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ERWINIA SPECIES IDENTIFICATION USING MATRIX-ASSISTED LASER DESORPTION IONIZATION-TIME OF FLIGHT MASS SPECTROMETRY

F. Rezzonico[1,2*], B. Duffy[1,2], T.H.M. Smits[1,2] and J.F. Pothier[1,2]

[1] *Environmental Genomics and System Biology Research Group, Institute of Natural Resource Sciences, Zürich University of Applied Sciences (ZHAW), Campus Grüental, 8820 Wädenswil, Switzerland*
[2] *Agroscope Changins-Wädenswil ACW, Plant Protection Division, Schloss, 8820 Wädenswil, Switzerland*

SUMMARY

Rapid and reliable identification of plant pathogenic bacteria is critical for effective implementation of phytosanitary measures. The genus *Erwinia* includes a number of economically important plant pathogens such as fire blight agent *Erwinia amylovora* or Asian pear pathogen *Erwinia pyrifoliae*, together with closely related plant epiphytes of unknown pathogenicity or even with a potential use for biological control like *Erwinia tasmaniensis* or *Erwinia billingiae*, respectively. Current laboratory methods to achieve satisfactory identification and discrimination between species within the *Erwinia* genus are based on the isolation on semi-selective media, serology, specific PCR and gene locus sequencing: these approaches are complicated and time-consuming, often requiring *a priori* assumptions over the identity of the isolates. Here we present a streamlined approach based on whole-cell Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) based on the AXIMA mass spectrometer of Shimadzu-Biotech Corp that demonstrates the potential of this technology for quick species identification in plant diagnostics within the genus *Erwinia*.

Keywords: bacterial identification, MALDI-TOF MS, taxonomy, fire blight.

Corresponding author: F. Rezzonico
E-mail: fabio.rezzonico@zhaw.ch

INTRODUCTION

Erwinia (Winslow *et al.*, 1920) is a genus of the *Enterobacteriaceae* family that was originally created to unite all Gram-negative, nonsporulating, fermentative, peritrichously flagellated plant-pathogenic bacteria (Kwon *et al.*, 1997). Since its inception, the genus has undergone several taxonomical rearrangements following refined phenotypic characterization or DNA sequence analysis, with many species being transferred to other genera such as *Pectobacterium* (Hauben *et al.*, 1998), *Dickeya* (Samson *et al.*, 2005) (both genera containing phytopathogenic pectinolytic bacteria causing soft rot diseases), *Pantoea* (Brady *et al.*, 2010), *Brenneria* (Hauben *et al.*, 1998), *Lonsdalea* (Brady *et al.*, 2012), or *Enterobacter* (Brenner *et al.*, 1986; Dickey and Zumoff, 1988). In addition, a number of new species were recently described and approved within the genus including *Erwinia piriflorinigrans* (López *et al.*, 2011) and *Erwinia uzenensis* (Matsuura *et al.*, 2012), two novel pathogens that affects European pear trees; *Erwinia typographica*, isolated from the gut of healthy bark beetles (Skrodenytė-Arbačiauskienė *et al.*, 2012); *Erwinia gerundensis* a cosmopolitan epiphyte originally isolated from pome fruit trees (Rezzonico *et al.*, 2016); and "*Candidatus* Erwinia dacicola", an olive fly endosymbiont (Estes *et al.*, 2009). As of January 2017, 19 species are listed in the genus.

Quick and reliable identification of plant pathogens and discrimination from closely related epiphytes is a critical requirement for disease control and implementation of the appropriate control measures (Montesinos, 2003). Several standardized procedures are available for the detection, identification and quantitation of the different *Erwinia* spp. in diagnostic laboratories (Bühlmann *et al.*, 2013; Gottsberger, 2010; Kim and Jock, 2001; López *et al.*, 2004; Pirc *et al.*, 2009). Most of these methods habitually target one single species and thus require some *a priori* assumption on the identity of the organism under investigation. Yet, not all *Erwinia* spp. are covered by these protocols, which are as well obviously unsuited for the discovery of new taxa. While sequencing of 16S rRNA does not offers resolution at species level, single housekeeping genes have previously been employed with variable success to classify the different species within *Erwinia*, although

relationships between the different species are often inconsistent when comparing phylogenetic trees obtained with different target genes (Palmer *et al.*, 2017). To overcome this problem, laborious and time-consuming methodologies such as multi-locus sequence analysis (MLSA) are necessary. Using the latter approach, *Erwinia* constitutes a monophyletic clade mostly composed by phytopathogenic, non-pectinolytic bacteria that cause wilts and dry necroses, but also includes non-pathogenic organisms, such as epiphytes and insect symbionts (Rezzonico *et al.*, 2016).

In the last two decades, whole cell Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) has emerged as a reliable tool for rapid microbial identification in clinical diagnostics (Dierig *et al.*, 2015; Patel, 2013). Identification is based on unique mass/charge ratio (m/z) fingerprints of proteins, which are ionized using short laser pulses directed to subcolony amounts of whole ("intact") bacterial cells embedded in a matrix. After desorption, ions are accelerated in vacuum by a strong electric potential and separated on the basis of their time of flight to the detector, which is proportional to the mass-to-charge ratio. The main benefit of this technique with respect to conventional molecular biological tools resides in the straightforward preparation of the samples, which can commonly be completed in less than a minute directly from a single bacterial colony, and in the fact that it does not require any *a priori* knowledge of the organism to be investigated. This technique has proven itself reliable across a broad range of conditions, being relatively unaffected by factors such as the cell-growth phase or the composition of the culture medium, thus displaying limited variability in mass-peak signatures within the typically designated mass range between 2,000 and 20,000 Daltons (Lay, 2001; Maier *et al.*, 2006; Rezzonico *et al.*, 2010). These features make whole cell MALDI-TOF MS an easy, rapid and cost-effective technique, which is extraordinarily suited for high-throughput routine analysis (Seng *et al.*, 2009).

A small number of MALDI-TOF MS platforms currently share the commercial market, the two most common being those from Bruker Daltonics, which includes a mass spectrometer along with the Biotyper software and database, and the Shimadzu Axima mass spectrometer with the Launchpad software and the SARAMIS™ (Spectral ARchive And Microbial Identification System, AnagnosTec GmbH) database created by AnagnosTec GmbH. The latter system was recently acquired by bioMérieux and is currently being redeveloped under the commercial name VITEK® MS. Both platforms have demonstrated an excellent accuracy in the identification of the isolates routinely scrutinized in medical diagnostic laboratories, with a percentage of high-confidence identifications of about 94% for the Bruker system compared to about 89% for Shimadzu (Cherkaoui *et al.*, 2010). However, reliable identification ultimately depends upon comparison with a validated and comprehensive database of reference peptide-mass fingerprints and commercial databases are still currently heavily skewed towards clinical taxa and rarely include environmental bacterial species relevant to plant, animal and public health (Rezzonico *et al.*, 2010). A proof of principle that MALDI-TOF MS can be employed for the identification of species within the genus *Erwinia* was provided on the Bruker Daltonics platform (Sauer *et al.*, 2008), yet only few species were considered and several of them were represented only by their type isolate, thus not embracing the full taxonomic diversity within each single species (Sauer *et al.*, 2008; Wensing *et al.*, 2012). Furthermore, since the two databases are not compatible, the data obtained through the Bruker system cannot directly be imported and used for *Erwinia* species identification using the Shimadzu platform.

The aim of this work was to expand the existing commercial SARAMIS™ database through new independent measurements to include the maximum available number of *Erwinia* species and to compare, at different taxonomic levels, the clustering of the strains generated by the analysis of MALDI-TOF MS spectra to the results obtained using MLSA, the current gold standard for phylogenetic analysis.

MATERIALS AND METHODS

Bacterial strains and biological sample preparation variables. A total of 109 strains representing 15 available *Erwinia* species (Rezzonico *et al.*, 2010, 2009), plus six so-called "Pinking" isolates (Born *et al.*, 2016; Stockwell *et al.*, 2008) were selected for clustering analysis and generation of MALDI-TOF MS SuperSpectra. Among the *Erwinia* strains, 35 belonged to the species of fire blight causative agent *Erwinia amylovora*, which was studied more in detail. To allow comparison with previously published genotypic data, we chose both *Rubus*- and Spiraeoideae-infecting strains taking care to select the latter within all major CRISPR genotypes (Mann *et al.*, 2012; Rezzonico *et al.*, 2011, 2012) in order to cover the maximum available diversity. Nine strains of species belonging to related genera, some of which were previously included in the genus *Erwinia*, were selected as outgroups. Reference strains were mainly obtained from commercial culture collections (Supplemental Table S1). For standardized spectral acquisition and generation of SuperSpectra™ in SARAMIS™, bacteria were routinely streaked on LB agar and grown at 28°C for 16 to 24 h.

MALDI-TOF MS spectrum acquisition. Intact cells from individual colonies were scraped from agar plates using a disposable loop and smeared onto a target spot of a polished ground steel 48-position MALDI sample target (Industrietechnik mab AG, Basel, Switzerland. For each strain, four distinct single bacterial colonies were spotted in

order to compare the reproducibility of masses within replicates. Cells were overlaid with 1 µl of a saturated solution of sinapinic acid (Sigma-Aldrich, Buchs, Switzerland) in 60% acetonitrile (Sigma-Aldrich, Buchs, Switzerland) – 0.3% trifluoroacetic acid (Sigma-Aldrich, Buchs, Switzerland) and air-dried for a few minutes at room temperature. Protein mass fingerprints were obtained using an AXIMA Confidence Linear/Reflectron MALDI-TOF Mass Spectrometer (Shimadzu-Biotech Corp., Kyoto, Japan), with detection in the linear, positive mode at a laser frequency of 50 Hz and within a mass range of 2,000 to 20,000 Da. Acceleration voltage was 20 kV and the extraction delay time was 200 ns. A minimum of 20 laser shots per sample was used to generate each ion spectrum. For each bacterial sample, a total of 50 protein mass fingerprints were averaged and processed using the Launchpad version 2.8 software (Shimadzu-Biotech Corp.). For peak acquisition, the average smoothing method was chosen, with a smoothing filtering width of 50 channels. Peak detection was performed with the threshold-apex peak detection method using the adaptive voltage threshold which roughly follows the signal noise level, and subtraction of the baseline was set with a baseline subtraction filter width of 500 channels. For each sample, a list of the significant spectrum peaks was generated that included the m/z values of the peaks, their mass deviations, and signal intensity. Every target plate was calibrated individually at the beginning of each plate acquisition using spectra of the reference strain *Escherichia coli* K-12 DSMZ 1576 (GM48 genotype) and a second measurement was performed at the end of the run as a control.

MALDI-TOF MS spectrum analysis and SuperSpectrum generation. Generated protein mass fingerprints were imported in SARAMIS™ and analyzed using the following presetting parameters: mass range, from 2,000 to 20,000 Da; allowed mass deviation, 800 ppm. For dendrogram generation, the complete list of detected masses was exported in Microsoft Excel as comma-separated values (csv) file. An Excel macro (J. Pothier, personal communication) was executed to convert the list of masses into a binary matrix indicating the presence/absence of each individual mass signal. For each isolate, only the 150 strongest mass signals present in at least 50% of the replicate spectra were retained. The resulting file was implemented for cluster analysis in the PAST (Paleontological Statistics, http://folk.uio.no/ohammer/past/) software package version 2.11 (Hammer *et al.*, 2001) using the PG (paired-group) algorithm with 500 bootstrap replicates and visualized by means of the phylogenetic trees graphical viewer FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/. Clustering of the different isolates in the tree based on MALDI-TOF MS data was compared to that obtained in the neighbor-joining phylogenetic tree constructed using MLSA sequences.

As reference spectra for a rapid identification, SARAMIS™ uses so-called "SuperSpectra" consisting of taxon-specific biomarkers. SuperSpectrum generation was based on recovered mass signal markers with an absolute intensity of at least 200 mV included in the 2,000- to 20,000-Da mass range. To create the SuperSpectrum of a species, the protein mass fingerprints of the strains clustering with the respective type strain in the MLSA tree were used. Using the SARAMIS™ SuperSpectrum tool, a subset of protein masses found in at least 90% of the strains of one species were selected and tested for their discriminatory power by comparing them to all of the database entries. Dependent on the amount of remaining species-identifying marker masses, each was given a numeric value in order to get a maximum total number of points not higher than 1,250. A set of 20 to 40 marker masses is normally sufficient to obtain a specific identification to the species level. The identification results obtained using MALDI-TOF MS were compared to those obtained using DNA sequencing combined with a BLAST similarity search.

Multilocus sequence analysis of housekeeping genes. Partial sequences of housekeeping genes *gyrB* (742 bp), *rpoB* (637 bp), *atpD* (642 bp), and *infB* (615 bp) were selected for multilocus sequence analysis in a subset of isolates composed by at least one strain per species. Wherever available, sequences were retrieved from NCBI and, if included in our study, the type strain of the species was selected. For novel strains and in case of missing data, amplification and sequencing of the aforementioned genes were performed as described previously (Brady *et al.*, 2008) by means of the HotStarTaq master mix kit (Qiagen, Basel, Switzerland) and the ABI PRISM BigDye Terminators version 1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), respectively. All 16S rRNA gene sequences were obtained from GenBank. DNA sequences were aligned with ClustalW (Thompson *et al.*, 1994). Sites presenting alignment gaps were excluded from analysis. The phylogenetic trees were generated on the basis of the concatenated sequences of the four housekeeping genes fragments or over 1162 positions of the 16S rRNA gene. The Molecular Evolutionary Genetics Analysis (MEGA) program, version 7 (Kumar *et al.*, 2016), was used to calculate evolutionary distances and to infer a tree based on the neighbor-joining (NJ) or minimum evolution (ME) method using the maximum composite likelihood (MCL) model. Nodal robustness of the inferred tree was assessed by 1,000 bootstrap replicates. GenBank accession numbers for sequences used in this work are listed in Supplemental Table S2.

RESULTS AND DISCUSSION

Confirmation of all *Erwinia* spp. The identity of all strains analyzed in this work was verified both using 16S rRNA gene sequences as well as at least one of the MLSA genes. Phylogenetic analysis confirms that the genus *Erwinia* is not monophyletic with respect to this

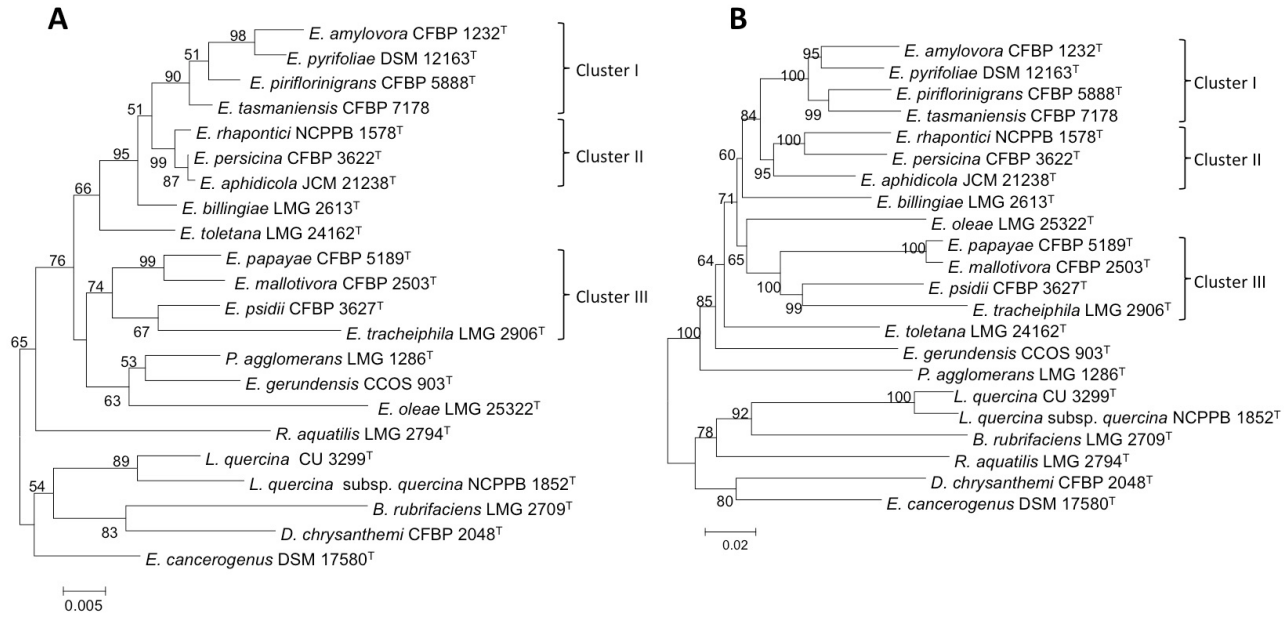

Fig. 1. Taxonomy of representative strains of the *Erwinia* species considered in this study based on 16S rRNA sequences (A) and the concatenated sequences of housekeeping genes *gyrB-rpoB-atpD-infB* (B) The trees were constructed with the Neighbor-Joining method and nodal supports were assessed by 1000-bootstrap replicates. Only bootstrap values greater than 50% are shown. The scale bar represents the number of base substitutions per site. Wherever possible sequences were obtained from the NCBI databases (http://www.ncbi.nlm.nih.gov), else the corresponding gene were sequenced according a protocol described previously for *Enterobacteriaceae* (Brady *et al*., 2008).

gene (Rezzonico *et al*., 2016) with its species that can be assigned to several clusters based on the 16S rRNA gene sequences (Fig. 1A): Cluster I represents the pathoadapted group of *Erwinia* (Kamber *et al*., 2012; Smits *et al*., 2013) and includes the type species of the genus, *E. amylovora*, together with *E. pyrifoliae*, *E. piriflorinigrans* and *E. tasmaniensis*; Cluster II comprises *E. rhapontici*, *E. persicina* and *E. aphidicola*; and Cluster III encompasses *E. papayae*, *E. mallotivora*, *E. psidii* and *E. tracheiphila*. *E. oleae* and *E. gerundensis* are joined in a fourth cluster that contains also outgroup *P. agglomerans*, whereas *E. billingiae* and *E. toletana* formed single deep-branching taxa not directly related to any of the above clusters (Rezzonico *et al*., 2010, 2009) (Fig. 1A). The presence of Clusters I-III, but of not of the fourth cluster containing *P. agglomerans* LMG 1286^T, could be confirmed by MLSA using concatenated sequences of the housekeeping genes *gyrB*, *rpoB*, *atpD* and *infB*, which allowed to place all investigated *Erwinia* species within a single monophyletic group that is distinct from the other *Enterobacteriaceae* genera evaluated as outgroups (Fig. 1B), thereby confirming previous findings (Palmer *et al*., 2017; Rezzonico *et al*., 2016). The peripheral position of *P. agglomerans* with respect to the *Erwinia* group is an artefact due to the imbalance in the number of taxa between the two genera.

MALDI-TOF MS analysis was able to discriminate strains within *Erwinia* and to segregate them into separate species with the same level of accuracy as MLSA. Strains belonging to the same species examined in this work (Supplemental Table S1) clustered together and with the respective type strain when using the MALDI-TOF data to build the cladogram, thus demonstrating the efficacy of this approach for rapid identification at species level (Fig. 2). In terms of species grouping, an outcome similar to that obtained using MLSA was reached when cluster analysis was performed on the basis of protein mass fingerprint patterns derived from MALDI-TOF MS measurements (Fig. 2). Although the topology between the different *Erwinia* species within the single clusters was not identical to the one obtained with the analysis of the concatenated housekeeping genes, the branching among the different clusters was similar to the one obtained using the MLSA approach, with Clusters I-III of *Erwinia* species being conserved in both trees. Both MALDI-TOF MS and the MLSA trees were concordant in locating *E. billingiae* next to Cluster I+II as well as *E. oleae* and *E. toletana* next to Cluster III on individual branches within the genus. On the other hand, *E. gerundensis* showed a somewhat divergent peak pattern, thereby clustering outside the genus, which is thus not monophyletic in the MALDI-TOF MS tree (Fig. 2). Even if a direct comparison of the respective MALDI-TOF MS datasets is not possible due to the different chemical matrices used in the two studies, it is interesting to notice that the protein mass fingerprints of *E. gerundensis* showed quite divergent patterns from both those of *Erwinia* and *Pantoea* placing this new taxon at a distance from both genera in the individual trees based on MALDI-TOF MS data (Fig. 2 and Rezzonico *et al*., 2010), respectively. This, together with the marginal position of the species in the genus *Erwinia* according to both MLSA

and phylogenomic analysis (Rezzonico *et al.*, 2010), suggests that *E. gerundensis* may be a member of a novel genus situated between *Erwinia* and *Pantoea*. All three analyses placed strains Cu 3298, Cu 3299^T and Cu 3300, received and described in the literature as '*Erwinia lupinicola*' (Bühlmann *et al.*, 2013; Poza Carrion *et al.*, 2008), into an individual branch within the former clade IV of necrogenic *Brenneria* species, thus confirming the results previously obtained on the basis of glyceraldehyde-3-phosphate dehydrogenase gene sequences (Brown *et al.*, 2000). These pathogenic strains, causing the drippy pod of white lupine, were later suggested to belong to a pathovar of *Brenneria quercina* (*i.e.*, *B. quercina* pv. *lupinicola*) (Lu and Gross, 2010), a species now reclassified into the novel genus *Lonsdalea* (Brady *et al.*, 2012).

Two distinct pathotypes within *E. amylovora*. A proof-of-principle of the ability of MALDI-TOF MS analysis to allow discrimination at sub-species level was provided by an in-depth investigation performed on 35 isolates belonging to the causative agent of fire blight *E. amylovora*, a destructive disease that affects rosaceous plants worldwide (Bonn and van der Zwet, 2000). Although *E. amylovora* is mainly recognized as a pathogen of *Malus* and *Pyrus* spp., the disease has been described for other taxa of the Spiraeoideae subfamily as well as for members of the Rosoideae subfamily belonging to the genus *Rubus*, such as raspberry or blackberry (Rezzonico *et al.*, 2012). Cross-infectivity between Spiraeoideae- and *Rubus*-infecting isolates of *E. amylovora* is limited and the two pathotypes can be distinguished molecularly in several ways, *e.g.*, by characterization of their lipopolysaccharide biosynthesis genes (Rezzonico *et al.*, 2012), of their CRISPR repeat regions (Rezzonico *et al.*, 2011), or by molecular fingerprinting techniques such as rep-PCR and PCR-ribotyping (McManus and Jones, 1995).

A total of 26 Spiraeoideae-infecting and 9 *Rubus*-infecting isolates were analyzed in this study, whereby the clustering obtained using MALDI-TOF MS closely matched the one previously achieved using *rpoB* gene sequences (Rezzonico *et al.*, 2012), with a single clade containing all Spiraeoideae-infecting isolates (S) and *Rubus*-infecting strains divided into three separate branches, one of which (R2) clustering basal to the Spiraeoideae clade (Fig. 3). Despite the ability of MALDI-TOF MS to distinguish *E. amylovora* pathotypes, it was not always possible to recognize single strains for this species as demonstrated for *P. agglomerans*, where repeated independent measurements of the same isolate typically clustered together in the tree generated by SARAMIS (Rezzonico *et al.*, 2010). The current distribution pattern of *E. amylovora* diversity is believed to have originated from two evolutionary bottleneck events, resulting in a genetically highly homogeneous population in Spiraeoideae-infecting strains, especially those isolated in Europe (Rezzonico *et al.*, 2011). The fact that replicate measurements of the same *E. amylovora* isolate were found

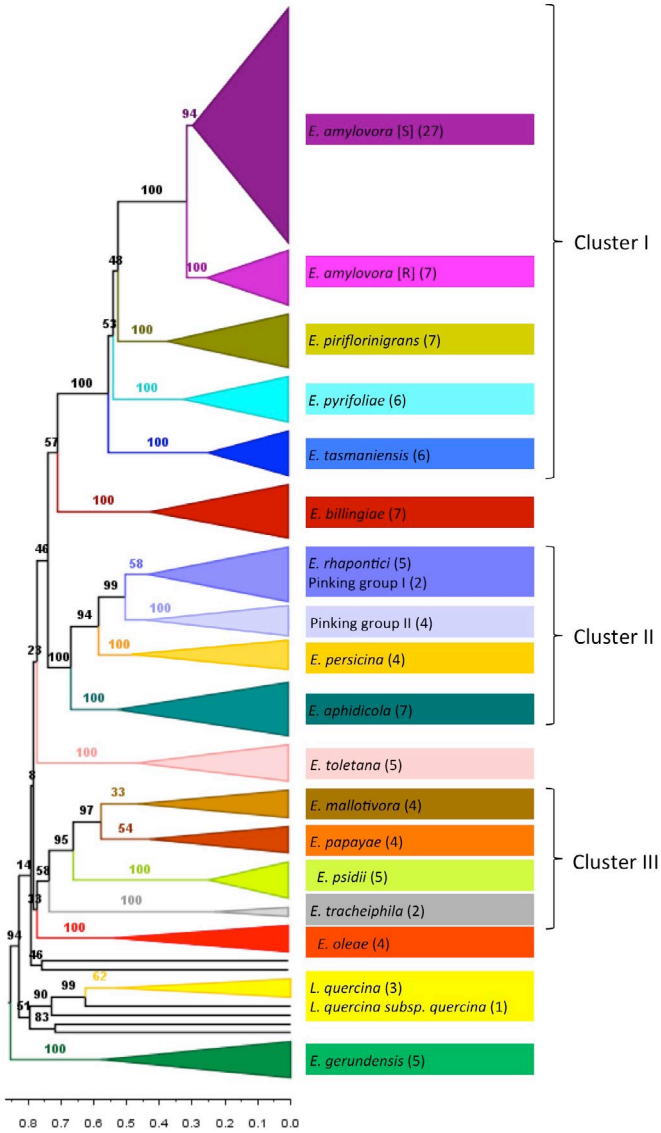

Fig. 2. Dendrogram of the strains belonging to the genus *Erwinia* based on MALDI-TOF MS protein mass fingerprints. The tree was constructed using the PG algorithm with 500 bootstrap replicates. All the branches containing strains belonging to the same species are collapsed, whereby their number is indicated between parentheses. Based on the obtained mass fingerprints within each cluster SuperSpectra for each species were created. Species *E. amylovora* and *E. rhapontici* are further subdivided into two clusters, demonstrating the ability of MALDI-TOF MS to allow discrimination at sub-species level (see Fig. 3 and Fig. 4)

to scatter along the SARAMIS tree, albeit remaining within the same pathotype, suggests that the measurable diversity within this species is smaller than the noise that is implicit in each MALDI-TOF MS measurement, thus placing strain typing for *E. amylovora* out of reach of our current protocol.

Species determination of isolates displaying the "pinking" phenotype. Iron (II) chelator proferrorosamine A (L-2-(2-pyridyl)-1-pyrroline-5-carboxylic acid) is

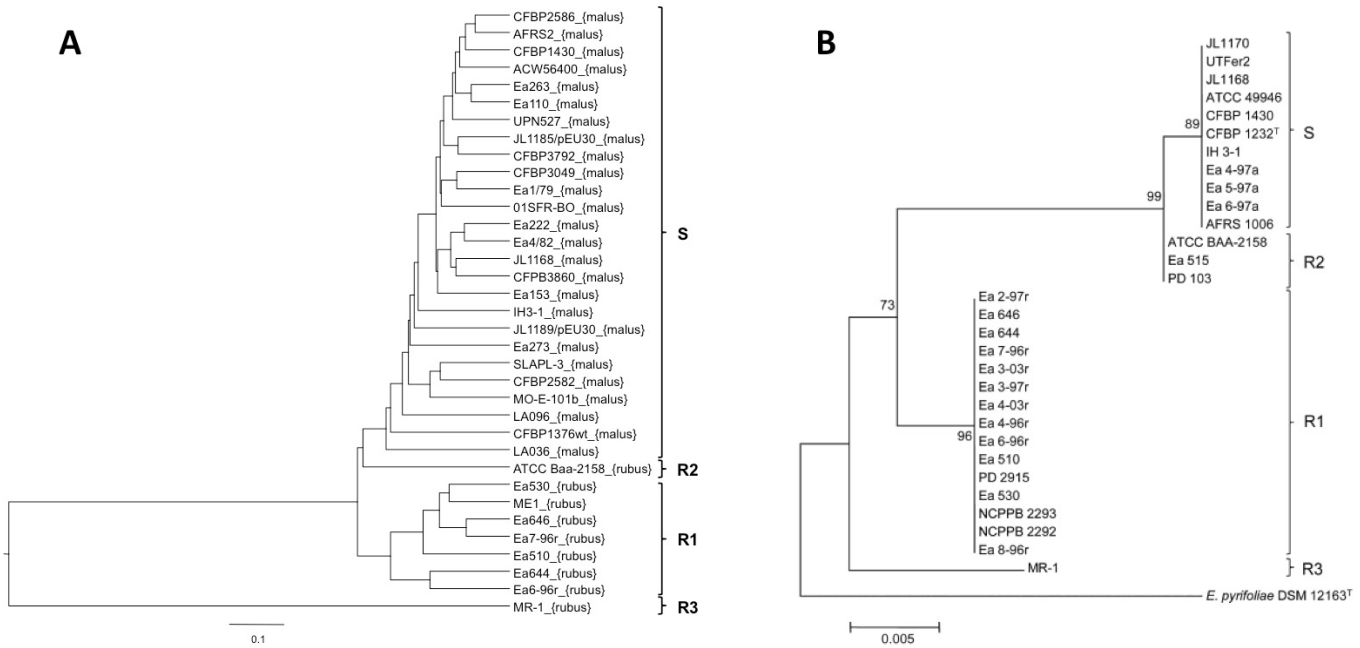

Fig. 3. Intraspecific clustering of the isolates belonging to *E. amylovora* based on MALDI-TOF MS protein mass fingerprints (A) and comparison with clustering formerly obtained on the basis of the *rpoB* gene (B) (Rezzonico *et al*., 2012). All Spiraeoideae-infecting strains are regrouped within a single clade (S), whereas the *Rubus*-infecting strains can be divided into three groups: a major cluster (R1) encompassing all Canadian and most of the US isolates, plus two single-strain groups: the first (R2) is represented by strain ATCC BAA-2158 (IL-5), previously found to be closely related to Spiraeoideae-infecting strains along with isolates PD103 and Ea515 (which were not included in this work), whereas the second single-strain group (R3) is constituted by strain MR-1, which shows the largest divergence in both trees.

a metabolite produced by *E. persicina* and *E. rhapontici* that causes pink colony coloration in culture media containing iron (Born *et al*., 2016; Feistner *et al*., 1997; Stockwell *et al*., 2008). Both *E. persicina* and *E. rhapontici* were reported as regular co-isolates during fire blight surveys and uptake of proferrorosamine A by colonies of *E. amylovora* can lead to false identifications of the latter species in the diagnostics (Stockwell *et al*., 2008). We tested here a number of "pinking" isolates routinely identified on King's B agar from plant samples during fire blight surveys performed by Agroscope (Wädenswil, Switzerland) and used MALDI-TOF MS to assess whether they belong to *E. persicina* or *E. rhapontici*. The results were then compared with those obtained using *gyrB* and *atpD* sequencing-based identification. Both sequence as well as MALDI-TOF analysis revealed that, unlike the result obtained in a previous study with North American isolates (Stockwell *et al*., 2008), none of the six Swiss "pinking" isolates tested belonged to *E. persicina*. Two isolates (ACW 44286 and ACW 41072) were found to cluster with *E. rhapontici* type strain NCPPB 1578^T (Pinking group I), whereas the other four (ACW 44214, ACW 43997, ACW 41558 and ACW 40945) formed a discrete group (Pinking group II) not associated to any other *E. rhapontici* strain included in this study (Fig. 2). However, the same segregation of the "pinking" isolates into two groups within *E. rhapontici* was found also when analyzing the trees based on the *gyrB* and *atpD* gene sequences, whereby the latter four Pinking group II isolates clustered together with *E. rhapontici* strains LMG 2645 and LMG 2648 (Fig. 4). This subspecies division within *E. rhapontici* is not apparent in studies based on 16S rRNA gene sequences (Kwon *et al*., 1997; Thapa *et al*., 2012), but confirms previous indications obtained on the basis of MLSA data (Brady *et al*., 2012).

CONCLUSIONS

This study demonstrates the accurateness and the ease of MALDI-TOF MS for the identification of isolates belonging to the genus *Erwinia*. Identification at species level was achieved on the basis of the generated protein profiles generated with minimal labor, time and materials directly from colonies grown on agar. This offers an attractive alternative to the relatively high investment required for single-locus validation, PCR amplification, and sequencing. The investment in a MALDI-TOF mass spectrometer is comparable to the one needed for a 16-capillary DNA sequencing machine, but it requires a fraction of the operating costs and consumables. In some cases, such as for fire blight causative agent *E. amylovora* or proferrorosamine-producing bacterium *E. rhapontici*, it is even possible to discern the individual pathotypes or the intraspecific groups, respectively, that compose the species and that are not evident based on 16S rRNA gene sequencing.

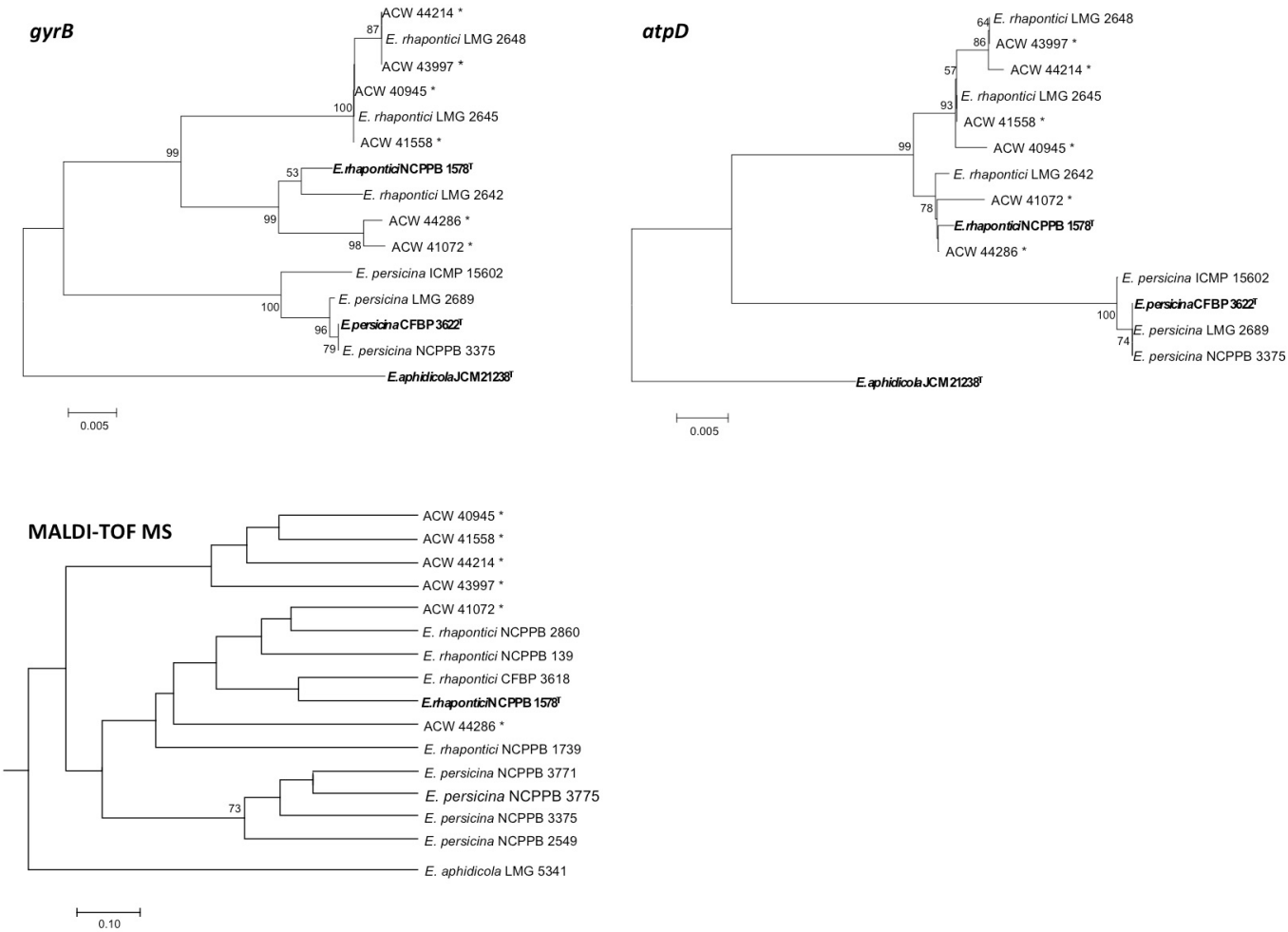

Fig. 4. Clustering of the isolates displaying the "pinking" phenotype (*) within the species *E. rhapontici* and *E. persicina* on the basis of *gyrB*, *atpD* gene sequences and MALDI-TOF MS. All tested "pinking" isolates-were found to belong to *E. rhapontici*. The *gyrB* and *atpD* trees were constructed with the Neighbor-Joining method and nodal supports were assessed by 1000-bootstrap replicates. Only bootstrap values greater than 50% are shown. The scale bar represents the number of base substitutions per site. The MALDI-TOF tree was exported from SARAMIS, the scale bar represents the percentage of divergence between the spectra. *E. aphidicola* was used as outgroup to root the trees.

MALDI-TOF MS offers thus an attractive alternative for identification of *Erwinia* isolates at species or subspecies level compared to the relatively high workload required for multi-locus PCR and sequencing.

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