction delay time was 40 ns.

For each spectrum, 500 laser shots were collected and analyzed (10×50 laser shots from different positions of the target spot). The spectra were calibrated externally using the standard calibrant mixture (Escherichia coli extracts including the additional proteins RNase A and myoglobin, Bruker Daltonics). Calibration masses were as follows: RL36, 4364.3 Da; RS22, 5095.8 Da; RL34, 5380.4 Da; RL33meth, 6254.4 Da; RS19, 10299.1 Da; RNase A, 13682.2 Da; myoglobin, 16952.5 Da.

Spectrum generation and data analysis

For automated data analysis, raw spectra were processed using the MALDI Biotyper 2.0 software (Bruker Daltonics, Leipzig, Germany) at default settings. The software performs normalization, smoothing, baseline subtraction, and peak picking, creating a list of the most significant peaks of the spectrum (m/z values with a given intensity, with the threshold set to a minimum of 1% of the highest peak and a maximum of 100 peaks). To identify unknown bacteria, each peak list generated was matched directly against reference libraries (3,476 species) using the integrated patterns matching algorithm of the Biotyper 2.0 software (Bruker Daltonics, GmbH, Germany). The unknown spectra were compared with a library of reference spectra based on a pattern recognition algorithm using peak position, peak intensity distributions and peak frequencies. Once a spectrum has been generated and captured by the software, the whole identification process was performed automatically, without any user intervention. MALDI-TOF MS identifications were classified using modified score values proposed by the manufacturer: a score value ≥ 2 indicated species identification; a score value between 1.7 and 1.9 indicated genus identification, and a score value < 1.7 indicated no identification.

For reference library construction, 36 independent spectra were recorded for each bacterial isolate (three independent measurements at twelve different spots each). Manual/visual estimation of the mass spectra was performed using Flex Analysis 3.0 (Bruker Daltonics GmbH, Germany) performing smoothing and baseline subtraction. Checking existence of flatlines, outliers or single spectra with remarkable peaks differing from the other spectra was done, taking into account that mass deviation within the spectra set shall not be more than 500 ppm. Finally, 20 spectra were selected, removing questionable spectra from the collection. To create peak lists of the spectra, the BioTyper software was used as described above. The 20 independent peak lists of a strain were used for automated “main spectrum” generation with default settings of the BioTyper software. Thereby, for each library entry a reference peak list (main spectrum) which contains information about average masses, average intensities, and relative abundances in the 20 measurements for all characteristic peaks of a given strain was created, so a main spectrum displayed the most reproducible peaks typical for a certain bacterial strain.

Cluster analysis was performed based on comparison of strain-specific main spectra created as described above. The dendrogram was constructed by the statistical toolbox of Matlab 7.1 (MathWorks Inc., USA) integrated in the MALDI Biotyper 2.0 software. The parameter settings were: ‘Distance Measure’ = Euclidian and ‘Linkage’ = complete’. The linkage function is normalized according to the distance between 0 (perfect match) and 1000 (no match).

Phylogenetic analyses

The results of MALDI-TOF MS analysis were compared with those obtained after rrs, recA, atpD and nodC gene sequence analyses. In this work we obtained some sequences of these genes that are absent in databases according to Rivas et al. [19] for rrs.
### Table 2. MALDI BioTyper identification results for family Rhizobiaceae strains.

| Source of isolation | Reference | Organism (best match) | score values* |
|--------------------|-----------|-----------------------|---------------|
| **A. Strains from different collections** |
| Ensifer fredii USDA 205T | Glycine nodules [53] | Ensifer fredii LMG 6217T | 2.585 |
| Rhizobium loessense LMG 23187T | Astragalus nodules [72] | Rhizobium loessense CECT 71908T | 2.335 |
| Rhizobium tropici CIAT 899T | Phaseolus nodules [82] | Rhizobium tropici DSM 11418T | 2.582 |
| Rhizobium radiobacter ATCC 19358T (NCBI 90427, CIP 1043257) | soil [28] | Rhizobium radiobacter DSM 30147T | 2.566 (2.524, 2.488) |
| Rhizobium rubi ATCC 13335T | Rubus tumours [28] | Rhizobium rubi DSM 6772T | 2.505 |
| **B. Species correctly reclassified in other species** |
| Agrobacterium tumefaciens ATCC 23308 (NCBI 13307, CIP 67.1), former type strains | Malus tumours [28] | Rhizobium radiobacter DSM 30147T (strain included in Biotype 2.0 database) | 2.522 (2.408, 2.405) |
| Ensifer xinjiangense LMG 17930 (CECT 4657), former type strains | Glycine nodules [92] | Ensifer fredii LMG 6217T | 2.413 (2.151) |
| Rhizobium trifolii ATCC 14480, former type strain | Trifolium nodules [29] | Rhizobium leguminosarum USDA 2370T | 1.218 |
| **C. Species erroneously included in other species** |
| Ensifer morelense Lc04T | Leucaena nodules [36,59] | Ensifer adhaerens LMG 20216T | 1.245 |
| Rhizobium phaseoli ATCC 14482T | Phaseolus nodules [29,32] | Rhizobium etli CFN42T | 1.991 |
| Rhizobium pisi DSM 30132T | Pisum nodules [29] | Rhizobium leguminosarum USDA 2370T | 1.782 |
| **D. Species with problems in their identity** |
| Ensifer kummerowiae CCBAU 71714T | Kummerowia nodules [56] | Ensifer meliloti ATCC 9930T | 2.261 |
| Rhizobium fabae LMG 23997T | Vicia nodules [66] | Rhizobium pisi DSM 30132T | 2.258 |
| Rhizobium indigoefera CCBAU 71042T | Indigofera nodules [56] | Rhizobium leguminosarum USDA 2370T | 2.219 |
| Rhizobium loessense CCBAU 17908T (LMG23187T) | Astragalus nodules [72] | Rhizobium gallicum R602spT | 2.283 (2.354) |
| Rhizobium mongolense USDA 1844T | Medicago nodules [75] | Rhizobium gallicum R602spT | 2.506 |
| Rhizobium yanglingense CCBAU 1623T | Gueldenstaedtia nodules [87] | Rhizobium gallicum R602spT | 2.314 |
| **E. Strains included in already described species** |
| RTM17 | Trigonella nodules [49] | Ensifer meliloti ATCC 9930T | 2.140 |
| GVPV12 | Phaseolus nodules [44] | Ensifer meliloti ATCC 9930T | 2.145 |
| RPA13 | Prosopis nodules [43] | Ensifer meliloti ATCC 9930T | 2.241 |
| RMP01 | Melilotus nodules [49] | Ensifer meliloti USDA 103T | 2.252 |
| RMP05 | Melilotus nodules [49] | Ensifer medicina USDA 103T | 2.114 |
| RPA08 | Prosopis nodules [43] | Ensifer medicina USDA 103T | 2.092 |
| RPA11 | Prosopis nodules [43] | Ensifer medicina USDA 103T | 2.177 |
| RPA20 | Prosopis nodules [43] | Ensifer medicina USDA 103T | 2.211 |
| FL27 | Phaseolus nodules [45] | Rhizobium gallicum R602spT | 2.405 |
| RH12 | Phaseolus nodules [45] | Rhizobium gallicum R602spT | 2.399 |
| RPA02 | Prosopis nodules [43] | Rhizobium giardinii H152T | 2.432 |
| RPA12 | Prosopis nodules [43] | Rhizobium giardinii H152T | 2.425 |
| RPVF18 | Phaseolus nodules [38] | Rhizobium leguminosarum USDA 2370T | 2.017 |
| RVS11 | Viciea nodules [39] | Rhizobium leguminosarum USDA 2370T | 2.152 |
| ATCC 14480 | Trifolium nodules [29] | Rhizobium leguminosarum USDA 2370T | 2.128 |
| P3-13 | Phaseolus nodules [48] | Rhizobium lusitanum P1-7T | 2.314 |
| USDA 1929 | Medicago nodules [75] | Rhizobium mongolense USDA1844T | 2.474 |
| ATCC 13332 | no data [93] | Rhizobium radiobacter DSM 30147T | 2.644 |
| 163C | Prunus tumours [47] | Rhizobium rhizogenes ATCC 11325T | 2.195 |
| IAM 13571 | no data [48] | Rhizobium rhizogenes ATCC 11325T | 2.267 |
| K84 | soil [46] | Rhizobium rhizogenes ATCC 11325T | 2.185 |
| Br859 | Leucaena nodules [82] | Rhizobium tropici CIAT 899T | 2.613 |
| **G. Strains do not belonging to described species** |
| Br816 | Leucaena nodules [34,35] | Ensifer americanum | 1.775 |
| RPVR32 | Phaseolus nodules [38] | Rhizobium leguminosarum USDA 2370T | 1.066 |
| CVII4 | Viciea nodules [39] | Rhizobium leguminosarum USDA 2370T | 1.288 |
gene, Gaunt et al. [20] for recA and atpD genes and Laguerre et al. [21] for nodC gene. The sequences were aligned using the Clustal W software [22]. The distances were calculated according to Kimura’s two-parameter model [23]. Phylogenetic trees were inferred using the neighbour-joining method [24] and the MEGA 4.0 package [25].

### Results and Discussion

#### Database setting

In Biotyper 2.0 database only three species of genus *Rhizobium* are included and none of genus *Ensifer* or *Shinella*. Therefore a database extension in order to include the species currently described in these genera is necessary before applying MALDI-TOF MS to the identification of rhizobial isolates.

Owing to the fact that in Biotyper 2.0 database the type strains of three species of genus *Rhizobium* are already included, *R. tropici* DSM 11418T, *R. rubi* DSM 6772T and *R. radiobacter* DSM 30147T, we verified the reproducibility of MALDI-TOF MS using the type strains of these species that were cultivated in two different media (YMA and TY) and incubated at 24 and 48 h.

The results obtained showed that the analysed strains matched with high score values (higher than 2.5) with each corresponding type strain already present in Biotyper 2.0 database when TY medium and 24 h incubation were used (Table 3). Lower score values were found with YMA medium incubated at 24 h and only *R. rubi* ATCC 13335T and *R. radiobacter* ATCC 19358T were correctly identified using YMA medium. Therefore best results for rhizobial species were obtained with TY medium and 24 h incubation, in spite of previous studies that have demonstrated high reproducibility of MALDI-TOF MS analysis in different culture media and growth phases [14,26,27].

Before the extension of Biotyper 2.0 database we also checked the suitability of MALDI-TOF MS system to differentiate the spectra of representative species from the three genera currently accepted in Family *Rhizobaceae*.

Firstly we compared the spectra of the type strains from the type species of the three genera currently included in family *Rhizobaceae*. The results obtained showed that the spectra of *Rhizobium leguminosarum* USDA 2370T, *Ensifer adhaerens* LMG 20216T and *Shinella granuli* DSM18401T were clearly distinguishable since there were not common peaks among their spectra (figure 1).

Subsequently, we analyzed the spectra of two phylogenetically close and one phylogenetically divergent species from each genus. We selected from genus *Rhizobium* the close species *R. leguminosarum* (type species of genus *Rhizobium*) and *R. pisi* as well as the species *R. cellulolyticum*, phylogenetically distant from them. From genus *Ensifer* we chose the close species *E. meliloti* and *E. medicae* and the species *E. adhaerens*, which is the type species of genus *Ensifer* and it is phylogenetically distant from the other two species. Finally, from genus *Shinella* we chose the close species *S. granuli*, type species of genus *Shinella*, and *S. kummerowiae* and the phylogenetically distant *S. fisca*.

All these spectra were quite different with almost any common peaks among those of species belonging to different genera as we previously observed for the type species of each genus. Within the same genus the spectra of close species were more similar than those from divergent species. For example, considering the mass tolerance

### Table 1. Comparison of identification results by MALDI-TOF MS with different conditions.

| Media and incubation times (extraction method) | Incubated in YMA during 24 h (best match) | Score value* | Incubated in TY during 24 h (best match) | Score value* |
|-----------------------------------------------|------------------------------------------|--------------|------------------------------------------|--------------|
| **Strain**                                    |                                          |              |                                          |              |
| *Rhizobium radiobacter* ATCC 19358T           | *Rhizobium radiobacter* DSM 30147T       | 2.388        | *Rhizobium radiobacter* DSM 30147T       | 2.566        |
| *Rhizobium tropici* CIAT 899T                 | *Rhizobium tropici* DSM 11418T           | 1.897        | *Rhizobium tropici* DSM 11418T           | 2.582        |
| *Rhizobium rubi* ATCC 13335T                  | *Rhizobium rubi* DSM 6772T               | 2.500        | *Rhizobium rubi* DSM 6772T               | 2.505        |
| **Strain**                                    |                                          |              |                                          |              |
| *Rhizobium radiobacter* ATCC 19358T           | *Rhizobium radiobacter* DSM 30147T       | 2.077        | *Rhizobium radiobacter* DSM 30147T       | 1.389        |
| *Rhizobium tropici* CIAT 899T                 | *Rhizobium tropici* DSM 11418T           | 1.399        | *Rhizobium tropici* DSM 11418T           | 1.547        |
| *Rhizobium rubi* ATCC 13335T                  | *Rhizobium rubi* DSM 6772T               | 2.333        | *Rhizobium rubi* DSM 6772T               | 1.522        |

* score value ≥2 indicates species identification; 1.7 < score value < 2 indicates genus identification, score value < 1.7 indicates no identification.

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**Table 2.** Comparison of identification results by MALDI-TOF MS with different conditions.

| Strain | Source of isolation | Reference | Organism (best match) | score values* |
|--------|---------------------|-----------|-----------------------|---------------|
| RTP05  | Trifolium nodules   | [94]      | *Rhizobium leguminosarum* USDA 2370T | 1.701         |
| CFN299 | Leucaena nodules    | [82]      | *Rhizobium tropici* CIAT 899T       | 1.191         |
| CS8    | Prunus tumour       | [95]      | *Rhizobium radiobacter* DSM 30147T  | 1.956         |

* score value ≥2 indicates species identification; 1.7 < score value < 2 indicates genus identification, score value < 1.7 indicates no identification.

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Figure 1. MALDI-TOF MS spectra of whole-cell extracts obtained from the type strains of two close and one divergent species from each genus analysed in this study: (A) *Rhizobium*, (B) *Ensifer* and (C) *Shinella*.

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±2 m/z for each peak as we have previously described [13], in genus Rhizobium, R. leguminosarum USDA 2370T and R. pisum DSM 30132T shared peaks at 3126, 4689, 6773, 7296 and 9308 Da that are not in R. etli CCM14480 ALAB21T (figure 1A). In genus Ensifer, peaks at 2502, 2605, 4652, 5005, 5211 and 9304 Da were present in E. fredii ATCC 9939T and E. medicace USDA 1037T and not in E. adhaerens DSM 20216T, although there are two common peaks in the three species compared (3741 and 7494 Da). (figure 1B). In genus Shinella, we found peaks at 5006, 4613 and 2504 Da in S. granuli and S. kummerowiae that were not present in S. fusca and, although S. fusca shared few peaks with S. granuli (3615, 3723, 7229, 7444 Da), this phylogenetically distant species had many specific peaks that were not in the other two species (2070, 2237, 2326, 2497, 3254, 5096, 6531, 6507 Da) (figure 1C).

These results showed that the spectra of both phylogenetically close and distant species from the same genus, as well as those of species of different genera within family Rhizobiaceae can be differentiated by MALDI-TOF MS. Therefore we extended the database MALDI BioTyper 2.0 with 56 type strains of species from genera Rhizobium, Ensifer and Shinella belonging to Family Rhizobiaceae (Table 1).

Comparison between MALDI-TOF MS and phylogenetic analyses

To compare the data obtained by MALDI-TOF MS analysis with those based on gene sequence analysis (figures 2, 3 and 4), a cluster analysis was performed based on a correlation matrix using the integrated tools of the MALDI Biotyper 2.0 software package. Figure 5 showed that the genus Rhizobium was divided into several clusters whose distribution basically coincided with that observed after rrs, recA and atpD gene analyses. The results evidenced that some reclassifications performed within genus Rhizobium are correct as occurs in the case of the former species Agrobacterium tumefaciens reclassified into A. radiobacter [28]. MALDI-TOF MS results confirmed that they are the same species since their type strains belong in different collections matched with score values higher than 2 (Table 2A and 2B). These results are congruent with those obtained from rrs, recA and atpD gene analyses since these strains presented nearly identical sequences (figures 3 and 4). After reclassification of the complete genus Agrobacterium into Rhizobium, the current valid name for these species is Rhizobium radiobacter [3].

MALDI-TOF MS analysis also confirmed the R. trifoli ATCC 14480 reclassification into R. leguminosarum [29], since the strain ATCC 14480 matched with R. leguminosarum USDA 2370T with a score value higher than 2 (Table 2B), and Blastobacter aggregatus DSM 1111T into R. aggregatum [30] since strain DSM 1111T clustered with species of genus Rhizobium (Figure 5).

On the contrary, some species of genus Rhizobium were erroneously reclassified. For example, R. phaseoli type strain was reclassified into R. leguminosarum [31]. Later the biovar phaseoli type I of this species was reclassified into R. etli [32], so it was not clear the location of the R. phaseoli type strain. A revision based on rrs, recA and atpD analysis showed that R. phaseoli is a valid species distinguishable from both R. leguminosarum and R. etli [29]. The results of the MALDI-TOF MS confirmed these results since R. phaseoli ATCC 14482T matched with R. etli CFN42T with score values lower than 2 (Table 2C).

Moreover, the MALDI-TOF MS cluster analysis showed, in agreement with rrs, recA and atpD gene analyses, that some current Rhizobium species are indistinguishable (figures 2, 3, 4 and 5). For example, the type strains of R. mongolense, R. loessense and R. yanglingense matched with R. gallicum R602sp with score values higher than 2 (Table 2D). In addition, R. indigoferson CCB1AU 71042T matched with R. leguminosarum USDA 2370T with a score value of 2.219 and R. fabae LMG 23997T matched with R. pisum DSM 30132T with a score value of 2.258 (Table 2D). Therefore the taxonomic status of all these species should be revised according to the current rules of bacterial taxonomy.

The genera Shinella and Ensifer MALDI-TOF cluster analysis was performed together (figure 6) since they are closely related on the basis of recA and atpD gene analyses (see figures 3 and 4). This closeness was confirmed after MALDI-TOF cluster analysis although the distribution of Shinella species was slightly different (figure 6). The species S. yambaruensis was the closest related species to S. granuli on the basis of MALDI-TOF MS analysis, whereas these two species were distant according to their rrs gene sequences (figure 2). However, S. yambaruensis DSM 18801T matched with S. granuli DSM 18401T with a score value lower than 2 corresponding to different species from the same genus.

The distribution of species in the genus Ensifer was coherent with those found after rrs analysis with E. medicace and E. melliloti forming the same group, E. morelense close to E. adhaerens and E. americanum (a not yet validated species) close to E. fredii (figure 6). In the genus Ensifer also MALDI-TOF MS analysis confirmed some reclassifications as that of species E. xinjiangense into E. fredii [33] since the former type strains E. xinjiangense LMG 17930 and CECT 4657 matched with E. fredii LMG 6217T with score values of 2.413 and 2.151, respectively (Table 2B). Also was confirmed the reclassification of the strain Rhizobium sp. Br816 as Ensifer sp. [34,35] since it clustered with E. americanum (figure 6). However, in agreement with rrs, recA and atpD gene analyses (figures 2, 3 and 4), strain Br816 does not belong to this species since it matched with E. americanum DSM 15007T with score values lower than 2 (Table 2G).

However, other reclassifications were not correct as occurs with E. morelense reclassified into E. adhaerens [36] since E. morelense Lc04T matched with E. adhaerens LMG 20216T with a score value of only 1.245 (Table 2C) in agreement with rrs, recA and atpD gene analyses (figures 2, 3 and 4).

In the genus Ensifer, also some species were indistinguishable, for example, E. kummerowiae CCB1AU 71714T matched with E. melliloti ATCC 9939T with a score value of 2.261 suggesting that they belong to the same species (Table 2D). Since this result coincides with the analysis of rrs, recA and atpD genes, the taxonomic status of E. kummerowiae should be revised.

All these findings showed that MALDI-TOF MS results are comparable to those obtained after the phylogenetic analysis of core genes from members of family Rhizobiaceae including that of rrs gene in which is currently based the classification within this family [1]. These results are in agreement with those previously reported for other bacterial groups [37] and therefore we analysed the potential of MALDI-TOF MS for identification of fast-growing rhizobia isolates.

Identification of rhizobial strains by MALDI-TOF MS

To prove the suitability of the extended MALDI Biotyper 2.0 database for routine identification and discrimination of fast-growing rhizobial species we analysed several strains previously identified by rrs and housekeeping gene sequencing belonging to different species and genera of family Rhizobiaceae (Table 2 and 2G).

The species R. leguminosarum contains some strains with identical rrs gene and divergent recA and atpD genes [29,38,39]. For
Identification of *Rhizobiaceae* with MALDI-TOF MS

Figure 2. Neighbour-joining phylogenetic rooted tree based on 16S rRNA sequences (about 1475 nt) showing the taxonomic location of the species included in this study. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Accession numbers from Genbank are given in brackets.

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Figure 3. Neighbour-Joining phylogenetic tree based on recA gene sequences (about 520 nt) showing the position of species included in this study. Bootstrap values calculated for 1000 replications are indicated. Bar, 2 nt substitution per 100 nt. Accession numbers from GenBank are given in brackets.
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Figure 4. Neighbour-joining phylogenetic tree based on atpD gene sequences (about 500 nt) showing the position of species included in this study. Bootstrap values calculated for 1000 replications are indicated. Bar, 2 nt substitution per 100 nt. Accession numbers from Genbank are given in brackets.
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example, the strains RPVF18, RVS11 and ATCC 14480 have housekeeping genes closely related to the type strain of this species USDA 2370\(^T\) and other strains have phylogenetically distant ones, such as RPVR31, CVIII4 and RTP05 (figures 3 and 4). Although all these strains clustered with \textit{R. leguminosarum} USDA 2370\(^T\) after MALDI-TOF MS cluster analysis (figure 5), only when the housekeeping genes were almost identical the score values were higher than 2 with respect to \textit{R. leguminosarum} USDA 2370\(^T\) (Table 2F). These results were congruent with those from \textit{recA} and \textit{atpD} gene analyses showing that, in spite of the complete identity of \textit{rrs} gene, \textit{R. leguminosarum} could contain several subspecies perfectly distinguishable by MALDI-TOF MS analysis as it has already been observed in other bacterial species [26,40,41].

Although housekeeping gene sequences present higher variability than those of \textit{rrs} genes, the ITS fragment located between 16S and 23S gene in fast growing rhizobia is the most hypervariable chromosomic region and has been proposed as a tool for species differentiation [42]. However, MALDI-TOF MS showed that strains with housekeeping genes nearly identical but different ITS sequences belong to the same species. For example, the strains RPA12 and RPA02 shared only 73\% identity in their ITS sequences with respect to \textit{R. giardinii} H152\(^T\) suggesting they can represent different species [43]. However, in agreement with \textit{rrs}, \textit{recA} and \textit{atpD} gene analyses, MALDI-TOF MS showed that they belong to \textit{R. giardinii} since they matched with the type strain of this species with score values higher than 2 (Table 2F).

The same was found for the genus \textit{Ensifer} strains RTM17 and GVPV12 that matched with \textit{E. meliloti} ATCC 9930\(^T\) with score values higher than 2 (Table 2F) in spite of the differences in the ITS region (95\% identity) [44] and in agreement with the results of the housekeeping gene analyses (figures 3 and 4).

Intraspecific variability in species of family \textit{Rhizobiaceae} could be also due to the presence of large plasmids codifying for symbiotic or virulence factors. Nodulating species may contain different biovars that carry different \textit{nodC} genes [21,38,44,45] and pathogenic species contain strains that carry plasmids involved in tumour (pTi) or hairy roots induction (pRi) [46,47]. Therefore we analysed strains with different combinations of chromosomal backgrounds and symbiotic or virulence plasmids by MALDI-TOF MS.

For example, within genus \textit{Rhizobium}, \textit{R. leguminosarum} contains three biovars: viciae, trifolii and phaseoli [31,38], perfectly distinguishable on the basis of their \textit{nodC} gene sequences (figure 7). However MALDI-TOF MS analysis showed that strains with housekeeping genes close to \textit{R. leguminosarum} USDA 2370\(^T\) (RVS11, RPVF18 and ATCC 14480) [29,38,39] matched with score values higher than 2 with this strain with independence to the biovar they belong to (figure 7). Likewise, the strains FL27 from biovar gallicum [45] and PhD12 from biovar phaseoli [21] carrying divergent \textit{nodC} genes (figure 7) matched with \textit{R. gallicum}.
R602sp1 with score values higher than 2 (Table 2F). The same was found in \textit{R. lusitanum} whose strains P1-7 T and P3-13 have phylogenetically distant nodC genes (figure 7) but they matched with a score value of 2.314 (Table 2F).

In genus \textit{Ensifer}, \textit{E. meliloti} also contains different biovars with divergent nodC genes (figure 7). However, the strains RPA13 and RTM17 from biovar meliloti and the strain GVPV12 from biovar mediterraneum [44] were matched with \textit{E. meliloti} ATCC 9930T with score values higher than 2 by MALDI-TOF MS (Table 2F).

Conversely, strains from the same biovar but divergent housekeeping genes were perfectly distinguished by MALDI-TOF MS in genus \textit{Rhizobium}. For example, the strain CVIII14 matched with a score value lower than 2 with \textit{R. leguminosarum} USDA 2370T, although both strains belong to the biovar viciae (Table 2G). To this biovar also belongs \textit{R. pisi} DSM 30132T that was correctly distinguished by MALDI-TOF MS from \textit{R. leguminosarum} USDA 2370T (Table 2G). Moreover, strains CFN299 and CIAT 89991, whose \textit{rs} and housekeeping genes showed they belong to different species [48], matched with score values lower than 2 (Table 2G) in spite of the complete identity of their nodC genes (figure 7).

In genus \textit{Ensifer}, \textit{E. meliloti} RPA13 and RTM17 and \textit{E. medicae} RMP01, RMP05, RPA08, RPA11 and RPA20 belong to the same biovar (meliloti) [49]. However, in agreement with \textit{rs} and housekeeping gene analyses (figures 2, 3 and 4), the strains of these both species were clearly distinguished by MALDI-TOF MS (Table 2F).

Finally, two species of genus \textit{Rhizobium}, \textit{R. rhizogenes} and \textit{R. radiobacter}, contain non-pathogenic strains, tumourigenic strains and hairy roots inducing strains (Table 2F). In both cases their strains were correctly identified by MALDI-TOF MS in agreement with the \textit{rs} and housekeeping gene analyses (figures 2, 3 and 4) in spite of the plasmidic content. In this way the non-pathogenic strain K84 [46], the tumourigenic strain 163C and the root inducing strain IAM 15571, matched with \textit{R. radiobacter} DSM 30147T with high score values (2.185, 2.195 and 2.158, respectively). The tumourigenic strain ATCC 23308 (type strain of the former species \textit{A. tumefaciens}) and the root inducing strain ATCC 13332 (erroneously named \textit{R. rhizogenes}) also matched with the non-pathogenic strain \textit{R. radiobacter} DSM 30147T with score values higher than 2 (Table 2F).

Conversely, although the pTi plasmids of the tumourigenic strains 163C and C58 are closely related [47], they belong to different species according to MALDI-TOF MS results (Table 2G) in agreement with the \textit{rs} and housekeeping gene analyses (figures 2, 3 and 4).

All these results showed that plasmids carried by fast growing rhizobial strains do not affect their identification by MALDI-TOF MS since strains of the same species carrying very different plasmids and strains from different species carrying similar plasmids were correctly identified by MALDI-TOF MS.
In conclusion, the results presented in this work clearly showed that MALDI-TOF MS is a reliable and rapid method for rhizobial identification comparable to housekeeping gene sequence analysis since it is able to discriminate between strains with identical \textit{rrs} gene sequences but divergent \textit{recA} and \textit{atpD}. This feature represents important advantages based on the rapidity and cost per sample with respect to gene sequencing. With this methodology, if the databases include all rhizobial species described in each moment, it will be possible to identify all isolates belonging to species already described as well as the detection of new species. Therefore, MALDI-TOF MS opens a new and very useful way for diversity and ecological studies applicable to analysis of large populations of isolates allowing the differentiation of strains, species and genera of fast-growing rhizobia with an effectiveness of 100% in the identification at species level.

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**Author Contributions**

Conceived and designed the experiments: LF PFM EMM JMGB EV. Performed the experiments: LF FSJ PGF RR EV. Analyzed the data: LF FSJ EV. Contributed reagents/materials/analysis tools: PGF EMM JMGB. Wrote the paper: LF EV.
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